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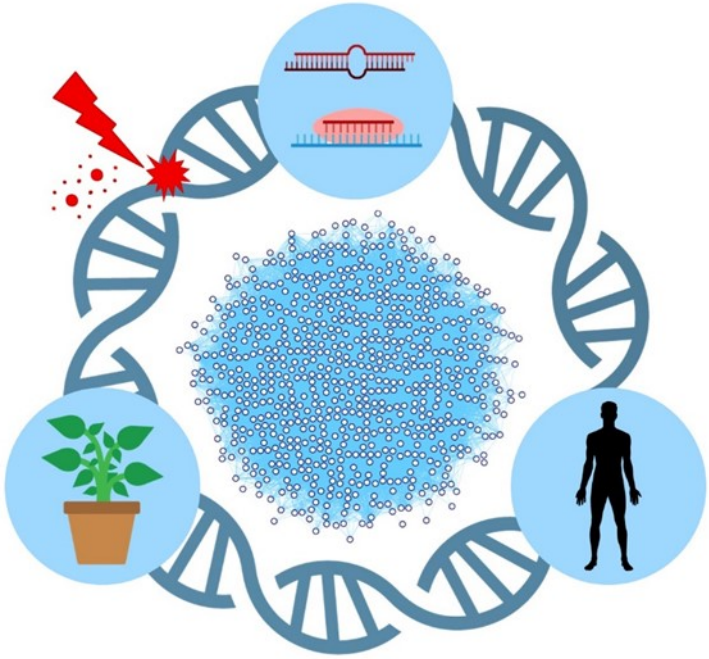
Shedding light on the role of plant miRNAs in DNA damage response (DDR) and *trans*-kingdom transfer



Carla Gualtieri

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXIII– A.A. 2017-2020

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"*Ciò che spesso non si dice è che sono le emozioni a guidarci verso il piu autentico dei desideri. Queste emozioni danno lo slancio affinché si compia*"

Carla Gualtieri

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…and do not object to me if in the end the greatest thank is to me. After so much investment I can finally say: CE L'HO FATTA!

Contents

Abstract

One of the challenges that living organisms face is to respond promptly to genotoxic stress to avoid DNA damage. To this purpose, they developed complex DNA damage response (DDR) mechanisms. These mechanisms are highly conserved among organisms, including plants, and need to be finely regulated to take place properly. In this scenario, microRNAs are emerging as active players, thus attracting the attention of the research community. The involvement of miRNAs in DDR has been investigated prominently in human cells whereas studies on plants are still scarce. Besides, recently, miRNAs started to be envisioned as *trans*-kingdom molecules able to exert regulatory functions in evolutionarily distant organisms. Particularly, attention is drawn to plant miRNAs ingested with the diet; the evidence is accumulating on their ability to regulate genes in organisms other than the one in which they were synthesized, including humans and pathogens.

In the present Ph.D. thesis, different bioinformatics approaches have been developed aiming at identifying plant miRNAs along with their endogenous and cross-kingdom targets to pinpoint conserved pathways between evolutionarily distant species. Alongside model organisms, the developed pipeline may find an application on any species of interest to address species-specific cross-kingdom interactions or to perform large-scale investigations involving several plant/animal species. The emergence of DDR-related miRNAs in plants and humans constitutes fundamental pieces of information obtained from these approaches.

To experimentally investigate the involvement of plant miRNAs in the regulation of DDR-associated pathways, an *ad hoc* system was developed, using the model legume *Medicago truncatula*. Specific treatments with camptothecin (CPT) and/or NSC120686 (NSC) targeting components of DDR, namely topoisomerase I (Top1) and tyrosyl-DNA phosphodiesterase 1 (Tdp1), were used. These treatments, imposed on *M. truncatula* seeds for 7 days, do not influence the germination process, but result in inhibition of seedling development, causing an increase in cell death and accumulation of DNA damage. To demonstrate that the imposed treatments affected DDR, the expression of *SOG1* (suppressor of gamma response 1) master-regulator was investigated by qRT-PCR. Importantly, a phylogenetic study demonstrated that *M. truncatula* possessed a small *SOG1* gene family, composed of *MtSOG1A* and *MtSOG1B* genes. The expression of both genes was significantly enhanced in a treatment-specific manner. Additionally, the expression of multiple genes playing important roles in different DNA repair pathways, cell cycle regulation, and chromatin remodeling, were differentially expressed in a treatment-specific manner. Subsequently, specific miRNAs identified from the bioinformatics approach as targeting genes involved in DDR processes were investigated alongside their targets, thus providing the first step in their function validation.

To investigate plant miRNAs *trans*-kingdom potential, additional studies were conducted using apple (*Malus domestica*) since it can be eaten raw and hence, can be a better system for feeding trials. As a proof of concept, artificial miRNAs

(amiRNAs, designed based on most express miRNAs identified in apple fruits) were delivered to human colorectal adenocarcinoma cells and the expression of these microRNAs and their *in silico* predicted targets were evaluated by qRT-PCR. Specifically, amiRNAs mimicking mdm-miR482a-3p and mdm-miR858 were transfected into HT-29 cell lines. After 72 h, amiRNAs were clearly detected inside the cells and the performed qRT-PCR analysis showed significant downregulation of the *IL4R* (Interleukin 4 Receptor) gene, involved in promoting Th2 differentiation, suggesting the possibility of apple miRNAs to regulate the activity of human genes *in vitro*.

Taken together, the results presented in the current PhD thesis demonstrate the involvement of plant miRNAs in DDR-associated processes as well as present evidence on the plant miRNAs *trans*-kingdom potential, by using both *in silico* approaches and specifically designed experimental *in vitro* systems.

Abbreviations

Act (actin)
ACYLTR (anthocyanin 5-aromatic acyltransferase)
AGO1A (argonaute protein 1A)
AP (apurinic/apyrimidinic)
ATM (Ataxia Telangiectasia Mutated)
ATR (Rad3-related)
ATUBC2 (ubiquitin-conjugating enzyme)
BER (Base Excision Repair)
BRCA1 (Breast cancer susceptibility gene 1)
CDKs (Serine/threonine cyclin-dependent kinases)
CHK1 (Checkpoint kinase 1)
CHK2 (Checkpoint kinase 2)
CPT (Camptothecin)
Cyc (Cyclin)
DDR (DNA Damage Response)
DMSO (Dimethyl sulfoxide)
DNAM (DNA methyltransferase 1-associated protein)
DPC (DNA-protein crosslink)
DR (Direct Repair)
DSBs (Double-Stranded Breaks)
Elf1α (ETS-related transcription factor)
E2F (E2F Transcription Factor 1)
ERCC1 (Excision Repair 1)
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)
H4 (H4 Histone)
HAT (Histone Acetyl Tranferase)
HDAC (Histone Deacetylase)
HT-29 (human colorectal adenocarcinoma cell line)
HR (Homologous Recombination)
IL4R (Interleukin 4 Receptor)
MMR (Mismatch Repair)
MRE11 (Meiotic Recombination 11)
MUS81 (methyl methansulfonate UV sensitive)
NAM (No Apical Meristem)
NBS1 (Nijmegen Breakage Syndrome 1)
NER (Nucleotide Excision Repair)
NHEJ (Nonhomologous end joining)
NSC (NSC120686)
PARP1 (poly(ADP-ribose)polymerase1)
PCD (Programmed Cell Death)
PDF2 (protodermal factor 2)
PLD (Phospholipase D family)
PPRep (prolyl endopeptidase)
PROM1 (Prominin 1)
PTMs (Post-Translational Modifications)

qRT-PCR (quantitative Real-Time Polymerase Chain Reaction)
RAD50 (Double Strand Break Repair Protein)
RAD54 ((Radiation sensitive 54)
RdDM (RNA directed DNA methylation)
RISC (RNA-induced silencing complex)
ROCK2 (Rho Associated Coiled-Coil Containing Protein Kinase 2)
ROS (Reactive Oxygen Species)
RPA (Replication protein A)
RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)
RXRα (Retinoid X Receptor Alpha)
SBSs (single-stranded breaks)
SCGE (Single Cell Gel Electrophoresis)
SMAD3 (SMAD Family Member 3)
SOG1 (Suppressor of gamma response)
TDP1 (Tyrosyl DNA phosphodiesterase 1)
TDP2 (Tyrosyl DNA phosphodiesterase 2)
TOR (Target Of Rapamycin Kinase)
Top1 (topoisomerase 1)
Top2 (topoisomerase 2)
Tub (tubulin)
Ubi (ubiquitin)
53BP1 (p53-binding prote

1. Introduction

1.1. DNA damage response (DDR)

Preservation of genome integrity is essential for all living organisms. However, cells are constantly at risk of DNA damage coming from either endogenous processes (replication, transcription, DNA metabolism) or external cues (UV radiation, high soil salinity, drought, chilling injury, air, and soil pollutants). Apart from DNA metabolism, metabolic by-products such as Reactive Oxygen Species (ROS), join the plethora of endogenous stressors that can cause DNA damage. In human cells, DNA damage induced by spontaneous hydrolysis or ROS arises at a frequency spanning from a few hundred to over 10^5 per cell, according to the type of damage (Bray and West, 2005). To cite some examples from plants, in maize seeds, the estimated number of abasic (apurinic and apyrimidic) sites generated in root tips during the first 20 h of imbibition was equal to 3.75×10^5 per genome and cell. Whole-genome sequencing of *Arabidopsis thaliana* lines obtained from single seed descent after 25–30 generations, revealed a genome-wide average mutation rate around 7×10^{-9} per site per generation; this could be translated as less than one single mutation per generation (Ossowski et al., 2010; Weng et al., 2019). This low error rate due to the replication machinery per single cell is strong evidence of the efficiency with which DNA lesions are recognized and repaired in plant cells. Indeed, even though plants lack mobility as known in animals, they are provided with incredible genomic plasticity. Plant genes and proteins have been considered as processing units with biochemical connections, forming an information-processing system referred to as "perceptron", since plants can select the most suitable options for coping with a changing environment (Scheres & van der Putten, 2017). Within this context, DNA Damage Response (DDR) is among the strategies used by plant cells to safeguard their genome and therefore their growth and development.

1.1.1. Causes of DNA damage and activation of DDR

During their lifespan, plants are continuously exposed to stress conditions that can compromise genome stability, physiological growth, and development. The DNA metabolism itself, both during the replication and repair processes, is among the major causes of genome errors and mutations. In 1953, in describing the structure of the DNA double helix, Watson and Crick wrote, "*It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.*" Since then, understanding the mechanisms through which DNA is copied and faithfully transmitted from one cell to another has been one of the main fields of interest in biology.

Apart from DNA metabolism, metabolic by-products such as ROS can cause DNA damage. This is likely to happen when there is an imbalance between ROS production and removal by scavenger mechanism, as in the case of biotic (pathogen

infection) and abiotic stresses (e.g. drought, salinity, extreme temperature, metal toxicity). The high oxidizing potential of ROS can damage a large variety of molecules, including the electron-rich bases of DNA by causing single and double-stranded breaks (SSBs, DSBs) (Amor et al., 1998; Dizdaroglu et al., 2002; Roldán-Arjona & Ariza, 2009; Yi et al., 2014). One problem is represented also by the fact that ROS can move between the different cellular compartments such as nucleus, cytosol, and organelles (Cimini et al., 2019). Alongside the endogenous stressors, external cues such as solar UV radiation, high soil salinity, drought, chilling injury, air, and soil pollutants (including heavy metals) contribute to impair plant growth and development. Plant DNA damage is also observed upon microbial infection and a family of DNA-damaging effectors was recently identified in plant-pathogenic oomycetes (Camborde et al., 2019).

1.1.2. Conserved DDR features between animals and plants

DDR is defined as a complex signal-transduction network consisting of DNA damage sensors, signal transducers, mediators, and effectors (Yoshiyama et al., 2013), evolutionarily conserved between organisms (**Fig. 1**). To take place properly, this system requires sophisticated regulatory mechanisms. Starting from the detection of a DNA lesion by the sensors, various pathways are activated leading to responses that span from the activation of cell-cycle checkpoints to programmed cell death (PCD) when the repair of the DNA lesion is not possible. Several studies have highlighted the evolutionarily conserved features of the core DDR machinery through eukaryotes, including plants and mammals (Yoshiyama et al., 2013; Nikitaki et al., 2018).

DDR sensors are proteins able to recognize DNA damage, and this in turn activates a series of events (e.g. phosphorylation cascades) that lead to the regulation of downstream processes (e.g. cell cycle checkpoint, DNA repair, programmed cell death) (Petrini & Stracker, 2003). In both animals and plants, the MRN complex, composed of MRE11 (Meiotic Recombination 11), RAD51, and NBS1 (Nijmegen breakage syndrome 1), as well as the RPA (Replication protein A) proteins, constitutes the main DDR sensor (**Fig. 1**). This complex is required for the recognition of strand breaks in pathways involving ATM (ataxia telangiectasia mutated) and ATR (Rad3-related) kinases (Yoshiyama et al., 2013). Specifically, the MRN complex is required for DSBs recognition in a pathway involving ATM (Yoshiyama et al., 2013). ATM and ATR are the main signal transducers of DDR. The role of these transducers is to amplify and transduce signals to downstream effectors. They are responsible for the phosphorylation of proteins such as the histone-variant H2AX (Dickey et al., 2009) which, in the phosphorylated form (γH2AX), acts as a DNA damage signal and recruiter of several proteins to DSB site (Petrini & Stracker, 2003; Yoshiyama et al., 2013).

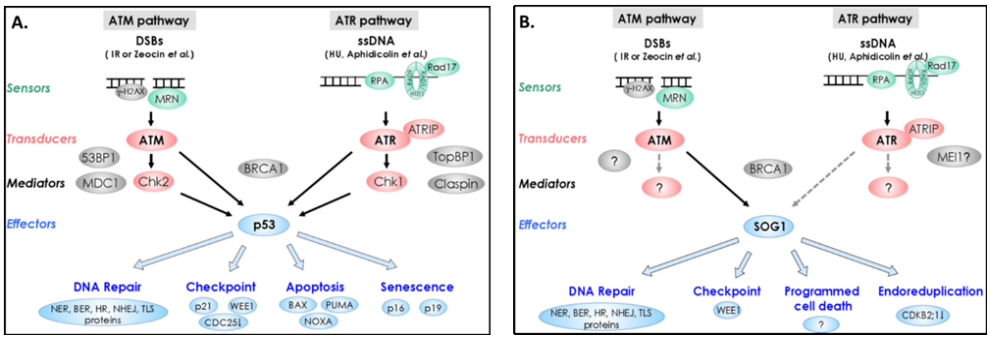

Fig. 1. DNA damage response pathways in animals **(A)** and plants **(B)**. Schematic representation of DNA damage signal through the sensors (green), signal-transducing kinases (red), mediators (gray), and effectors (blue), leading to the activation of downstream pathways. Dashed lines denote hypothetical situations (Yoshiyama et al., 2013).

While ATM is recruited at the DSB sites, ATR responds primarily to lesions associated with DNA replication (Cimprich & Cortez, 2008). In this case, RPA is the sensor binding to single-stranded DNA (ssDNA). Like ATM, ATR initiates a phosphorylation-mediated signal transduction cascade that leads to cell-cycle arrest and repair of DSBs or eventually to apoptosis (Balestrazzi et al., 2011a). In yeast and mammals, the CHK1 (Checkpoint kinase 1) and CHK2 (Checkpoint kinase 2) are the main factors that receive signals from ATR and ATM (Bartek et al., 2001; Chen & Sanchez 2004). Apparently, *A. thaliana* has no CHK1 and CHK2 ortholog. Considering that substrates of CHK1 and CHK2, such as the mediator BRCA1 (Breast cancer susceptibility gene 1), and E2F (E2 promoter binding Factor), are present also in plants (Lafarge, 2003; Inzé & de Veylder, 2006), it is hypothesized that other kinases may work as functional homologs of CHK1 and CHK2 in plants (Yoshiyama et al., 2013).

Mediators are temporal-spatial regulators and activators of the different factors involved in DDR. They work to recruit additional substrates and control their association with damaged DNA (Stewart et al., 2003; Stracker et al., 2009). Several mediators are known in human cells, such as MDC1 (mediator of DNA-damage checkpoint protein 1), 53BP1 (p53-binding protein), BRCA1, TOPBP1 (topoisomerase 2-binding protein 1), and CLASPIN involved in the co-regulation of the ATR pathway (**Fig. 1A**). Differently, the knowledge about mediators in plants is still scanty (**Fig. 1B**).

Signals from transducers activate downstream effectors which then stimulate appropriate responses. The most important effector in animals is the p53 protein, known as a tumor suppressor (Lavin & Kozlov, 2007). In animal cells, p53 decides the fate of the cell after DNA damages, namely cell-cycle arrest and DNA repair or apoptosis (Helton & Chen, 2007). The SOG1 (suppressor of gamma response 1) protein, a component of NAC (NAM, ATAF1/2, and CUC2) family, is the p53 functional homolog in plants (Yoshiyama et al., 2013). SOG1 acts as a DDR key regulator that governs the transcriptional response of DNA damage and coordinates the responses to several stimuli. Like p53, its activity decreases if the cell undergoes cell-cycle arrest and DNA repair or programmed cell death (PCD). Unlike human

cells, plant cells can undergo endoreduplication (**Fig. 1B**), a process in which the nuclear genome is replicated without cell division with a consequent increase of genomic DNA content. It is thus clear that most of the DDR factors are well preserved between animals and plants. However, various key components are unique in plants.

1.1.3. Peculiar DDR features in plants

Although carcinogenesis is not an equally compromising phenomenon in plants, accumulation of DNA damage is still a significant cause for growth inhibition and developmental defects. For example, *A. thaliana* seedlings subjected to DNA damage-inducing agents exhibit a significant loss of biomass (Hartung et al., 2006, 2007). Similarly, endogenous DNA damage derived from deficient DNA repair machinery leads to aberrant organogenesis and development (Wang & Liu, 2006; Cools & De Veylder, 2009; Boltz et al., 2012; Leehy et al., 2013). Therefore, increasing efforts have been dedicated to examining the mechanisms that help plants to cope with DNA damage. Interestingly, although some of the DDR components are conserved across species, the sessile nature of plants may have prompted them to develop a subset of unique DDR regulators, in particular at the level of cell cycle control. Checkpoint control in plants in response to different types of DNA stress is controlled by components that are conserved in other eukaryotes as well as by elements that are plant-specific (**Fig. 2**).

As mentioned before, although the general roles of ATM and ATR kinases are conserved in plants and mammals, some differences exist. While ATM/ATR loss-of-function in mammalian cells leading to enhanced carcinogenesis (Awasthi et al., 2015), in *A. thaliana* plants lacking ATM or ATR no developmental abnormalities had been evidenced in the absence of genotoxic stress (Culligan et al., 2006; Cools & De Veylder, 2009). However, the *atr* mutant displays sensitivity to replication stress-inducing agents such as aphidicolin (DNA polymerase inhibiting drug) and hydroxyurea (HU, deoxynucleoside triphosphate depleting drug), whereas the *atm* mutant is susceptible to DSB-inducing agents such as ionizing radiation and methyl methanesulfonate (MMS) (Garcia et al., 2003; Culligan et al., 2004). ATM is activated in response to DSBs and its activation is related to the MRN complex, as indicated by the fact that γ-H2AX foci are not observed in *rad50* and *mre11* mutants (Amiard et al., 2010). Again, although the loss of any component of the MRN complex results in embryonic lethality in vertebrates, *A. thaliana mre11* and *rad50* mutants are viable and display only hypersensitivity to genotoxic compounds (Gallego & White, 2001; Bundock & Hooykaas, 2002; Gherbi et al., 2001). Moreover, the fact that *mre11* and *rad50* mutants are fully sterile suggests that the corresponding genes play an essential function during meiosis, similar to their role in *Saccharomyces cerevisiae*. Orthologs of subunits of the RPA complex have also been described in plants. Though, differently from yeast and most animal species that possess a single copy of each of the three RPA complex subunits, plants have multiple RPA1, RPA2, and RPA3 subunits. Genetic analysis of the five *Arabidopsis* RPA1 subunits showed that they can be functionally distributed in two groups:

RPA1a, RPA1c, RPA1e, involved in DNA repair and recombination, and RPA1b, RPA1d, responsible for the control of DNA replication in the absence of stress (Aklilu et al., 2014). This data derives from DNA damage sensitivity tests demonstrating that only *rpa1c* and *rpa1e* mutants are sensitive to ionizing radiation and that only *rpa1c* mutants are sensitive to camptothecin which blocks DNA replication by inhibiting DNA topoisomerase I.

Despite the conserved features of ATM and ATR, increasing evidence indicates that how the DNA stress signaling pathways affect the cell cycle seems to be different in plants than in yeast and mammals, which might be an outcome of their sessile lifestyle (Nisa et al., 2019). Unlike mammals, where mutations in checkpoint regulators often result in embryo-lethal phenotypes, the presence of this type of mutations in plants is still compatible with life and drive only to conditional phenotypes. This features the exclusive possibility to isolate the downstream components of the ATM and ATR signaling cascades through genetic approaches. These approaches are useful to identify signaling cascade components that link plant development and environmental stresses to DNA checkpoint control. Moreover, the absence of embryo-lethal phenotypes permits to study the consequences of defective checkpoints over multiple generations. The mechanisms that regulate DNA damage response-dependent cell cycle arrest, are well described in mammals (Harper and Elledge., 2007; Ciccia & Elledge., 2010), mostly because of their relevance in preventing carcinogenesis.

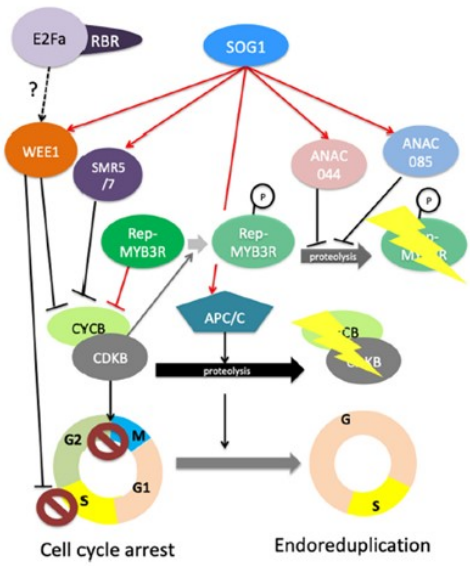

Fig. 2. Overview of the plant DDR. ATM and ATR signaling converge to the SOG1 transcription factor that controls the expression of hundreds of genes involved in cell cycle regulation, cell death control, and DNA repair. E2Fa/RBR complexes also control DNA repair by regulating DNA repair genes and by recruiting RAD51 and BRCA1 at DNA damage sites. The role of E2F/RBR complexes in DDR depends on CYCB1/CDKB and ATM/ATR activity, but the exact molecular mechanisms are unknown (Nisa et al., 2019).

Although the mechanisms of DNA damage detection are well preserved across species, plants seem to lack orthologous genes for most of the mammalian checkpoint signaling components such as p53 and the Chk1/2. Nonetheless, plants have a set of specific proteins to block the cell cycle in response to DNA damage, including SOG1, which is considered as the p53 counterpart (Yoshiyama et al., 2009; Yoshiyama et al., 2014). SOG1 was initially identified in a screen for mutants avoiding the γ-irradiation-induced G2 arrest typical of *uvh1* (UV hypersensitive 1) mutants (Preuss & Britt, 2003). The block of these mutants in G2 is the result of a SOG1-mediated delay of progression into mitosis probably through indirect suppression of M-phase-specific genes such as *CDKB2;1* and *KNOLLE* (Yoshiyama et al., 2009; 2013). The mechanism by which SOG1 stops G2/M has not been yet totally clarified, but some hypotheses exist (**Fig. 2**). The plant-specific CDK inhibitors SIAMESE/SIAMESE-RELATED (SIM/SMR) are regarded as ideal candidates for G2/M arrest. Thirteen SIM/SMR family members have been found in *Arabidopsis* (Yi et al., 2014). These members are divided into two different biochemical groups according to their interaction with the canonical A-type CDKA;1, which operates at the G1/S to mid-M phase, or with the plant-specific B-type CDKB1;1, active at the G2/M transition point (Boudolf et al., 2004; Inagaki & Umeda, 2011).

1.1.4. Suppressor of gamma response 1 (SOG1) as master-regulator of plant DDR

As already mentioned, SOG1 is the p53 functional homolog in plants and hence, the key-regulator of DDR. SOG1 is a transcription factor (TF) belonging to the NAC (NAM, ATAF1/2, and CUC2) family. The protein was first identified in the *Arabidopsis sog1-1* mutant characterized by a missense mutation resulting in the substitution of a highly conserved amino acid residue in the NAC domain (Preuss & Britt, 2003; Yoshiyama et al., 2009). In *Arabidopsis*, more than 100 genes belonging to this family of TFs have been identified and divided into ten major groups, making this protein family one of the largest in plants. Although it was demonstrated that NAC TFs play critical roles in different processes such as environmental stress responses, xylem cell specification, lateral root formation, or the establishment of the shoot apical meristem, the function of most NAC proteins is still uncertain (He et al., 2005; Olsen et al., 2005). Orthologs have been found in most land plants as well as in gymnosperms.

Aside from the well-conserved NAC domain, the C-terminus of SOG1 is characterized by the presence of five serine-glutamine (SQ) motifs, which are preferential targets for phosphorylation by ATM and ATR. The DNA damage-dependent SOG1 hyperphosphorylation detected in wild-type plants disappeared in transgenic plants bearing mutant SOG1, which encodes serine-to-alanine substitutions at all five SQ motifs, suggesting that one or more of the SQ motifs are effective targets for the hyperphosphorylation. Considering that these motifs are conserved in eudicots, monocots, an ancient flowering plant (*Amborella trichopoda*), and gymnosperms, Yoshiyama et al. (2014) proposed that SOG1 had

already been acquired starting from gymnosperms. More sequence data on ferns is necessary to perform detailed analyses to find out when the SOG1 genes appeared in the evolution of plants. The presence of SOG1 in mosses is still a matter of discussion as this protein possesses the conserved NAC domain but a structurally different region for protein-protein interaction (Yoshiyama et al., 2014).

SOG1 is the first TF whose function was associated to DDR in plants. When DSBs occur, SOG1 is activated through ATM-mediated phosphorylation, similarly to animal p53. As a master-regulator, it drives the cell fate towards cell cycle arrest, DNA repair, apoptosis or senescence, and endoreduplication (**Fig. 2**). Despite its similar function to the mammal p53, these transcription factors lack significant amino acid sequence similarity and are examples of divergent proteins. As reported by Hu et al. (2015), SOG1 plays a key role in the replication checkpoint activated by the deficiency of RTEL1 (an ortholog of human Regulator of Telomere Length 1). This implies an involvement not only in DDR but also in the replication checkpoint.

To expand the knowledge of plant-specific DDR, it is necessary to study SOG1 in greater detail. The identification of genes regulated by SOG1 is essential to understand how signal transduction takes place in response to DNA damage. Furthermore, the identification of factors that interact with SOG1 may contribute to understanding how SOG1 activity is regulated. Another aspect to investigate is whether SOG1's functions differ in different species. It is already known that in *A. thaliana* SOG1 plays different functions in different cell types. Addressing these open questions will have important implications for understanding the evolution of DDR in plants, and how plants' specific responses to DNA damage have helped them to overcome stressful environments.

1.1.5. DDR in seed germination

Seeds are propagation vectors of quiescent embryos which, under favorable conditions, germinate to give rise to a new plant. They are part of the array of strategies that plants adapt to ensure their survival. In close interconnection with their role in plant reproduction, seeds enclose the versatility and adaptability of the plant to different types of stresses, including genotoxic stress (Haak et al., 2017). Their vigor, influenced by the environment, time of harvest, and storage conditions, and thus their ability to generate a robust plant, rely on the capacity to safeguard the integrity of their genome (Rajjou et al., 2012; Waterworth et al., 2019). A decrease in nuclear size and chromatin condensation occurs to maintain genome stability during seed maturation, conditions that persist through desiccation, and germination (van Zanten et al., 2011).

In mature, desiccated seeds, the embryo enters in a state of quiescence characterized by reduced metabolism, although transcriptional and post-transcriptional modifications in response to environmental signals remain partially active (Holdsworth et al., 2008). Quiescent orthodox seeds possess a specialized glassy structure and molecules accumulated during maturation that protects the embryo. After imbibition, the reactivation of seed metabolism, leading to ROS

production, requires the *ex novo* synthesis of antioxidant molecules and the activation of DNA repair mechanisms (Kranner et al., 2010).

Molecular studies have recognized the crucial role of DDR and DDR-related pathways in maintaining the viability of the quiescent seed (Rajjou et al., 2012; Sano et al., 2016; Waterworth et al., 2015, 2019). The activation of such pathways influences seed longevity and there is evidence that plants are capable of adapting to environmental changes to promote seed viability over a relatively short timescale (Mondoni et al., 2014). Precisely, cellular survival relies on the coordinated action of several DNA repair pathways. Stress-dependent accumulation of DNA damage and regulation of DDR and DDR-related genes are featured during early seed imbibition in the plant model *A. thaliana* (Waterworth et al., 2010, 2015) but also in the model legume *M. truncatula* (Macovei et al., 2010, 2011; Balestrazzi et al., 2011b; Pagano et al., 2017, 2019). For instance, the expression levels of *OGG1* (8-Oxoguanine glycosylase) and *FPG* (Formamidopyrimidine-DNA glycosylase) genes, belonging to Base Excision Repair (BER) pathway are increased in response to copper- and polyethylene glycol (PEG)-induced stress in *M. truncatula* plantlets (Macovei et al., 2011). Other featured DNA repair components are associated with seed quality. This is the case of DNA ligase IV and the plant-specific DNA ligase VI, both involved in the processing of DSBs and essential to maintaining seed longevity in *A. thaliana* (Waterworth et al., 2010). Genes involved in Nucleotide Excision Repair (NER), such as *TFIIS* (transcription initiation factor S-II), are activated during seed germination in both *M. truncatula* (Macovei et al., 2011) and *A. thaliana* (Grasser et al., 2009). Interestingly, the *TDP1* (Tyrosyl DNA phosphodiesterase 1) and *TopI* (Topoisomerase 1) genes are also upregulated during seed imbibition in *M. truncatula* (Macovei et al., 2010; Balestrazzi et al., 2011b). The crucial role of ATM kinase in maintaining genome stability in seeds has been demonstrated as well (Waterworth et al., 2016). The master kinases ATM and ATR control the cellular response to DNA damage also in seeds through activation of downstream responses at the transcriptional and post-transcriptional levels (**Fig. 3**). ATM controls the advancement of germination in aged seeds, based on the transcriptional control of the cell cycle inhibitor SMR5. Both ATM and ATR influence seed viability but the molecular mechanism is still not well understood; however, it is believed that this is due to the transcriptional DDR which includes hundreds of genes encoding proteins involved in DNA repair, chromatin remodeling, and DNA metabolism (Waterworth et al., 2019). In the early stages of imbibition, seeds exhibit a large and rapid ATM-dependent transcriptional DNA damage response and DNA repair synthesis. It is believed that during seed aging the radicle emergence is delayed resulting in a lag phase that is accompanied by an ATM-mediated delay of cell cycle activation in the root apical meristem and extension of DNA repair activities (**Fig. 3**).

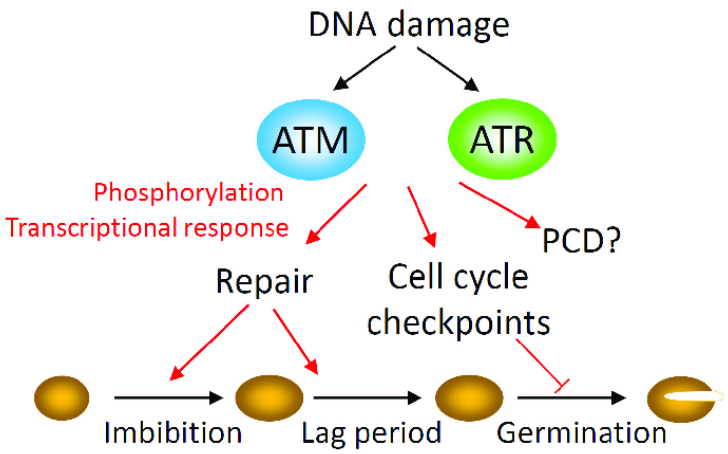

Fig. 3. The DNA damage response (DDR) in seeds. Activation of ATM and ATR following DNA damage leads to the activation of phosphorylation cascades and transcriptional responses coordinating downstream pathways (e.g. DNA repair, cell cycle checkpoints, programmed cell death). In damaged seeds, the inhibition of cell cycle checkpoints leads to delayed or even failed germination (Waterworth et al., 2019).

To conclude, seed quality and vigor are essential features for crop productivity. These properties are responsible for the fast and uniform seed germination. The endogenous metabolism of seeds together with environmental stresses and improper storage conditions enhance cellular and DNA damage and require an extended repair period with a consequent delay of germination that is characteristic of low-vigor seeds (Powell & Matthews, 2012). Within this context, the availability of molecular hallmarks of seed vigor, associated with DDR function, is expected to positively impact seed technology both in public and private sectors (Paparella et al., 2015; Araújo et al., 2016; Macovei et al., 2017).

1.2. A focus on DDR downstream pathways

As mentioned, DDR effectors relate to a series of pathways that comprise DNA repair, cell cycle checkpoints, programmed cell death (PCD), and endoreduplication. Although well-connected among themselves, in the present chapter, these pathways will be described separately, focusing on their implication and recent discoveries within the plant kingdom.

1.2.1. DNA repair pathways

The main DNA repair pathways include photoreactivation (PR) or direct repair (DR), mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), DNA-protein crosslink (DPC) repair, and double-strand break repair (DSBR), which comprises nonhomologous end-joining (NHEJ) and the homologous recombination (HR) (**Fig. 4**). Gaining knowledge about the regulation of DNA repair

systems is essential to understand the biological importance of DNA repair mechanisms in plant resistance to the cytotoxic and mutagenic effects of environmental and endogenous DNA-damaging agents.

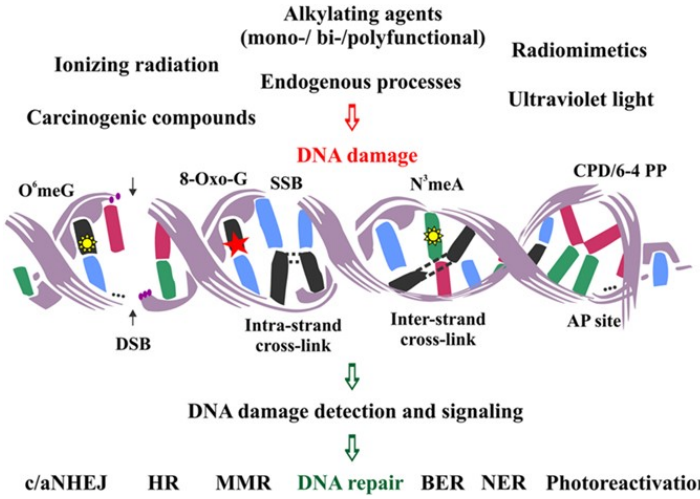

Fig. 4. Diagram of the major DNA lesions induced by different external and endogenous factors, and the types of DNA repair mechanisms activated to remove them from the plant genome (Manova & Gruszka, 2015).

PR is a light-dependent pathway in which the damaged DNA is reverted to a normal configuration through the activity of enzymes called photolyases. These enzymes are highly specialized in recognizing a specific substrate. All photolyases contain the two electron-reduced forms of FAD (FADH¬) as photocatalyst (Sancar, 2003). After specific binding to the DNA lesion, the enzymes remove the damage through the absorption of blue light in the 300-600 nm range (Tuteja et al., 2009).

The MMR mechanism corrects replication and genetic recombination errors that result in poorly matched nucleotide. During replication, the DNA polymerase enzyme first exerts a proof-reading action removing the incorrect nucleotide and then continues the polymerization. Alternatively, the cell is equipped with enzymatic complexes that act at the post-replication level. These complexes can recognize the error and remove it through an endonucleolytic cut on the neo-synthetized strand, thus restabilizing the correct sequence through the action of specific polymerases (Marti et al., 2002).

DSBs repair involves the HR and NHEJ pathways. HR takes place only when two DNA duplexes contain extensive regions of homology, while NHEJ allows the repair of DSBs without using sequence homology. HR is restricted to the S and G2 phases of the cell cycle due to the requirement for the sister chromatid as a template, while NHEJ is active throughout the cell cycle and does not rely on a template (Brandsma & Gent, 2012). The balance between both pathways is essential for genome stability. In NHEJ, the Ku70-Ku80 heterodimers (Ku) are the first players in the repair of DSBs. The lack of homology sequence leads to an error-prone type of repair, frequently resulting in small insertions, deletions, or substitutions at the

break site. On the other side, HR is highly error-free and acts through the activity of RAD51 recombinase (Chapman et al., 2012).

NER pathway is responsible for the repair of major DNA lesions causing more relevant distortion in the helical DNA structure, such as UV-products and bulky covalent adducts (Kunz et al., 2005; Balestrazzi et al., 2011a). While NER mechanism removes the extended DNA lesions, BER is responsible for the repair of single damaged-base residues in DNA (Tuteja et al., 2009). This process is known to remove the most frequent types of damage such as deamination, alkylation, oxidized bases, AP (apurinic and/or apyrimidinic,) sites, and SSBs. Basically, it consists of the damaged base excision by a DNA glycosylase followed by substitution that requires the consecutive action of at least three enzymes, an AP endonuclease, a DNA polymerase, and a DNA ligase (Stivers & Jiang, 2003). Other enzymes with functions in BER include poly(ADP-ribose)polymerase1 (PARP1). PARP1 is involved in the recruitment of additional BER components to the damaged site (Doucet-Chabeaud et al., 2001). Studies in *Arabidopsis thaliana* demonstrated that most of the animal BER proteins have functional and/or structural homologs in plants (Roldán-Arjona & Ariza, 2009).

Another important DNA repair mechanism, although not enough investigated in plants, is the DPC repair. DPC intervenes when proteins become covalently blocked to DNA and, due to their bulky size, these cause DNA impairment. To maintain cell viability and access to important genetic regions, plants have evolved at least three independent pathways to repair this kind of highly toxic lesion (Enderle et al., 2019). DPC is subdivided into four different classes, depending on the presence and type of DNA adjacent breaks (Hacker et al., 2020) (**Fig. 5**).

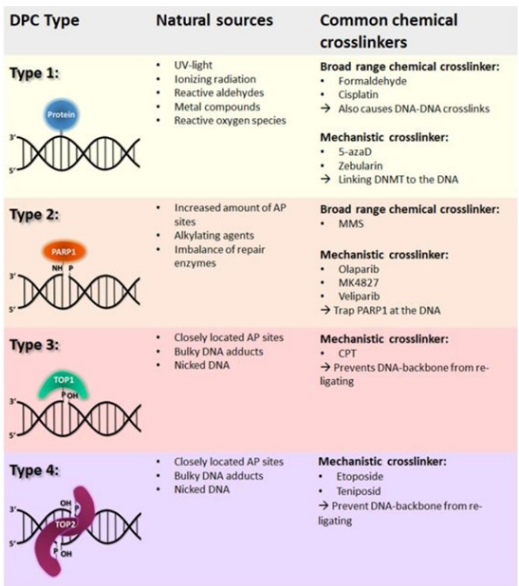

Fig. 5. Schematic representation of different types of DNA-protein crosslink (DPC) (Hacker et al., 2020).

Type 1 DPC is not associated with DNA breaks and the protein is bound to an intact DNA strand. The formation of this adduct is caused by UV light, irradiation (IR), accumulation of ROS, or reactive aldehydes. Relating to the chemical compounds that can be used to induce its formation, formaldehyde, cytosine analogs, like 5-azacytidine (5-azaC) and zebularine, can be cited. Type 2, 3, and 4 consists only of enzymatic DPC. These are spontaneous products caused by failed enzymatic reactions in which covalent DNA-protein intermediates get stabilized. Type 2 DPC are formed next to an AP site, which arises during BER in the course of the activity of either PARP1 or Polβ (Polymerase β) when they remain trapped on the DNA. Type 3 and 4 DPC consist of trapped topoisomerase cleavage complexes, which can occur spontaneously during processes like DNA replication, recombination, transcription, or chromosome segregation. In type 3 DPC, TopI remains trapped to DNA via a tyrosyl-phosphodiester bond at the 3′-end of an SSB. In contrast, in the type 4 DPC, TopII remains trapped with the DNA via two tyrosyl-phosphodiester bonds at the 5′-ends of a DSB (Hacker et al., 2020).

The presence of DPC results in steric hindrance to DNA replication and transcription machinery and can lead to blocked replication forks, chromosomal aberrations, or even cell death if not repaired in time. Cells possess specialized and canonical mechanisms to repair this kind of lesion. Canonical mechanisms mainly include NER and HR, where HR seems to contribute to the repair of bulkier but also small DPC during S- and G2-phase of cell-cycle (Vaz et al., 2017). Zebularine-induced DNA damage in *Arabidopsis* demonstrated the involvement of NER and HR in DPC repair (Pecinka et al., 2009). The mechanisms directly involved in the repair of DPC in plants include endonucleolytic cleavage, proteolytic degradation, and enzymatic hydrolysis (Hacker et al., 2020). The endonucleolytic cleavage of DPC is mainly performed by MUS81 (methyl methanesulfonate UV sensitive). MUS81 belongs to the family of XPF/MUS81 endonucleases and is highly conserved in the eukaryotic kingdom (Interthal & Heyer, 2000). The mechanism of action of MUS81 provides the formation of DSBs which requires the activity of other repair pathways, such as HR or replication fork regression (Regairaz et al., 2011). Another endonuclease that might contribute to DPC repair in plants is MRE11, which is part of the conserved MRN-complex. MRE11 is responsible for 3′-end resection and RAD50 is needed for the induction of HR or long-range tethering of two DNA ends (Gallego et al., 2001). The proteolytic degradation is performed by the metalloprotease WSS1 (weak suppressor of SMT protein 1) which specifically degrades the protein part of a DPC (Stingele et al., 2014). After proteolysis of the DPC, a small peptide remnant remains attached to the DNA and it is thought to be subsequently removed by NER (Stingele et al., 2017). The crosslink bond itself can also be directly resolved by enzymatic hydrolysis. It has been demonstrated that in the animal kingdom, the aforementioned TDP1 enzyme, capable of hydrolyzing 3′-adducts of bulky lesions, is involved in the repair of both Type 3 and Type 2 DPC (Mao et al., 2001). To allow the access of TDP1 in TopIcc (cleavage complex) a preceding partial degradation by the proteasome is needed before TDP1 can hydrolyze the phosphodiester bond (Interthal & Champoux, 2011). The 3′-phosphate

remains at the DNA after the hydrolysis preventing relegation, meaning that further processing of the lesion is required. Studies of the TDP1 enzyme in *Arabidopsis* indicated a similar function in plants as in yeast (Lee et al., 2010a). TDP2 is another specialized enzyme whose function is associated with the hydrolysis of the two phosphotyrosyl bonds between TopII and the DNA. TDP2 prefers to process the 5′-phosphotyrosyl termini of Type 4 DPC and plant homologs were found in *Medicago truncatula* (Confalonieri et al., 2014; Faè et al., 2014).

1.2.1.1. DNA topoisomerases

Replication, transcription, and recombination cause the formation of supercoils in DNA which leads to topological stress. These changes in topology are resolved by members of a ubiquitous family of enzymes known as DNA topoisomerases (Ghilarov & Shkundina, 2012). Topoisomerases bind to DNA through the tyrosine residue of their catalytic site, forming a transient phosphodiester bond, cleaving either one or both strands of the double helix (**Fig. 6**). Among topoisomerases, TopI breaks and binds a single-strand DNA forming a covalent DNA-enzyme intermediate that allows the broken strand to rotate around the intact one until DNA supercoiling is dissipated.

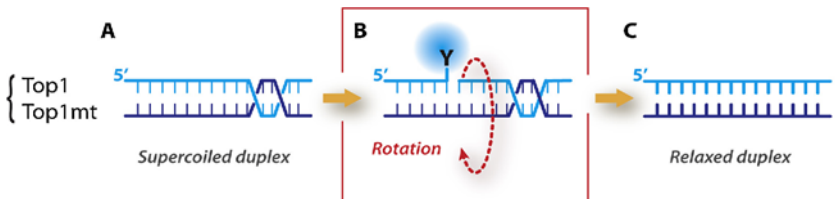

Fig. 6. Schematic representation of the Topoisomerase 1 mechanism of action (Pommier et al., 2014).

Protein sequence information acquired about topoisomerases helped to identify conserved motifs in eukaryotic organisms. All conserved motifs of animal type I topoisomerases are present in the same spatial arrangement in the plant type I topoisomerases, suggesting evolutionary conservation of the overall structure among TopI gene family (Caron & Wang, 1994). Type 1 topoisomerases are monomeric proteins, further divided into two subfamilies, namely, type IA and type IB, based on their mode of action. The members of each family subtype are entirely unrelated in terms of primary amino acid sequence and tertiary structure; however, they perform similar functions. Type IA topoisomerases are attached to the 5' phosphate, while type IB topoisomerases are attached to 3' phosphate of the nicked DNA strand. Importantly, in plants, two *TopI* genes had been identified, namely *TopIα* and *TopIβ* (Balestrazzi et al., 2000), with *TopIβ* being the most active isoform (Balestrazzi et al., 2003). Studies on the expression levels of *TopI* during the cell cycle were reported in tobacco cell cultures, showing an increased expression from G1 phase onwards, attaining a maximum during S phase (Mudgil et al., 2002). The *Top1β* gene activity was studied in carrot protoplasts where it was shown that it was arrested by

starvation in G0/G1 and became activated when protoplasts are induced to re-enter the cell cycle during the G1/S phase (Balestrazzi et al., 2003).

Considering their important function in DNA repair and maintaining genome integrity, topoisomerases are widely studied both in plants and animal systems. Many of the studies regarding topoisomerases were carried out using topoisomerase inhibitors, which, in mammalian cells, are considered also as important medicinal drugs, widely used in anticancer therapies. Molecular pharmacology and structural studies of topoisomerase inhibitors have led to the conceptualization and demonstration of the interfacial inhibitor concept. Indeed, these drugs can trap topoisomerase cleavage complexes by forming ternary complexes with a drug molecule bound at the interface of the enzymes and the cleaved DNA (Pommier & Marchand, 2011). TopI inhibitors reversibly stabilize the enzyme cleavage complexes by inhibiting their relegation. Drugs bind at the interface of the enzyme and DNA break by stacking with the DNA bases immediately flanking the cleavage site, altering the enzyme-DNA interactions, and preventing their dissociation. After the collision with replication forks, both DNA and RNA synthesis convert reversible cleavage complexes into DNA lesions causing DSBs (Pommier et al., 2006). The most studied TopI inhibitor is camptothecin (CPT), isolated from the bark of the Chinese tree *Camptotheca acuminata*, and developed by the US National Cancer Institute (NCI) (Wall & Wani, 1996). CPT intercalates between the DNA base pairs flanking the TopI cleavage site (**Fig. 7**). These TopI cleavage complexes (ccTopI) are reversibly trapped by CPT or other pharmaceutical derivatives. It was seen that high levels of cellular ccTopI can accumulate owe to DNA modifications (Pourquier et al., 2001; Pommier et al., 2003, 2006) or apoptosis (Sordet et al., 2004). The mechanism of CPT targeting has been shown in yeast cells that become resistant to the agent when the TopI gene is removed (Eng et al., 1988). Moreover, vertebrate cell lines, selected for resistance to CPT, presented specific point mutations in the catalytic site of TopI (Pommier et al., 1999). CPT penetrates vertebrate cells readily and targets the TopI enzyme within minutes of exposure. In plants, studies in maize cells shown that CPT was able to inhibit cell growth and induce genomic DNA degradation (Sánchez-Pons & Vicient, 2013). Moreover, several studies in *A. thaliana* have implemented the use of CPT to characterize the function of plant topoisomerases. For instance, the *Arabidopsis top3α-2* mutant was described as having fragmented chromosomes during mitosis and high sensitivity to camptothecin, providing an important role in chromosome segregation of this type IB topoisomerase (Hartung et al., 2008). In another study, CPT was used to target *Arabidopsis* TOP1α resulting in reduced DNA methylation and H3K9me2 levels (Dinh et al., 2014). Considering the effect of CPT on plant growth, initial studies have shown that the addition of concentrations higher than 50 nM resulted in the abortion of both roots and shoots in wild type *Arabidopsis* plants at the seedling stages, although the seedlings of the *top1α-1* mutant presented a reduced sensitivity to the treatment (Takahashi et al., 2002).

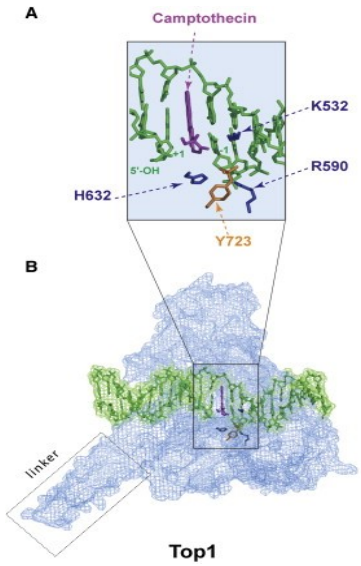

Fig. 7. Mechanism of camptothecin (CPT) binding to topoisomerase I. **(A)** Intercalation of CPT in the DNA break between the base pairs flanking the TopI cleavage complex. **(B)** Overview of the TopI-DNA cleavage complex. TopI is shown in blue, DNA is shown in green, and CPT is shown in purple (Pommier et al., 2010).

Hence, CPT is a very important tool to explore replication-mediated (and transcription-mediated) DNA damage in various organisms, providing a powerful way to study the genetic factors that are implicated in checkpoint regulation and DNA repair in response to TopI-mediated DNA damage (Pommier et al., 2006).

1.2.1.2. Tyrosyl DNA phosphodiesterase 1

The repair of trapped TopI-DNA covalent complexes is mediated by Tyrosyl DNA phosphodiesterase 1 (TDP1). This enzyme catalyzes the hydrolysis of the covalent linkage between the catalytic tyrosine residue of topoisomerase I and the 3′ end of a DNA phosphodiester bond. TDP1 protein belongs to the phospholipase D family (PLD), is characterized by the presence of two catalytic HKD (histidine, lysine, aspartic acid) motifs (Interthal et al., 2001). Inhibition of TDP1 enzyme can provide a convenient approach in exacerbating the sensitivity of cells to CPT (Kawale and Povirk, 2018). By releasing the tyrosyl-linked covalent topoisomerase peptides from the DNA, TDP1 enzymes accompany the activation of the repair pathway. Thus, TDP1 helps in rescuing the genome from the threat of atypical relaxation brought about due to aberrant topoisomerase activity.

The TDP1 function was first identified in the yeast *Saccharomyces cerevisiae* and shown to repair TopI-covalent complexes (Pouliot et al., 1999). TDP1 genetic inactivation had been shown to confer hypersensitivity to CPT both in *S. cerevisiae* and human cells (Vance & Wilson, 2002; Interthal et al., 2005). In human cells, it was demonstrated that TDP1 hydrolyses the 3’ end of tyrosine residues in two sequential reactions, as shown in **Fig. 8**. It can also resolve the 5’end albeit with poor

efficiency (Nitiss et al., 2006). TDP1 can process 3' peptides ranging from one to more than 100 residues and it induces the digestion or denaturation of TopI (Debéthune et al., 2002). TDP1 can also hydrolyze a wide range of physiological and pharmacological 3' blocking lesions. For example, it hydrolyses 3'-deoxyribose lesions resulting from base alkylation after AP lyase processing and the hydrolyzation of synthetic DNA adducts such as biotin and fluorophores (Murai et al., 2012).

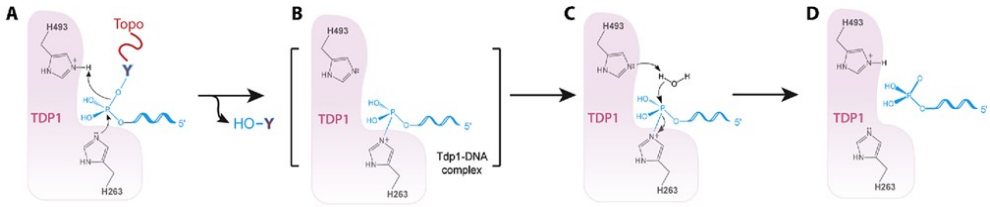

Fig. 8. The TDP1 catalytic mode of action as described in human cells. (A) Nucleophilic attack of the phosphodiester backbone by the imidazole N2 atom of H263. H493 donates a proton to the tyrosyl moiety of the leaving group. (B) Phosphohistidine covalent intermediate. (C) Second nucleophilic attack via an activated water molecule by H493. (D) Generation of a final 3'-phosphate product and free TDP1 (Pommier et al., 2014).

When the crystal structure of the human TDP1 protein (hTDP1) was solved in 2002, it was confirmed that the two-step catalytic mechanism (specific for the PLD family) is followed with a unique approach since it possesses a chemically asymmetric active site where H263 acts as a nucleophile in the first step reaction (Davies et al., 2002). To briefly summarize the catalytic mechanism, the first step consists of a nucleophilic attack of the Top1-DNA phosphotyrosyl bond by H263 residue (HKD motif 1), resulting in a transient covalent phosphoamide bond. Subsequently, the H493 residue (HKD motif 2) hydrolyzes the covalent intermediate using an activated water molecule and generating a product with a 3' phosphate end that will be further processed by the endogenous DNA repair machinery (Pommier et al., 2006). Though the course of time, TDP1 had been extensively studied in human cells due to its implication in cancer progression (Dexheimer et al., 2008; Perego et al., 2012). This is based on the idea that TDP1 is a target for several DDR (DNA damage repair) kinases (Zhou et al., 2005), involved in the activation of cell cycle checkpoints, apoptosis, and DNA repair pathways (Jackson & Bartek, 2009). For instance, the 3'phosphatase activity is carried out by PNKP (polynucleotide kinase 3'-phosphatase) (Jilani et al., 1999) forming a complex with TDP1, and other DNA repair enzymes such as XRCC1 (Excision repair cross complementation group 1), PARP1, Polβ, and Lig3 (Ligase III) (Plo et al., 2003). Orthologues of these human genes were also functionally characterized in *Arabidopsis* (Waterworth et al., 2009; Kim et al., 2012; Martínez-Macías et al., 2013; Li et al., 2015; Spampinato, 2017) but their specific mechanism are not yet completely understood. The N-terminus regulatory domain of TDP1 has been shown to directly bind the catalytic domain of PARP1 in human cells (Das et al., 2014). PARylation stabilizes TDP1 in response to ccTopI-induced DNA damage, and recruits both TDP1 and XRCC1 to the DNA

damage sites. In this way, the N-terminal domain acts as a molecular switch that determines whether ccTopI are repaired by TDP1 when PARP1 is activated or by other alternative endonuclease pathways (Das et al., 2014).

As in the case of topoisomerase I, two isoforms of TDP1 gene (TDP1α and TDP1β) had been identified in plants (Macovei et al., 2010). The two genes, first found in *M. truncatula*, contain the two HKD motifs essential for the catalytic activity of the enzymes. TDP1 was investigated in *A. thaliana*, where a *tdp1* mutant was shown to be sensitive to vanadate and CPT (Lee et al., 2010a; Kim et al., 2012). This loss-of-function mutation resulted in a dwarf phenotype with developmental defects and reduced fertility. Despite the low level of sequence conservation with hTdp1, both components of the active site and those of the DNA-binding groove are present in the plant TDP1 proteins. A unique feature of the Tdp1β protein is the presence of a HIRAN (HIP116, Rad5p, N-terminal) domain flanked by the two HKD catalytic sites (Macovei et al., 2010). *M. truncatula MtTdp1α*-depleted plants revealed different levels of transcriptional modulation in genes involved in DNA damage sensing, DNA repair, and chromatin remodeling. Up-regulation of senescence-associated genes and telomere shortening was observed. Because the *MtTdp1α*-depleted cells showed altered nucleolar architecture, it is supposed that the gene may be involved in the nucleolar checkpoint (Donà et al., 2013). Additionally, even if the *MtTPD1β* gene resulted in up-regulated in the *MtTDP1α*-depleted plants, this was not sufficient to rescue the altered phenotype, suggesting that the two genes do not have an overlapping function. This is supported also by the different expression patterns of the two genes in response to several types of stresses, indicating that the *TDP1β* had an immediate response while *TDP1α* is activated later on (Sabatini et al., 2017). It has been hypothesized that this might be related to the presence of the HIRAN domain, previously predicted to function as a DNA-binding domain that may recognize several features associated with DNA damage and stalled replication forks (Iyer et al., 2006).

Similar to the role of CPT in the study of TopI functions, the use of TDP1 inhibitors have the potential to assist in the characterization of TDP1 multilevel activities. The strongest inhibitors of the hTdp1 so far identified are classified as Tdp1 phosphotyrosine substrate mimetics since they share the same structural features of the natural phosphotyrosine substrate (Huang et al., 2011). The NSC120686 (2-chloro-6-fluorobenzaldehyde 9H-fluoren-9-ylidenehydrazone) compound has been identified by Weidlich and colleagues (2010) as a pharmacophore able to inhibit hTdp1 activity. The biological effects of NSC120686 had been tested in different human ovarian carcinoma cell lines selected for resistance to the CPT-derivative gimatecan. In these lines, increased *hTdp1* gene expression was registered, confirming the involvement of Tdp1 in the cell response to the treatment (Perego et al., 2012). The effectiveness of a combinational therapy including hTdp1 inhibitors and TopI inhibitors was also tested in human carcinogenic lines (Al-Keilani, 2013). The NSC120686 molecule was supplied to the malignant glioma cell line U87 in the presence/absence of different topoisomerase inhibitors. When delivered alone, the NSC120686 treatment revealed strong dose-dependent toxicity levels whereas no significant correlations were

observed between the *hTdp1* gene expression level and cell resistance to the inhibitor. On the other hand, NSC120686 treatments of *M. truncatula* calli resulted in similar effects as the *MtTdp1α*-depletion when considering cell growth inhibition and gene expression profiles (Macovei et al., 2018).

1.2.2. Cell cycle regulation/checkpoints

The cell cycle is a process controlled by a series of events that eventually leads to the reproduction of two daughter cells. The cell cycle is subjected to regulation by environmental cues, such as hormones, nutrients, light, temperature, and developmental cues. It is also a way to protect the cell if DNA damage is detected through the stopping or slowdown of cell proliferation. In plants, as in all eukaryotes, the four basic phases of the mitotic cell cycle are conserved. The widely conserved cyclin-dependent kinases (CDKs) and their cyclin (Cyc) partners are the driving forces of cell cycle progression regulating mitosis (M), cytokinesis, postmitotic interphase (G1), DNA synthetic phase (S) and post-synthetic interphase (G2) (Wang et al., 2004). In plants, CDK/cyc complexes activate the retinoblastoma-related protein (RBR) through its phosphorylation. RBR activates a set of genes that are regulated by the E2F/DP transcription factor and are necessary for S-phase entry and DNA replication. In G2 phase, the activity of CDK/cyc complexes induces entry into mitosis. Degradation of mitotic cyclins and inhibition of kinase complexes permit exit from mitosis (Francis, 2007).

The major effectors that control the cell cycle are the serine/threonine cyclin-dependent kinases (CDKs). Specifically, CDKs bind one or more cyclins, a family of proteins involved in the control of the cell cycle. Due to extracellular and intracellular signals, they modulate gene transcription and cell division (Malumbres, 2014). Two classes of CDKs, CDKA, and CDKB, have been found in plants. CDKBs are plant-specific kinases and comprise four members divided into two subfamilies, CDKB1 and CDKB2. CDKB1s are involved in the control of M phase (Nowack et al., 2012) whereas CDKB2 is associated with the progression from G2 to M (Yoshiyama et al., 2013). CDKA is of central importance in controlling both the G1-S and G2-M transitions. It was seen that the expression of the CDKA gene is crucial during root stem cell function. Indeed, in the *A. thaliana cdka* mutants, the growth of both primary and secondary roots has been almost completely stopped (Nowack et al., 2012).

Cyclins (Cyc) are abundant proteins in both animal and plant kingdom where 10 and 13 classes of cyclins were identified in *Arabidopsis* and human genomes. Five types (A, B, C, H, and L) are common to both species. Cyclins D are present in animals and plants, but no affinity was shown in phylogenetic analysis (Wang et al., 2004). Three classes, G1/S cyclins, S cyclins, and M cyclins, are directly involved in the control of cell cycle events. The fourth class, the G1 cyclins, controls the entry into the cell cycle in response to extracellular growth factors or mitogens. During the G1 phase, G1 cyclins (belonging to the cyclin D family) are stimulated by growth factors. In the G1/S phase, G1/S cyclins (E family) drive the cell in proceeding to DNA synthesis even if the growth factors are withdrawn. During G2, the A-type

cyclins are degraded by ubiquitin-mediated proteolysis, whereas B-type cyclins are actively synthesized (Yang, 2012). Analysis of the completed genome sequence of *Arabidopsis* reveals a total of 10 genes belonging to the CycD family. There are a single CycD1 gene, three genes in the CycD2, and three CycD3 genes. Also, three cyclin genes do not lie in these groups and may arise from separate CycD classes with single members. Both CycD2 and CycD3 interact with CDKA and are stimulated by sucrose. After sucrose removal, the CycD3 protein disappears rapidly while CycD2 protein is relatively stable and appears to be regulated post-translationally or by protein associations (Oakenfull et al., 2002). Plants lack the E-type cyclins which are involved in G1/S checkpoint control in animals; although, it was reported that CycA3;2 can control cell division and differentiation (Yu et al., 2003), functions that are analogous to those of cyclin E in animals. In plants, it was also shown that CycB1;1 is specifically activated after DNA damage, and this activation is directly controlled by SOG1. Moreover, RAD51 is a substrate of CDKB1-CycB1 complexes and they are involved in blocking the cell-cycle activity after DNA damage and in mediating HR (Weimer et al., 2016).

Another master regulator, present in both plants and animals, is TOR (Target of Rapamycin). In plants and animals, TOR signaling regulates conserved and specialized cellular and developmental processes (Shi et al., 2018). In *Arabidopsis*, TOR expression is strongly induced in meristematic regions and associates with photosynthesis-derived glucose energy signals specifically dedicated to controlling the proliferation of stem cells (Xiong et al., 2013; Li et al., 2017). TOR directly phosphorylates and activates the transcription factor E2Fa, playing an essential role mainly in root meristem regulation. Moreover, glucose-TOR signaling governs the transcriptional reprogramming of an impressive amount of genes involved in central and secondary metabolism, cell cycle, transcription, signaling, transport, and protein folding (Van Leene et al., 2019).

1.2.3. Programmed cell death (PCD)

PCD is a very important mechanism to protect organisms from accumulating mutations. The capacity of specific cells to activate PCD emerged very early during evolution, as different genes and molecules engaged in PCD are common to distant phyla, belonging to different kingdoms. Intriguingly, although PCD had been initially related to eukaryotic multicellular organisms, it also takes place in eukaryotic unicellular organisms and in bacteria, where the death of a single cellular organism correlates with the multifunctionality of the colony (Ameisen, 2004). Generally, the term PCD presupposes a "genetically programmed" process activated by cells in response to “appropriate” stimuli as opposed to "accidental cell death” caused by severe insults (Galluzzi et al., 2015). Currently, mainly three types of animal PCD are recognized: apoptosis, autophagic cell death, and necrosis. Although, it must be considered that agents that cause apoptosis can also cause necrosis, and the distinction between the two forms of cell death in cultures depends on the severity of the insult (Lennon et al., 1991; McCabe et al., 1997; O’Brien et al., 1998; Mammone et al., 2000). Among the three, apoptosis is probably the best-

characterized typology of PCD. It is defined by specific morphological markers, such as cell shrinkage, chromatin condensation, and nuclear destruction, followed by the breakup of the cell into fragments known as "apoptotic bodies." This definition of cell death attributed to animals is only partially applicable to plant cells. In fact, in plants the cells and their nuclei are not always fragmented into separate bodies and, the generated fragments are never engulfed by adjacent cells. These differences are due to the presence of specific cell compartments and to the absence of external phagocytosis events (Greenberg, 1996; Pennell & Lamb, 1997). In plants, this type of death is referred to as apoptotic-like PCD or simply PCD. The PCD morphotype commonly observed in plant cell cultures under abiotic stress is characterized by the presence of cytoplasm shrinkage, condensation of chromatin, and rupture of nuclei (De Pinto et al., 2012; Reape & McCabe, 2008).

Based on plant cytological events, the point of no return, ensuring the death of a plant cell, is that in which not only mitochondria but also chloroplasts are involved (van Doorn, 2005). From a biochemical point of view, there is evidence that some plant proteases digest the substrates of caspases, master regulators of animal PCD, and that plant PCD is inhibited by caspase inhibitors. Currently, in plants some groups of caspase-like, the metacaspase I e II are known (Woltering, 2004). The plant caspase-like enzymes share some similitude with animal caspases in terms of their activities. In fact, they both are involved in PCD, their proteolytic cutting occurs always on aspartate residues and they are not affected by generic inhibitors of cysteine and serine proteases. In mammals, PCD is activated during physiological cell development but also in severely damaged cells, thus reducing the risk of accumulating cells with a damaged genome (Borges et al., 2008). This is also true for plants. Plants activate PCD in a series of physiological processes such as embryogenesis or the emergence of new plants during mature seed germination (Domínguez & Cejudo, 2014). Selective removal of cells by PCD is also required to prevent the propagation of negative genetic characteristics in progeny such as in the self-incompatibility mechanism used by several plant species to hinder inbreeding problems, thus promote outbreeding. This mechanism consists of PCD activation in the pollen tube to evade self-pollination in plants with bisexual flowers (Bosch & Franklin-Tong, 2008).

It is thus clear that PCD is involved in a plethora of processes spanning from common and specific organ shaping and morphological adaptive responses to defense strategies activated against abiotic and biotic injuries.

1.2.4. Endoreplication

In addition to stimulating cell cycle arrest, DNA repair, or cell death, cell cycle checkpoints can activate the endocycle as a DNA stress response. Activation of endoreplication has been observed when plants accumulate DNA DSBs (Adachi et al., 2011). Contrary to the induction of cell death, ATM and ATR kinases play a redundant role in regulating endoreplication. Transcriptome analysis demonstrated that the SOG1-dependent onset of endoreplication following DNA stress, take place together with a suppression of G2/M-specific cell cycle genes (including mitosis-

specific cyclins) and activation of cell cycle-inhibitory genes such as SIM, SMR1, SMR5, and WEE1 (Adachi et al., 2011). The reasons why plants activate endoreplication in response to DNA lesions remain to be elucidated. It is assumed that an endoreplication cell only rarely proceeds to cell division, thus preventing the transmission of DNA errors to tissues or offspring. In addition to preventing the spread of mutations, endoreplication could explain the persistent growth in the absence of cell division. This is true in UVB stress responses, where the initiation of endocycle is likely mediated by the atypical transcription factor DP-E2F-LIKE1/E2Fe (DEL1/E2Fe). Six E2F transcription factors, divided into two subclasses, were identified in the model plant *A. thaliana*. Transcription factors such as E2Fa, E2Fb, and E2Fc, play an essential role in the transcriptional control of processes associated with replication and chromatin (Lammens et al., 2009). Other E2F factors include DEL1/E2Fe, DEL2/E2Fd, and DEL3/E2Ff, acting as transcriptional repressors that link cell cycle control with cell differentiation. In the case of UVB stress, the specific expression of DEL1/E2Fe is suppressed and this in turn determines the transcriptional activation of its CCS52A2 target gene, which is an endocycle activator. The endocycle-driven cell enlargement might help plants adapt to UVB stress. For example, an increase in the endoploidy might compensate for a drop-in leaf cell number. Indeed, DEL1/E2Fe-overexpressing plants with low DNA ploidy levels are hypersensitive to UVB while knockout plants with high endoploidy are more tolerant (Radziejwoski et al., 2011). Beyond the repression of endocycle onset, DEL1/E2Fe represses the promoter activity of the gene encoding for PHR1 photolyase, a photoreactivation enzyme involved in the removal of cyclobutane pyrimidine dimers, which are the primary lesions induced by UVB, that block the replication fork. Thus, the endocycle onset is accompanied by increased DNA repair activity, which might be essential to allow the cell to perform endoreplication, possible when DEL1/E2Fe is repressed or lost.

1.3. Chromatin remodeling in the context of DDR

A critical aspect to consider when studying DDR is to ensure that the DNA repair complexes and checkpoint proteins can have access to the DNA damage sites. Although the basic principles of DDR are phylogenetically similar, it must be taken into account that eukaryotic DNA is organized in compact and dynamic chromatin structures, with nucleosomes as basic units followed by multiple high-order levels of organization. Specifically, chromatin structure is constituted by the nucleosomes in which a segment of DNA (146 bp) is wrapped around eight histone proteins forming the so-called octamers. Each octamer is made up of two molecules of the four histone types H2A, H2B, H3, and H4. A histone H1, not part of the nucleosome itself, stabilizes the internucleosomal DNA (Donà & Mittelsten Scheid, 2015). The electrostatic interactions between the opposite charges of the DNA and the histones mediate the association between them. These associations may be weakened or strengthened through the introduction of specific histone modifications, leading to chromatin relaxation or condensation (Murr et al., 2007). The best-known chromatin remodeling processes include the activity of ATP-consuming chromatin remodellers

that shift or remove nucleosomes, the replacement of histones with histone variants, and the introduction of post-translational modifications in the histone subunits. These processes affect DNA accessibility, thus influencing the activity of DNA repair machinery.

1.3.1. Chromatin modifications involved in DDR

Chromatin remodeling is among the evolutionarily conserved pathways that contribute to efficient DNA repair in eukaryotes (Gursoy-Yuzugullu et al., 2016). Histone acetylation, methylation, phosphorylation, and ubiquitination are among the most widely studied post-translational modifications (PTMs). These chemical modifications are added or removed from histone amino acid residues by specific complexes and strongly influence chromatin architecture, nucleosomal positioning, and access to DNA during many processes, including cell cycle regulation, cell proliferation, apoptosis, DNA replication, transcription, and repair (Donà & Mittelsten Scheid, 2015; Zhang et al., 2017). The acetylation of specific lysine residues on the N-terminal of H3 and H4 tails that extend out from the nucleosome, adds a negative charge inducing repulsion of the wrapped DNA and thus, chromatin relaxation (Murr et al., 2007). The dynamic acetylation/deacetylation process is regulated by the Histone Acetyl Transferase (HAT) and Histone Deacetylase (HDAC) complexes, that promote chromatin decondensation and condensation, respectively. A genome-wide study of histone acetylation in maize under stress conditions revealed the hyperacetylation is correlated with slower cell cycle progression and subsequent growth inhibition (Zhao et al., 2014). In *Arabidopsis*, the HAT complexes HAM1 and HAM2 (histone acetyltransferase of the MYST family, orthologs of the mammalian TIP60) are known for their involvement in stress response and developmental processes, while MSI4, a WD-40 repeat-containing protein, part of HAM complexes, was shown to be phosphorylated by ATM and ATR in the context of DDR (Xiao et al., 2013; Roitinger et al., 2015). Links between DDR and histone acetylation has been observed in other plant systems as well, including *M. truncatula* and *Petunia hybrida*. For example, Pagano et al. (2017, 2019, 2020), demonstrated that TRRAP (transformation/transcription domain associated protein) gene, encoding a transcriptional activator required for the assembly of different HAT complexes involved in DNA repair, was upregulated during seed germination. In animal cells, TRRAP has been proposed as a shared element between DNA repair and chromatin remodeling (Murr et al., 2006).

Although the mechanisms that determine histone acetylation and therefore chromatin decondensation are clear, the relationships between histone modification and DDR show different and peculiar facets. For example, it has been demonstrated that X-ray treatments of *Arabidopsis* plants result in H3 histone acetylation and H4 histone deacetylation (Drury et al., 2012) while the opposite effect was induced by γ-rays in *Triticum aestivum* plants (Raut & Sainis, 2012).

In addition to the histone acetylation/deacetylation processes, histone methylation/demethylation by methylase and demethylase can modify histones with opposite effects based on the amino acid residues that are methylated. Cytosine

methylation of DNA is another epigenetic modification that influences gene expression and silences the activity of transposable elements (TE). In plants, cytosine methylation is found in three distinct sequence contexts: CG, CHG, and CHH (where H stands for C, A, or T) (Law & Jacobsen, 2010). The RNA-directed DNA methylation (RdDM) pathway is responsible for all types of de novo cytosine methylation, especially within small TEs or at the boundaries of large TEs. *Arabidopsis* mutants of components belonging to the RdDM pathway were shown to be viable and fertile. This is not true in crops with large genomes, where TEs are also abundant in gene-rich chromosome arms. To date, knowledge about the associations between RdDM and agronomic traits is still scanty (Kawakatsu & Ecker, 2019).

1.3.2. DNA damage signaling and repair in the context of chromatin

The activation of eukaryotic DDR to sense or suppress DNA damage and allow DNA repair must be considered in the context of chromatin (**Fig. 9**) since chromatin mobility contributes to, and equally jeopardize, genome stability (Dion & Gasser, 2013). Thus, understanding chromatin dynamics is crucial in regulating DDR in eukaryotes (Nair et al., 2017). Both ATM and ATR kinases are activated by chromatin perturbations for the actuation of DDR and its downstream pathways, such as DNA repair, cell cycle checkpoints, cell death, and senescence (Bakkenist & Kastan, 2015).

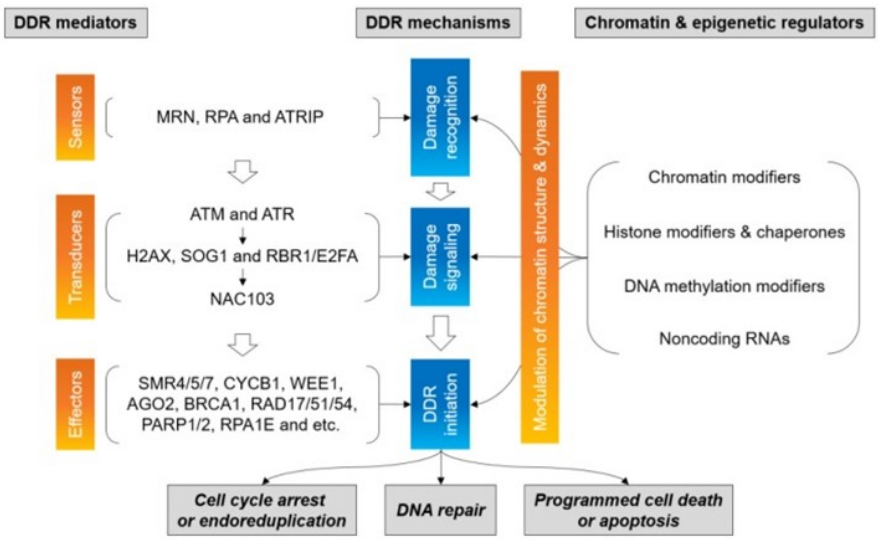

Fig. 9. Signaling pathway of DDR in the context of chromatin. Chromatin structure and dynamics are regulated by chromatin remodeling and epigenetic modifications to mediate DNA damage recognition, signaling, and repair (Kim, 2019).

The recognition of nucleosomes by DDR sensors and transducers induce or mediate DNA damage signaling and repair within the chromatin (Agarwal & Miller, 2016). The MRN complex senses the DNA ends and chromatin at DSB sites, whereas the ATM bound to the MRN is induced by DNA ends and acts as an initial signal transducer (Dupré et al., 2006). Conversely, ATR is exclusively activated when the ssDNA and ssDNA/dsDNA terminals at the DNA damage sites are recognized by DDR sensors, namely RPA and ATRIP (Cortez et al., 2001; Zou & Elledge, 2003). Once activated, ATM and ATR phosphorylate and/or regulate the secondary downstream transducers H2AX, SOG1, and RBR1(RETINOBLASTOMA RELATED1)/E2FA (Kim, 2019). As the key DDR player, SOG1 phosphorylation leads to transcription of the tertiary downstream transducer NAC103, and the effectors SMR4/5/7, CycB1, WEE1, AGO2 (Argonaute 2), BRCA1, RAD17/51/54, PARP1/2, RPA1E (Ogita et al., 2018). During DDR, chromatin remodeling rearranges nucleosomes and higher-order chromatin structures. The occurrence of chromatin remodeling as well as the binding of specific chromatin proteins, such as H2AX and H2AZ at DNA damage sites, may influence the damage recognition, signaling, and repair processes (Widlak et al., 2006; Rossetto et al., 2010). Increasing evidence demonstrates how chromatin remodellers modulate DNA damage signaling and repair in eukaryotes. The human NuRD chromatin-remodeling complex accumulates within DSB-flanking chromatin and coordinates proper signaling and repair of DSBs. This accumulation allows the histone ubiquitylation at DSB sites to aid the accumulation of BRCA1 and the E3 ubiquitin ligase RNF168 (Smeenk et al., 2010). The interaction between the chromatin remodeler SMARCA5/SNF2H and RNF168 in DNA damage- and PARP-dependent manner is required for the RNF168-dependent signaling of DSBs to trigger H2AX ubiquitylation and BRCA1 accumulation at DSB sites (Smeenk et al., 2013). The yeast INO80 (Inositol-requiring mutant80) complex binds H2AX at DSB sites and affects the dynamics of both H2AX- and H2AZ-containing nucleosomes surrounding the DSBs for signaling and repair (Morrison, 2017). In plants, six major subfamilies of ATP-dependent chromatin remodellers (ACRs) have been recognized: INO80, SWI2/SNF2 (SWitch2/Sucrose NonFermentable 2), CHD1 (Chromodomain helicase DNA 1), ISWI (Imitation SWitch), RAD54 (Radiation sensitive 54), and SNF2 (Donà & Mittelsten, 2015; Han et al., 2015). Among them, the INO80, SWR1, and RAD54 chromatin remodeling complexes play a pivotal role in plant DDR.

1.3.3. Chromatin remodeling and seed germination

Chromatin remodeling, required to allow the access of DNA repair enzymes at the damaged sites, is part of the versatile seed repair response. This aspect is still poorly explored in plants, although it is already known that major transcriptional changes and chromatin rearrangements mark the developmental transition from dry seed to germinated seed (Tanaka et al., 2008; Boychev et al., 2014; Wang et al., 2016a). The molecular events that characterize early seed germination represent an

intriguing model for exploring the link between chromatin remodeling and DNA repair in plants.

Crucial players in chromatin remodeling are HDACs that remove acetyl groups from histones, resulting in chromatin condensation and consequently gene silencing (Grandperret et al., 2014) and HATs that transfer acetyl groups to the lysine residues at the N-terminal region of histones and interact with transcription factors, promoting gene expression (Boychev et al., 2014). The association of specific HDACs to the molecular networks underlying seed germination and early seedling development has been described as in the case of HDA19/HD1 which takes part in the transcriptional repression of the AtABI3 (Abscisic acid Insensitive) gene promoter during early seedling development in *Arabidopsis*. Therefore, the ABA (abscisic acid) signaling pathway is suppressed, granting the establishment of young seedlings (Ryu et al., 2014). A significant reduction in the nucleus size and/or a notable chromatin condensation have been observed in dehydrating *Arabidopsis* seeds, indicating an adaptive response to dehydration and drought stress also at the chromatin level (van Zanten et al., 2011; Waterworth et al., 2015).

To address chromatin remodeling in the context of seed germination and seedling development, Pagano et al. (2017) investigated the *TRRAP* gene in *M. truncatula*, codifying for a transcriptional adaptor known in humans for its role in the recruitment of HAT complexes to chromatin during DNA repair. It has been speculated that DDR components might preferentially recruit the TRRAP-containing HAT complexes at the DSBs sites. DSBs-induced DDR networks probably result in chromatin alterations, such as the presentation of methylated lysine 79 of histone H3, that facilitate the binding of TRRAP-containing HAT complexes at the damaged site (Huyen et al., 2004). In *M. truncatula*, the biological significance of chromatin rearrangements has been investigated through the induction of genotoxic stress resulting from the administration of the HDAC inhibitors like trichostatin A (TSA) and sodium butyrate (NaB) (Pagano et al., 2017, 2019). The *TRRAP* gene, along with its predicted interacting partners HAM2 (Histone Acetyltransferase of the MYST family) and ADA2A (Transcriptional Adaptor), displayed tissue- and dose-dependent fluctuations in transcript levels. Furthermore, correlation analyses suggested a new putative link between DNA repair and chromatin remodeling involving OGG1 and TRRAP genes, in the context of seed germination. Intriguing correlations also connected DNA repair and chromatin remodeling with antioxidant players and proliferation markers. Alongside processes that favor chromatin decondensation, other mechanisms are activated to promote chromatin condensation in seed-specific contexts. Similarly, in *P. hybrida* seedlings exposed to NaB enhanced expression of HAT/HDAC genes along with repression of genes involved in DNA repair was observed, suggesting the involvement of chromatin modification- and DNA repair-associated pathways in response to NaB exposure during seedling development (Pagano et al., 2020). Moreover, a metabolomic analysis carried out in *M. truncatula* seedlings exposed to NaB, revealed significant changes in seed nucleotide, amino acid, lipid, and carbohydrate metabolism along with the up-regulation of antioxidant, DNA repair, and polyamine biosynthesis genes (Pagano et al., 2019). Significant changes in N^1-

methyladenosine and N^1-methylguanine metabolites resulted associated with the up-regulation of ALKBH1 (alkylation repair homolog) gene. These analyses provided a comprehensive picture of metabolic changes happening in seeds challenged with this specific HDAC inhibitor.

1.4. microRNAs: master-regulators of gene expression

To take place properly, DDR system requires sophisticated regulatory mechanisms. In this context, microRNAs (miRNAs) may contribute to the implementation of this essential mechanism. Although more pieces of information exist in the mammalian system, this specific topic is substantially less investigated in plants, thus requiring more dedicated attention. This chapter will provide a general overlook of the processes related to the evolution, biogenesis, mechanisms of action, and prediction tools, culminating with the general relevance of miRNAs in plants, and specifically end with the few information related to their possible involvement in DDR.

1.4.1. Evolution, biogenesis, and mechanism of action in animals and plants

MicroRNAs are defined as small (18–22 nucleotides), endogenous, non-coding, single-stranded ribonucleic acids, acting as regulators of biological and physiological processes (Jones-Rhoades & Bartel, 2004; Voinnet, 2009). Indeed, miRNAs can regulate the expression of specific target genes by post-transcriptional silencing or translational inhibition, thus regulating specific processes. In plants and animals, the regulation of gene expression by miRNAs constituted a step towards the development of more complex gene regulatory networks (Jones-Rhoades & Bartel, 2004; Bartel, 2009). Various authors theorized miRNAs as drivers to a multicellular state allowing the evolution of complex organisms (Bartel, 2004; Peterson et al., 2009; Erwin et al., 2011). Common and unique features characterize miRNA biogenesis among kingdoms. These similarities and differences are taken into account to understand if plants and animal miRNA pathways evolved in a common ancestor or independently. The predominant view is that the miRNA evolved convergently in plants and animals (Jones-Rhoades et al., 2006; Axtell et al., 2011; Tarver et al., 2012, 2015). This would indicate that miRNA pathway evolved independently at least nine times (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2002; Molnár et al., 2007; Zhao et al., 2007a; Grimson et al., 2008; Huang et al., 2012; Robinson et al., 2013). However, Moran et al. (2017) suggested an alternative explanation according to which it might be possible that because of the high sequence turnover rates in plants and non-bilaterian animals, no trace of shared miRNA sequences between contemporary lineages could be found (**Fig. 10**). The fact that plant miRNA genes are born and lost at high rates support this hypothesis (Fahlgren et al., 2010; Cuperus et al., 2011), hence only a few miRNA families are conserved among distant plant lineages (Axtell et al., 2007).

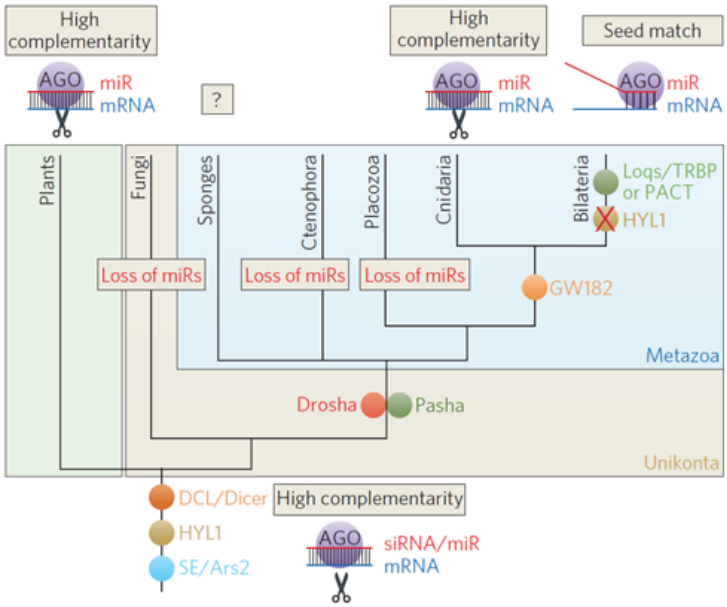

Fig. 10. Possible scenario of miRNA evolution in plants and animals in which the last common ancestor possessed a miRNA system. Gaining and losses of proteins and traits are shown in the relevant branches (Moran et al., 2017).

Comparison between *A. thaliana* and *A. lyrata* revealed that only 33% of the miRNA families are not conserved and were gained or lost throughout the ~10 million years (Myr) since they diverged (Cuperus et al., 2011). Genome data analysis and small RNA sequencing from *Capsella rubella,* a very close relative of *Arabidopsis*, indicated that the net flux rate (birth-death) for miRNA genes in *Arabidopsis* is 1.2–3.3 genes per Myr (Fahlgren et al., 2010). In animals, a recent study demonstrates that despite the notable examples of conservation, miRNA loss is much more represented than previously thought (Thomson et al., 2014). However, while the turnover of most animal miRNAs might be higher than previously thought, it is still lower than in plants. For example, in *Drosophila* it has been estimated around 0.8–1.6 per Myr (Lu et al., 2008; Berezikov et al., 2010).

These small molecules are transcribed by POLII (RNA POLYMERASE II) from MIR genes mainly located between protein-coding genes. While in animals 30% of MIR genes are located in introns, only three tested examples of intronic miRNAs are known so far in plants (Rajagopalan et al., 2006; Joshi et al., 2012). Moreover, in animals, approximately 50% of miRNA genes are found in clusters, often consisting of several mature miRNAs (Kim & Nam, 2006; Axtell et al., 2011). In plants, fewer cases of miRNA clusters are described, mostly codifying miRNAs from the same family with evident homology (Merchan et al., 2009; Axtell et al., 2011). However, some clusters of non-homologous miRNAs were identified in plants, which are expected to target related proteins.

A schematic representation of the biogenesis of miRNAs in both animals and plants is shown in **Fig. 11**. After transcription, the stem-loop regions of long primary

transcripts are processed by specific enzymes to give rise to a mature miRNA (Reinhart et al., 2002; Xie et al., 2005). In animals, the first step is performed by a specialized microprocessor complex consisting of the RNAse III Drosha with the cooperation of the RNA binding protein Pasha (DGCR8 in vertebrates). The second step of cleavage is conducted by the RNAse III Dicer (Kim et al., 2009). Differently, in plants, a Dicer homolog, DICER-LIKE 1 (DCL1), oversees both processing steps mandatory for miRNA maturation (Jones-Rhoades & Bartel., 2006; Voinnet, 2009). In both animals and plants, Dicer enzyme is essential for processing the precursor miRNA (pre-miRNA) into mature miR/miR* dsRNA duplexes (Cerutti & Casas-Mollano, 2006; Jong et al., 2009; Kim et al., 2009; Voinnet, 2009). In plants, the two processing events conducted by DCL1 occurs in the nucleus. Otherwise, in animals, the first processing event performed by Drosha takes place in the nucleus while the second step by Dicer takes place in the cytoplasm (Bartel, 2004; Axtell, 2011). Nevertheless, different studies in animals reported the presence of Dicer in the nucleus (Burger et al., 2015). Whether the nuclear localization of Dicer in animals is a relic of an ancient miRNA-processing pathway or a secondary adaptation is still a matter of debate. Both in animals and plants Dicer necessitates protein partners to accurately cleave pre-miRNAs (Kim et al., 2009; Voinnet et al., 2009). In plants, DCL1 is supported by the RNA binding proteins SERRATE (SE) and HYPONASTIC LEAVES1 (HYL1), both fundamental in miRNA biogenesis and development (Han et al., 2004; Vazquez et al., 2004; Voinnet et al., 2009). In animals, Ars2 (arsenite resistance gene 2, a Serrate homolog) has been identified as a partner of the microprocessor and Dicer (Sabin et al., 2009). As concern HYL1 protein, no animal homologs have been identified. Plant miR/miR* duplexes are very similar to those of animals. In fact, they are both ~ 22 nt long with imperfect complementarity between the two strands and a 2-nt 3′ overhang (Bartel, 2004; Kim, 2009; Voinnet, 2009). Despite these similarities, the stem-loop precursor in plants appears longer and more variable (Axtell et al., 2011).

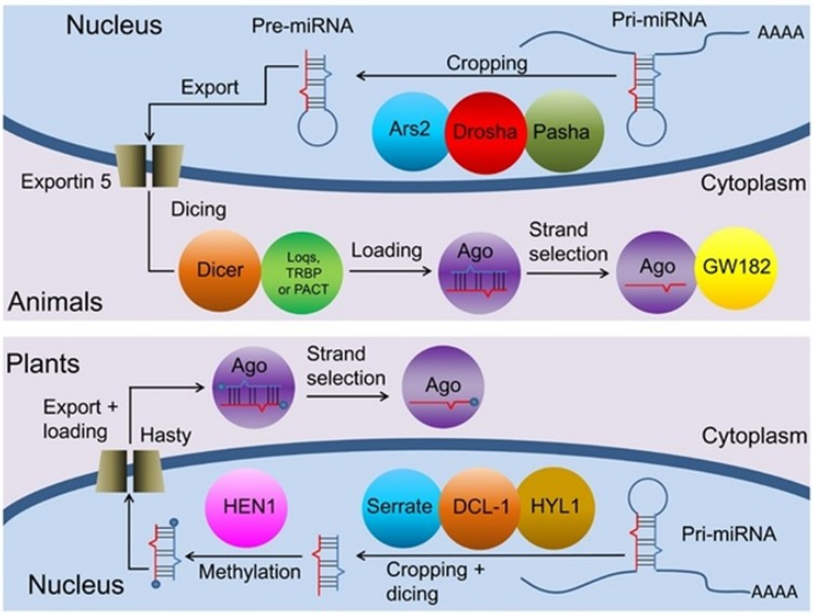

Fig. 11. Schematic representation of canonical miRNA biogenesis pathways in animals and plants. Protein homologs carrying similar functions such as Dicer of animals and DCL-1 of plants are represented in the same colour (Moran et al., 2013).

In both animals and plants, the binding of small RNAs widely complementary RNA targets leads to degradation of the small RNA by adenosine or uracil addition ('tailing') and 3′-to-5′ exonucleolytic decay ('trimming') (Ameres et al., 2010; Ameres & Zamore, 2013). Plant miRNAs are protected from these processes by 2′-*O*-methylation of their 3′ ends performed by the methyltransferase Hua enhancer 1 (HEN1) (Yu et al., 2005; Voinnet et al., 2009; Ameres et al., 2013).

MicroRNAs are responsible for translational repression or gene silencing by binding to the target mRNA transcripts through sequence complementarity, thus regulating their target genes at the post-transcriptional level (Jones-Rhoades et al., 2006). In both animals and plants, miRNAs necessitate a class of AGO (Argonaute) proteins, a core component of the RNA-induced silencing complex (RISC), to exert gene regulatory functions (Drinnenberg et al., 2011; Swarts et al., 2014). Once processed, the mature miRNA duplex is loaded onto AGO and the guide RNA strand selected by AGO directs RISC to the complementary RNA transcript (Bartel, 2004). AGO proteins are evolutionarily conserved from *Archaea* and *Bacteria* to *Eukarya*, where they take part mostly in small non-coding RNA related mechanisms. In plants, miRNA-directed cleavage is mediated primarily by AGO1. The binding of a plant miRNA to the target necessitates a nearly-full complementarity between the miRNA and its mRNA target which generally allows the endonucleolytic cleavage of the target by AGO between position 10 and 11 of the miRNA, often with a strong effect on a small number of targets (Qi et al., 2006; Luo & Chen, 2007). In contrast, every animal miRNA potentially regulates a large number of targets because the recognition by the miRNA of the correspondent target does not require perfect

complementarity but only a seven-nucleotide seed sequence, placed in positions 2–8 of the miRNA. Differently from plants, in animals, the vast majority of animal AGO proteins do not lead to miRNA target cleavage. Preferably, animal RISC brings to translational repression of targets through the block of translation initiation or elongation or deadenylation (Hutvagner & Simard, 2008; Ameres & Zamore 2013). This mode of action leads to a relatively weak modulation of less than twofold both at the RNA and protein levels (Baek et al., 2008; Selbach et al., 2008). This mechanism of action is advantageous as it is reversible thus allowing rapid expression of existing blocked mRNAs in a specific condition, time, or location (Bhattacharyya et al., 2006; Muddashetty et al., 2011). The degree of complementarity between the miRNA and its mRNA target is considered a major factor influencing the mode of target repression. High complementarity, as seen in plants, induces target cleavage by AGO while seed-matching promotes translational inhibition as mostly seen in animals (Hutvagner & Simard, 2008; Huntzinger & Izaurralde, 2011).

The existing differences between plant and animal miRNA mode of action are becoming blander considering that there is more translation inhibition in plants than previously appreciated (Brodersen et al., 2008; Iwakawa & Tomari, 2013; Liu et al., 2014; Reis et al., 2015). However, even this type of inhibition necessitates nearly-perfect complementarity as short matches limited to the seed region do not result in target inhibition in plants (Iwakawa & Tomari, 2013; Liu et al., 2014).

1.4.2. Advances and limitations of bioinformatics databases and tools to investigate plant miRNAs

Staring from the first miRNA cloning examples in plants, several additional miRNAs, both conserved and non-conserved, have been identified through a combination of computational predictions, forward genetics, and genome-wide miRNA profiling, especially in *Arabidopsis* (Jones-Rhoades & Bartel, 2004; Adai et al., 2005; Allen et al., 2005; Fahlgren et al., 2007). Because miRNAs interact with their target mRNA by sequence complementarity, public tools and databases available online were developed to support miRNA research. For example, miRBase and psRNATarget are most used by plant scientists. miRBase is a public repository for all published miRNA sequences and associated annotations, while psRNA target is a plant small RNA target analysis server (Dai et al., 2018; Kozomara & Griffiths-Jones, 2019). Sequencing analysis in plant systems different from *Arabidopsis*, including additional eudicots, dicots, basal plant lineages but also the unicellular algae such as *Chlamydomonas reinhardtii*, allowed the identification of both deeply conserved and species-specific miRNAs in different other species (Axtell & Bartel, 2005; Willmann & Poethig, 2007; Axtell et al., 2007; Zhao et al., 2007a). High-throughput pyrosequencing of miRNAs contributed to the knowledge of the regulatory roles played by plant miRNAs within diverse pathways.

As regards the mRNA targets, for many of the identified miRNAs, they have been computationally predicted and subsequently validated through molecular analysis. The validations indicated that some miRNA-target relationships are

ancient, while others have evolved more recently (Axtell, 2013). Molecular techniques may be employed to validate mRNA targets. For example, a modified 5' RACE allowed researchers to map the 5' ends of RNAs containing a 5' monophosphate and not a cap. Both modifications are characteristics of the 3' RNA cleavage products originated after miRNA-directed cleavage. The modified technique skips the 5' cap removal step that normally proceeds RNA adaptor ligation, RT-PCR, cloning, and sequencing. miRNA targets commonly are cleaved between the positions that pair to nucleotides 10 and 11 of the miRNAs, and this pattern of cleavage is characteristic for most miRNA targets (Llave et al., 2002; Kasschau et al., 2003). In plants, the transient or stable overexpression of MIR genes results in reduced accumulation of their full-length target mRNAs. Thus, the increased accumulation of short, cleavage fragments, is indicative of miRNA-directed cleavage. Techniques such as RNA gel blot, quantitative reverse transcription-polymerase chain reaction (qRT–PCR), or transcriptome analyses are used to monitor the accumulation of full-length target mRNAs. Caution is needed when using this technique because false-positive miRNA targets might be detected when miss expressing or overexpressing MIR genes. MIR gene mutations responsible for a decrease in accumulation of miRNAs results in increased target mRNA accumulation, sometimes in a tissue-specific manner, that may be visualized by RNA gel blot, qRT–PCR but also in situ hybridization and transcriptome analyses (Sieber et al., 2007). The accumulation of RNAs and the phenotypes resulting from the expression of transgenes holding predicted miRNA binding sites or miRNA resistant binding sites where miRNA complementarity has been disrupted may be monitored *in vivo*. This technique may be coupled with reporter gene fusions and MIR gene overexpression to follow the changes in both mRNA and protein accumulation (Mallory & Vaucheret, 2006).

Despite enormous advances at the computational and molecular level, there are still limitations in the techniques applied for a more in-depth study of miRNAs. The unavailability of the target genome in bioinformatics tools is an example. Many of the hitches require the construction of tailor-made pipelines which, even when decisive, lead to greater consumption of time. Another difficulty in the analysis of miRNAs is due to their small size and their fluctuations even within the same species due to interspecific variability or environmental conditions. Ligation of adapters and strategies to increase amplicon size contributes to their specific capture and synthesis. Considering the advantages but also the limitations of current techniques, new efforts are needed to improve the understanding of the role of miRNAs and its effects in the most disparate fields of biology and beyond.

1.4.3. The multifaced role of plant miRNAs

MicroRNAs are known for their involvement in a myriad of processes in plant life. Their implication in various aspects of plant developments, cellular processes, response to stresses, signal transduction, and even their biogenesis, is ascertained (**Fig. 12**). Organ maturation (Juarez et al., 2004; Guo et al., 2005), hormone signaling (Liu et al., 2009), developmental timing (Achard et al., 2004), and responses to

pathogens (Sullivan & Ganem, 2005; Navarro et al., 2016) as well as to environmental abiotic stresses such as drought (Zhao et al., 2007b), salinity (Zhao et al., 2009), heavy metals (Huang et al., 2009), and cold (Zhou et al., 2008) are among the biological and metabolic processes regulated by plant miRNAs. More than 50% of the targets of miRNAs belonging to conserved families are transcription factors and proteins implicated in developmental programs and cell differentiation (Jones-Rhoades et al., 2006; Mallory & Vaucheret, 2006). Among these, miR171 is among the plant conserved miRNAs playing multiple roles in plant development. This miRNA is involved not only in leaf morphogenesis and shoot branches but also in pollen ontogenesis. MiRNAs also participate in the growth of the primary and lateral roots constructing the architecture of the root system, particularly by modulating the signaling of plant hormone auxin (Wang et al. 2005; Curaba et al. 2014). There is increasing evidence that miRNAs regulate not only plant development but also responses to biotic or abiotic stresses (Shriram et al., 2016) and that environmental stresses lead to the synthesis of new miRNAs (Khraiwesh et al., 2012). To date, more than 40 miRNA families have been observed to be involved in responses to abiotic stresses in plants (Sunkar, 2007, 2010), many of which are involved in responses to salt and drought stresses (Xiong et al., 2006; Peng et al., 2014).

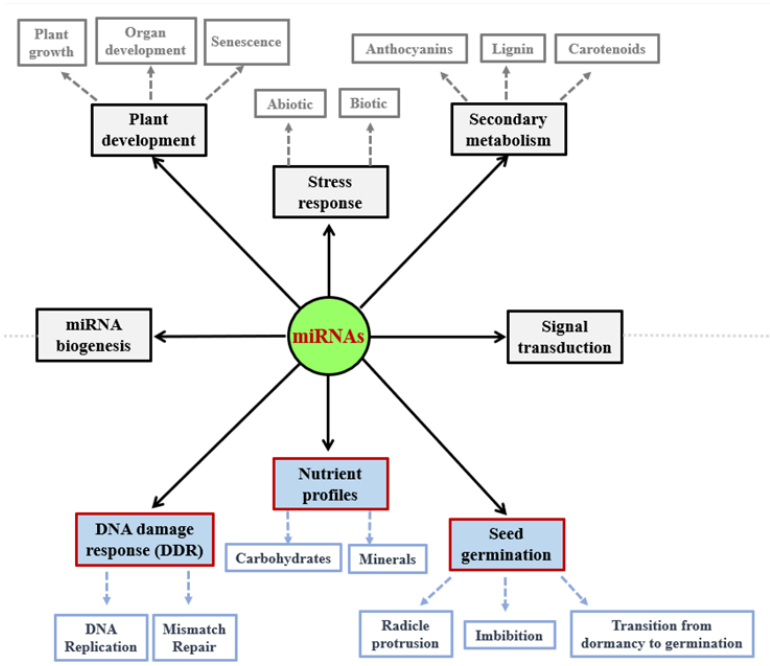

Fig. 12. Schematic representation of the roles that miRNAs play in plants.

Among the numerous conserved and novel miRNAs identified, many are implicated in the regulation of nutrient deprivation (Zhao *et al.*, 2012; Panda and Sunkar, 2015), and trace element toxicity (Huang et al., 2016; Cakir *et al.*, 2016). In fact, miRNA abundances are influenced by nutrient availability such as phosphorus

(P) and copper (Cu). Four different miRNAs are currently known to be Cu responsive, namely miR397, miR398, miR408, and miR857, three of which (miR397, miR398, miR408) belonging to conserved plant miRNA families. Another function recently assigned to miRNAs is the regulation of secondary metabolite productions such as flavonoids, lignins, terpenes and terpenoids, and alkaloids (Wagner & Kroumova, 2008; Gupta et al., 2017, Araùjo et al., 2018). For example, anthocyanin accumulation is regulated by the miR156 targeted SPL9 (Squamosa promoter binding protein-like 9) gene in *Arabidopsis* (Gou et al., 2011).

Plant miRNAs are also capable of regulating genes necessary for the miRNA pathway, such as DCL1 and AGO1 (Xie et al., 2003; Vaucheret et al., 2004, 2006; Rajagopalan et al., 2006). Plant miRNAs also constitute key regulatory modules in the process of seed development, dormancy, and germination. It is already known that some miRNA families (e.g. miR156, miR159, miR164) are down-regulated, while others (e.g. miR398, miR408, miR528) are up-regulated (Li et al., 2013; Araùjo et al., 2018) during seed development and germination. Other examples include miR159 and miR160, demonstrated to contribute to the regulation of seed germination by influencing seed sensitivity to ABA and auxin (Reyes & Chua, 2007; Nonogaki, 2010).

Taken together, all the above-mentioned processes regulated by miRNAs have vast implications in the agricultural field and the development of modern technologies for its improvement. Considering their importance, the unknown regulatory properties and functions of many miRNAs deserve to be deeply explored.

1.4.3.1. Regulatory properties of miRNAs in the context of DDR

MiRNAs are emerging as new players in DDR and DNA repair. The involvement of miRNAs in the regulation of DDR players is quite recent and insufficiently explored, especially within the plant kingdom. Conversely, studies in human cells have already shown that miRNAs are involved in the regulation of DDR-associated genes and their activity is intricately weaved with traditional elements such as ATM and p53 (Kato et al., 2009; Landau & Slack, 2011; Wan et al., 2011).

DNA lesions influence miRNA expression at the transcription and post-transcription levels as well as miRNA degradation, whereas miRNAs regulate the components of DDR machinery such as sensors, transducers, and effectors (Zhang & Peng, 2015). It has been demonstrated that microRNAs, in both plants and animals, are responsive to IR-induced oxidative stress and may be responsible for the epigenetic regulation of some DDR genes (Joly-Tonetti & Lamartine, 2012; Kim et al., 2016). In animals, miR-24, miR-138, miR-182, miR-101, miR-421, miR-125b, and miR-504 are crucial regulators of H2AX, BRCA1, ATM, or P53, (Joly-Tonetti & Lamartine, 2012; Lhakhang and Chaudhry, 2012). Additionally, miR-96, miR-155, miR-506, miR-124, miR-526, and miR-622b are involved in HR or NHEJ repair by targeting RAD51 or KU70/80, respectively (Choi et al., 2016a, 2014; Thapar, 2018). Plant specific miRNAs responsive to genotoxic stress includes the IR-induced *Arabidopsis* miR840 and miR850, which remain to be further characterized in terms

of their roles in DDR and DNA repair (Kim et al., 2016). Some miRNAs (e.g. osamiR414, osa-miR164e, and osa-miR408), were demonstrated to target specific helicases playing roles in recombination, replication and translation initiation, DSB repair, NER, or maintenance of telomere length. These miRNAs and their target genes were differentially expressed under different stress conditions, like salinity and irradiation, in the expected negative-correlation manner; when miRNAs are upregulated the genes are downregulated, and vice versa (Macovei & Tuteja, 2013).

It is thus clear that research on miRNAs involved in plant DDR is currently poorly represented in the scientific literature. Considering the implications of the DDR on the viability and quality of plants and seeds, it is also worth investigating these fine-tuning aspects in order to gain new and useful insights on this compelling topic.

1.4.3.2. miRNAs and chromatin remodeling

Chromatin remodeling processes are fundamental for gene activation/repression. Among the chromatin modifications, histone acetylation plays an important role in chromatin remodeling and is required for gene activation. By analyzing the accumulation of miRNAs in mutants of *Arabidopsis*, Kim and co-workers (2009) showed that the histone acetyltransferase GCN5 (General control non-repressed protein 5) induce repression on miRNA production, whereas it is necessary for the expression of a specific subset (*e.g.* stress-inducible) of *MIRNA* genes. The repressive effect of GCN5 in miRNA production is probably due to indirect repression of the miRNA machinery genes including *DCL1, SE, HYL1*, and *AGO1*. Chromatin immunoprecipitation assays showed that GCN5 targets a group of *MIR* genes and is needed for acetylation of histone H3 lysine 14 at these loci. Furthermore, inhibition of histone deacetylation by TSA treatment or in histone deacetylase gene mutants compromised the accumulation of specific miRNAs. These data suggest that GCN5 affects the miRNA pathway at both the transcriptional and post-transcriptional levels and that histone acetylation/deacetylation is an epigenetic mechanism implicated in the regulation of miRNA synthesis.

Another example of a chromatin modifier is constituted by the ATP-dependent SWR1 chromatin remodeling complex SWR1-C. It is involved in changing the histone H2A-H2B dimer with the H2A.Z-H2B dimer, leading to variant nucleosomes. In *Arabidopsis*, SWR1-C participates in the active transcription of many genes, but also the silencing of genes responsive to environmental and developmental stimuli. SWR1-C is required for miRNA-mediated developmental regulation. In SWR1-C mutants, miR156 and miR164 levels are reduced at the transcriptional level resulting in the accumulation of target mRNAs and associated morphological changes. MiRNA sequencing proved that the level of many miRNAs, including miR156, decreased in specific SWR1-C mutants, though some miRNA levels increased. Thus, SWR1-C contributes to transcriptional activation via nucleosome dynamics. Furthermore, SWR1-C contributes to the fine-tuning of plant developmental processes by generating a balance between miRNAs and target mRNAs at the transcriptional level (Choi et al., 2016b).

1.5. The *trans*-kingdom valence of plant miRNAs

Aside from the well-known, canonical functions of miRNAs, a new hypothesis started to emerge recently, where miRNAs appear as molecules that can be transferred from one species to another (Knip et al., 2014; Han & Luan, 2015) in a *trans*-kingdom fashion. The knowledge on this aspect is rapidly expanding, offering unique opportunities to identify miRNAs shared between the animal and plant kingdoms. This chapter focuses on the mobility of miRNA molecules from the perspective of *trans*-kingdom gene silencing. The mobility of miRNAs within the organism is a well-known phenomenon, but recent studies started to investigate also examples of miRNA exchange occurring between organisms of different kingdoms. These examples are predominantly found in interactions between hosts and their pathogens, parasites, and symbionts; although, recently, novel evidence was provided showing that that food-derived exogenous plant miRNAs can be passed to and influence the expression of mammalian genes, including humans.

1.5.1. From plants to humans

Plenty of evidence showing that plant miRNAs are present in human/animal plasma and can target cross-species genes, with potential beneficial or detrimental effects was reported (Vaucheret & Chupeau, 2012; Liu et al., 2017). The first study showing the assimilation of miRNAs through the diet and their potential interaction with human/mammal genes was published by Zhang et al. (2012), who observed the permanence of the miR168a in the sera and tissues of animals and humans. This miRNA was demonstrated to target the liver low-density lipoprotein (LDL) receptor adapter protein 1 (LDLRAP1), subsequently resulting in decreased removal of LDL from plasma. On the contrary, Dickinson et al. (2013) did not detect measurable uptake of any rice miRNAs in mouse feeding trials. Other recent studies pointed the attention to the miRNA cross-kingdom inhibition of cancer growth. Studies performed using synthetic plant miRNAs showed miRNA absorption at the gastrointestinal level along with a reduction of tumor burden in mice. The plant miR159 was abundantly found in human sera and associated with a decreased incidence and progression of breast cancer. This was explained by the specific binding of miR159 to the TCF7 (a Wnt signaling transcription factor) gene encoding a protein that interacts with MYC (Avian Myelocytomatosis Viral Oncogene Homolog) proteins, playing essential roles in cell cycle progression (Chin et al., 2016). Another example is the *Atropa belladonna* aba-miRNA-9497, shown to be highly homologous to *Homo sapiens* hsa-miRNA-378and thus being able to target the 3′-UTR of the mRNA encoding for a neurologically relevant protein called ZNF-691 (Zinc-Finger Transcription Factor). It is believed that this could explain the potent neurotoxic actions of the alkaloids found in *A. belladonna* neuroregulatory activity, induced by modulating the expression of ZNF-691-sensitive genes (Avsar et al., 2019).

Wet lab (Philip et al., 2015; Cavalieri et al., 2016; Liu et al., 2017) *versus* dry lab (Shu et al., 2015; Zhang et al., 2016a) experiments are being designed to prove or disprove this hypothesis. These compelling studies highlight the particular role of plant miRNAs in cross-kingdom communication. To date, plant miRNAs detected in human plasma belong to evolutionary conserved families (e.g. mi168, miR156, miR166, miR319, miR167) (Liang et al., 2014; Yang et al., 2015). These miRNAs were found to be resistant to RNase A activity, whereas they can also resist processing and cooking. Probably, this ability results from miRNAs association with other molecules such as proteins or lipids which protect them against degradation (Liang et al., 2014). Moreover, the methylated configuration of plant miRNAs grants them higher stability against degradation (Zhang et al., 2012). By these means, exogenous plant miRNAs can be released into mammalian cells where they can regulate multiple target genes based on sequence complementarity, similarly to how endogenous miRNAs act (Liu et al., 2017). This concept may envision plant miRNAs as natural bioactive compounds with potential health-promoting benefits.

1.5.2. From plants to pathogen and symbionts

Many of the existing examples of miRNA cross-kingdom transfer come from plant-pathogen interactions (Zhang et al., 2016b; Wang et al., 2017; Zhang et al., 2019; Gualtieri et al., 2020). The cross-kingdom transfer of endogenous plant miRNAs to pathogens is associated with the inhibition of their invasive attributes while, on the other side, miRNA transfer from parasitic eukaryotes to plants may suppress the immunity of the host plants. In the case of symbiotic and/or mutualistic relations, miRNA transfer from plants may influence the growth and development of these organisms (Zhu et al., 2017). Indeed, miRNAs have been observed to move in a cross-kingdom fashion from plants to fungi and vice versa. The transfer of miRNAs from plants to pathogenic fungi has been observed in the case of *Gossypium hirsutum* miR159 and miR166 transferred to *Verticillium dahliae* (Zhang et al., 2016b). These specific miRNAs, found to be present in fungal hyphae isolated from infected cotton tissues, were predicted to target two specific proteins related to fungal virulence, namely the isotrichodermin C-15 hydroxylase (HiC-15) and Ca^{2+}-dependent cysteine protease (Clp-1). To prove this relation, experiments were conducted by transiently expressing miRNA-resistant HiC-15 and Clp-1 in tobacco and *V. dahliae*, showing that the transfected plants acquired resistance to the fungal pathogen (Zhang et al., 2016b). The delivery of small RNA molecules from fungi to plants was also observed, as in the case of the novel miRNA-like RNA, Pst-milR1, from *Puccinia striiformis* f.sp. *tritici* (Pst), the agent causing the wheat stripe rust disease (Wang et al., 2017). This miRNA was identified by high-throughput analysis and predicted to target the 1,3-glucanase SM638 (pathogenesis-related 2) gene in wheat. This prediction was subsequently confirmed by co-transformation analyses and RACE (rapid amplification of the cDNA ends) validation in tobacco leaves.
The cross-kingdom transfer of miRNAs has been investigated also for its communication role between plants and plant-feeding insects, like in the case of *Plutella xylostella* (Zhang et al., 2019), and, most importantly, in pollinator insects.

Several studies reported prominently on the dietary intake of plant miRNAs by honey bees (Ashby et al., 2016; Gismondi et al., 2017; Zhu et al., 2017). In bees, the dietary intake of pollen-derived miR162a was proven to regulate caste development at the larval stage (Zhu et al., 2017). It was shown that miR162a specifically targeted TOR mRNA, thus downregulating its expression at the post-transcriptional level. This mechanism was found to be conserved also in *Drosophila melanogaster*, although this is a non-social type of insect (Zhu et al., 2017). As in the case of miRNAs transfer from plants to humans/mammals, contrasting results were reported also in this situation. As an example, Masood et al. (2016) shown that although plant miRNAs are accumulated after pollen ingestion in adult bees, no biologically relevant roles could be associated with them.

In the future, the study of this particular phenomenon of miRNA transfer may help researchers to develop increasingly sophisticated agricultural technologies based on miRNAs. For example, artificial miRNA (amiRNAs) may represent a valuable tool to combat current and future challenges in the agricultural sector (Chen et al., 2013; Mitter et al., 2016). Given this, miRNA-based strategies exploiting the potential of plant miRNAs to move across kingdoms and silence specific genes in distantly related organisms could prove to be useful, efficient, and sustainable solutions.

2. Aims of the research

Although involved in a significant number of relevant processes, the regulatory properties, and functions of many miRNAs are still unknown and deserved to be explored, especially when regarding miRNA trans-kingdom valence and involvement in DDR pathways, as evidenced by the cited literature (see Introduction).

Given the miRNA vastness of functions and their relevance to both plants and animals/humans, this study aimed to investigate the potential of plant miRNA *trans*-kingdom valence along with their involvement in DDR. In this variegated scenario, the present research proposes to:

1. Identify miRNAs targeting common biological processes between plant and human cells focusing on a particularly conserved pathway, namely DDR, by performing in silico bioinformatic analyses;
2. Develop an appropriate experimental working system to inhibit specific DDR pathways;
3. Analyze the expression of plant DDR-related genes and their putative miRNAs in the developed system.
4. Investigate the cross-kingdom valence of plant miRNAs predicted to target human genes in an *in vitro* system.

The model legume *Medicago truncatula* (barrel medic) has been chosen as a target to achieve the objectives from 1 to 3 because, aside from being a model legume system, it is also economically relevant as a forage crop and it has gain attention as having nutraceutical properties (Tava et al., 2011). To achieve objective 4, *Malus domestica* cv Golden Delicious was chosen as the target. Along with olive oil, red wine, greens, and a plethora of vegetables and other fruits, apples are part of the Mediterranean diet associated with health benefits such as lower cancer incidence (Berrino & Muti, 1989; Gallus et al., 2004). The fruit, available all year round in a variety of forms, is perceived as healthy food and consumed fresh or in the form of derivatives. Apples are rich in phenolic compounds (especially flavonoids and hydroxycinnamates), pectin, sugar, macro and microelements that give it antioxidant, antimicrobial, antitumor properties, and many other beneficial effects with potential applications in the food, pharmaceutical, and cosmetic industries.

3. Materials and methods

3.1. *In silico* analyses

3.1.1. Datasets used for the bioinformatics analyses

The list of *M. truncatula* miRNAs used in this study was obtained from the public database miRBase (http://www.mirbase.org/, Kozomara et al., 2019) and consisted of 756 sequences (426 unique).

The human 3'UTRome sequence dataset and the *M. truncatula* transcript dataset (Mt4.0 v1) were obtained from the psRNATarget (http://plantgrn.noble.org/psRNATarget/) and amounted to 21,233 sequences (18,167 unique human genes) and 62,319 transcripts (50,894 unique barrel medic genes), respectively.

The target gene and protein sequences were recovered from the NCBI RefSeq database (https://www.ncbi.nlm.nih.gov/refseq) for human targets and from the *Medicago* Genome Database (www.medicagogenome.org).

For the *de novo M. truncatula* network construction, six microarray datasets were retrieved from the ArrayExpress repository (https://www.ebi.ac.uk/arrayexpress):

(i) E-MEXP-1097 – describing the transcription profiling of all major organ systems to generate a gene expression atlas, developed by Benedito et al. (2008);

(ii) E-MEXP-3719 – containing the transcription profiling by array of seven developmental stages of *M. truncatula* cultivar A17 during seed maturation (from 24 DAP to dry seeds), developed by Verdier et al. (2013);

(iii) E-MEXP-2883 – containing transcription profiling of border cells, root tips, and whole roots (Tang, 2014);

(iv) E-MEXP-3190 – consisting of transcriptome profiling of *irg1* mutant leaf of *M. truncatula*, developed by Uppalapati and colleagues (2012)

(v) E-MTAB-3909 – enclosing a transcriptome analysis of secondary cell wall development, published by Wang et al. (2016b);

(vi) E-GEOD-43354 – containing the cell- and tissue-specific transcriptome analyses of root nodules, published by Limpens et al. (2013).

These amounted to a total of 117 raw expression samples that were used for *M. truncatula* co-expression network reconstruction.

3.1.2. Tools and parameters for miRNA target prediction

To predict miRNA targets in *M. truncatula* and *H. sapiens*, the tools used were psRNATarget (Dai et al., 2018) and RNAhybrid (Kruger and Rehmsmeier, 2006), respectively. The list of *M. truncatula* unique miRNAs was used as input for both tools, together with the *M. truncatula* transcript dataset or the human 3′ UTRome.

The parameters of the two target prediction tools were set to obtain a balanced number of network nodes (about 700 for *A. thaliana* and *H. sapiens*) in the network-based pipeline, and of unique target transcripts (about 1,700 for *M. truncatula* and *H. sapiens*) in the alignment-based pipeline.

A highly specific hybridization in seed region (typical for plants) was set in psRNATarget, which was used to find plant target genes for the network-based pipeline with the following parameters: number of top targets = 50, Expectation = 2.5, Penalty for G:U pair = 0.5, Penalty for other mismatches = 1, Extra weight in seed region = 1.5, Seed region = 2–13 nucleotides, Mismatches allowed in seed region = 0, HSP size = 19. For the alignment-based pipeline, the list of targets was obtained using the same parameters, except the number of top targets set to 15.

The predicted target list from RNAhybrid was filtered by fine-tuning the Minimum Free Energy (MFE) at a threshold of −36.5 kcal/mol for the network-based approach, and at −34.7 kcal/mol for the alignment-based pipeline. In both cases, a maximum of 50 targets per miRNA was considered, as previously reported as necessary for accurate analysis (Zhang et al., 2016a).

3.1.3. Development of a bioinformatics pipeline to assess plant miRNAs *trans*-kingdom valence

Taking advantage of miRNAs ability to cleave mRNA targets according to sequence complementarity in both plant and animal, bioinformatic approaches are useful tools to predict cross-species targets. The bioinformatic pipeline was designed and performed in collaboration with Dr. L. Pasotti's group from the Bioinformatics, Mathematical Modelling and Synthetic Biology (BMS) Laboratory (University of Pavia). Several strategies have been developed and implemented to achieve the common goal of identifying shared features (miRNAs targeting similar processes) between these evolutionary distant organisms:

(1) a gene network-based approach was used to compare the targeted biological processes in plants and human, using an *A. thaliana* homology-based system for plant network reconstruction;

(2) an alignment-based approach was used to identify nucleotide and protein similarities between *M. truncatula* and *H. sapiens* putative targets;

(3) another network-based approach using a *de novo* reconstructed *M. truncatula* gene network was used to further assess the common biological processes targeted in human and barrel medic.

A schematic representation of the employed strategies, steps, and methodologies used is shown in **Fig. 13**.

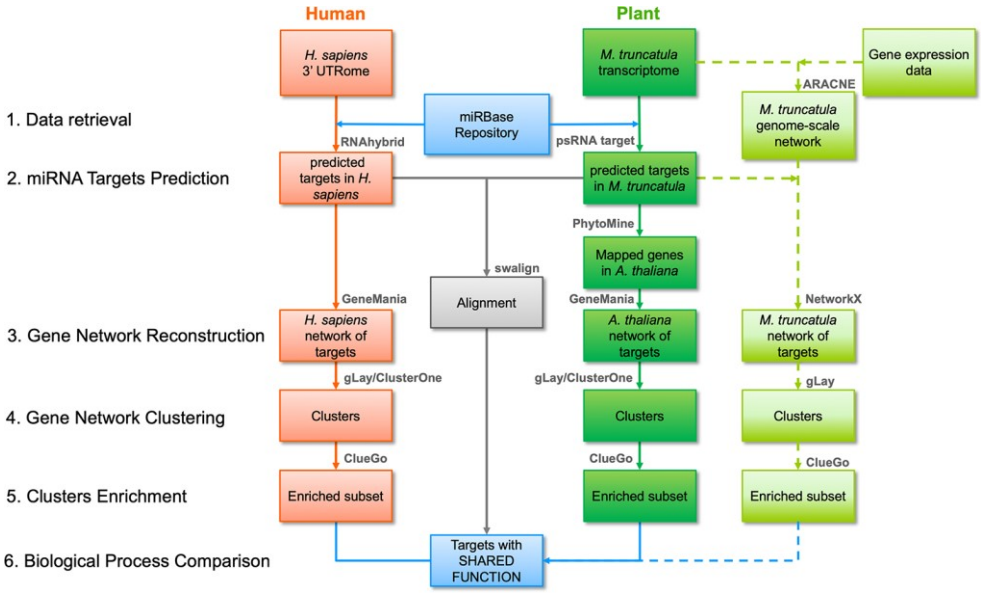

Fig. 13. Schematic representation of the pipeline used to conduct bioinformatics analyses to investigate plant miRNAs trans-kingdom valence (Bellato et al., 2019).

For the development of the network-based approach, the obtained predicted targets were used to construct the plant and human target networks using GeneMania (https://genemania.org/, Warde-Farley et al., 2010) taking into consideration all the genetic and co-expression interactions available within the tool. Since GeneMania does not contain *M. truncatula* among the available organisms, this procedure was used to construct a genetic interaction/co-expression network of *A. thaliana* by mapping the homologous genes of the *M. truncatula* predicted targets list using the Phytomine tool (https://phytozome.jgi.doe.gov/phytomine/begin.do, Goodstein et al., 2012). The relative threshold similarity between the species was set above 85%. Human and plant networks were imported and analyzed using Cytoscape (v.3.7.1) (https://cytoscape.org/, Shannon et al., 2003) and its applications. Clustering was carried out using the gLay (Su et al., 2010) and ClusterOne (Nepusz et al., 2012) algorithms. The parameters set for ClusterOne were as follow: minimum size = 50, minimum density = 0.25, unweighted edges, node penalty = 2, haircut threshold = 0, merging method = Multi-pass, Jaccard similarity, overlap threshold = 0.15, seeding method from unused nodes. The gLay algorithm does not have free parameters. For each cluster, enrichment analysis was carried out using ClueGO (Bindea et al., 2009) to find statistically overrepresented Gene Ontology (GO) terms in the Biological Process (BP) category, using a right-tail test with the Benjamini-Hochberg correction for multiple testing (75% detail level). GO terms having a p-value < 0.05 were considered for further analysis.

For the development of the alignment-based approach, the nucleotide and protein sequence of the predicted transcript targets found in *M. truncatula* and *H. sapiens* were compared by sequence alignment for each miRNA. A custom MATLAB R2018a (MathWorks, Natick, MA, USA) script was programmed to

automatically carry out this analysis and to evaluate the statistical significance of each comparison. The Smith-Waterman method (Smith and Waterman, 1981) was used to perform local alignment *via* the *swalign* function and get the optimal alignment score (in bits) as output. A random permutation-based statistical analysis was used to evaluate the significance of each alignment and to obtain a sequence length-independent scoring value (*p*-value) (Tiengo et al., 2015). For each sequence comparison, 200 random permutations were considered for the human nucleotide/protein sequence and an alignment was performed for each randomization. The resulting distribution was used to obtain the final *p*-value. Low *p*-values correspond to statistically significant alignments with a considered threshold of 0.05.

For the reconstruction of *M. truncatula* co-expression network, raw expression values were globally normalized using the Robust Multichip Average (RMA) method (Irizarry et al., 2003), and annotated using the MedtrA17_4.0 reference genome assembly. Co-expression analysis of the obtained expression panel was performed using ARACNE (Margolin et al., 2006). All the analyses were performed in the R environment, using *limma* (Ritchie et al., 2015) and *biomaRt* (Durinck et al., 2009) packages for the expression data preparation, and *minet* (Meyer et al., 2008) for the co-expression estimation. The obtained matrix was used to reconstruct a co-expression network for the miRNA targets of *M. truncatula*, with a Python (v.2.7) script, exploiting the *NetworkX* package (Hagberg et al., 2008) to create networks in a Cytoscape-compatible format. The miRNA targets of *M. truncatula*, were mapped into the network to extract their co-expression interactome. The resulting sub-network was filtered to eliminate the smallest units composed of single nodes or less than ten nodes because these are not informative in terms of interactions. The remaining giant component was analyzed using gLay clustering procedure and the obtained clusters were subjected to the ClueGO enrichment step.

3.1.4. Identification and *in silico* characterization of SOG1 in *M. truncatula*

The *A. thaliana* AtSOG1 (AT1G25580) peptide sequence was retrieved from Phytozome (vs. 12.1) and used to recover SOG1 homologs in the *M. truncatula* genome (Mt4.0v1) by using the BLASTP tool, with default parameters, of the same database. Apart from the *M. truncatula* genome, AtSOG1 peptide sequence was also used to retrieve SOG1 homologs in other plant species by blasting this sequence against all the plant genomes accessible in Phytozome database (vs. 12.1).

The genomic, transcript, coding, and peptide sequences of *M. truncatula* SOG1 (MtSOG1) were retrieved from Phytozome portal. PhytoMine, (https://phytozome.jgi.doe.gov/phytomine/begin.do) was used to find the precise positions of the exons on genomic sequences, average length, and distribution. Pfam (http://www.pfam.xfam.org) and InterPro (http://www.ebi.ac.uk/interpro/) were used to confirm and locate the NAM/NAC characteristic domain. Subsequently, the coding and the peptide sequences of MtSOG1 were aligned by CLUSTALW tool (https://www.genome.jp/) to find common and different features. The STRING (https://string-db.org/) online database (vs.11.0) was used to identify AtSOG1 and

MtSOG1 putative interactors. STRING is a database of known and predicted protein-protein interactions, both direct (physical) and indirect (functional). The AtSOG1 and MtSOG1 peptide sequences together with the organism of origin were used as STRING input to obtain the functional protein-protein interaction networks.

3.1.5. Phylogenetic tree construction

To generate the phylogenetic tree, the retrieved SOG1 peptide sequences with an E-value < -100 were first selected and aligned with MAFFT (Multiple Alignment Fast Fourier) (Katoh et al., 2002), using FFT-NS-i algorithm (Katoh et al., 2002). After alignment and trimming, the sequences were used to build a phylogenetic tree through MEGAX (Molecular Evolutionary Genetics Analysis) (Kumar et al., 2018) and using neighbor-joining (NJ) as a statistical method. The following parameters were imposed: *(i)* bootstrap method with 1000 replications; *(ii)* evolutionary distances calculated with Jones–Taylor-Thornton substitution model; *(iii)* rates among sites were gamma distributed with a value of 1 for the parameter (Carocha et al., 2015; Soler et al., 2014).

Sequences from the following species were used to construct the phylogenetic tree: *Amaranthus hypochondriacus*, *Amborella trichopoda*, *Ananas comosus*, *Aquilegia coerulea*, *Arabidopsis halleri*, *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Boechera stricta*, *Brachypodium distachyon*, *Brachypodium stacei*, *Brassica oleracea capitata*, *Brassica rapa*, *Capsella grandiflora*, *Capsella rubella*, *Carica papaya*, *Chlamydomonas reinhardtii*, *Citrus clementina*, *Citrus sinensis*, *Coccomyxa subellipsoidea*, *Cucumis sativus*, *Daucus carota*, *Dunaliella salina*, *Eucalyptus grandis*, *Eutrema salsugineum*, *Fragaria vesca*, *Glycine max*, *Gossypium raimondii*, *Kalanchoe fedtschenkoi*, *Kalanchoe laxiflora*, *Linum usitatissimum*, *Malus domestica*, *Manihot esculenta*, *Marchantia polymorpha*, *Micromonas pusilla*, *Micromonas sp.*, *Mimulus guttatus*, *Musa acuminata*, *Oropetium thomaeum*, *Oryza sativa*, *Ostreococcus lucimarinus*, *Panicum hallii*, *Panicum virgatum*, *Phaseolus vulgaris*, *Physcomitrella patens*, *Populus trichocarpa*, *Prunus persica*, *Ricinus communis*, *Salix purpurea*, *Selaginella moellendorffii*, *Setaria italica*, *Setaria viridis*, *Solanum lycopersicum*, *Solanum tuberosum*, *Sorghum bicolor*, *Sphagnum fallax*, *Spirodela polyrhiza*, *Theobroma cacao*, *Trifolium pratense*, *Vitis vinifera*, *Volvox carteri*, *Zea mays*, *Zostera marina.*

3.2. Wet-lab analyses

3.2.1. Experimental setup

M. truncatula seeds (cv. Jemmalong, M9-10a genotype), gently provided by Fertiprado L.d.a. (http://www.fertiprado.pt/en/), Portugal, were used for this study. Seeds were treated with different concentrations of camptothecin (CPT, 25 µM, 50 µM, 100 µM) (Sigma-Aldrich, Milan, Italy), and 25 µM NSC120686 (NSC, 2-chloro-6-fluorobenzaldehyde 9H-fluoren-9-ylidenehydrazone, provided by National Cancer Institute, Bethesda, USA). Following the selection of the most appropriate CPT dose, a synergistic CPT+NSC treatment was implemented. The concentrations

were selected based on preliminary phenotypic results in the case of CPT treatments and previous studies in the case of NSC (Macovei et al., 2018). Because these compounds are dissolved in 100% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy), specific DMSO controls, corresponding to each concentration used in the indicated treatments, were set up. Specifically, DMSO 0.29% (subsequently denominated as DMSO_C) corresponds to the concentration used for the CPT treatments, DMSO 0.17% (subsequently denominated as DMSO_N) corresponds to the concentration used for the NSC treatments, and DMSO 0.23% (subsequently denominated as DMSO_CN) corresponds to the concentration used for the CPT+NSC treatments. A non-treated control (NT) was also used for all experiments carried out. A schematic diagram of the proposed experimental design is shown in **Fig. 14**. The designated treatments were applied to *M. truncatula* seeds placed in Petri dishes (30 seeds per dish) containing a filter of blotting paper moistened with 2.5 mL H_2O (NT, non-treated control) or indicated solutions (CPT, NSC, CPT+NSC, DMSO_C, DMSO_N, DMSO_NC). Petri dishes sealed with parafilm were kept in a growth chamber at 22°C under light conditions with a photon flux density of 150 µmol $m^{-2}s^{-1}$, photoperiod of 16/8 h, and 70–80% relative humidity. To keep the seeds moistened, 1 mL solution was added for each treatment on the third day. The experiment was followed for a time period of seven days and subsequently the plant material was used fresh or frozen in liquid nitrogen for subsequent analyses.

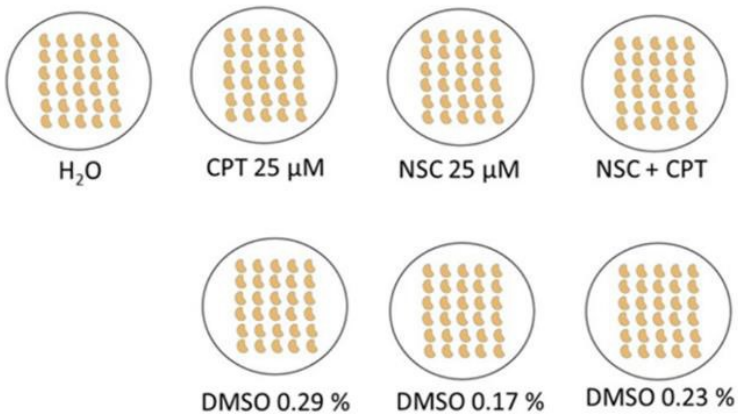

Fig. 14. Schematic representation of the experimental design set up to evaluate the effect of selected treatments on *M. truncatula* seed germination and seedling development. CPT, camptothecin; NSC, NSC120686; DMSO, dimethyl sulfoxide.

3.2.2. Phenotypic evaluation

M. truncatula seeds subjected to the above-mentioned treatments were monitored every 24 h for seven days. At the end of the experiment (7th day), several biometrical parameters were measured:

- germination percentage (%), consisting of the percentage of total germinated seeds; a seed is considered germinated when the radicle protrusion reaches at least 1 mm of length;
- seedling length (millimeters, mm), measured using millimetric paper;
- seedling fresh weight (FW, grams, g), measured using an analytical weight scale (Mettler AJ100, Mettler Toledo, Germany).

Data are represented as mean ± standard deviation (SD) of at least three independent measurements.

3.2.3. Single Cell Gel Electrophoresis (SCGE)

Nuclei were extracted from *M. truncatula* radicles isolated from 7-days old seedlings (corresponding to each of the imposed treatments). For nuclei extraction, radicles were placed into 2 mL Eppendorf tubes with 50 µL Tris HCl EDTA (0.4 M Tris HCl pH 7.0, 1 mM EDTA pH 8) and frozen in liquid N_2 were chopped in a Petri dish placed on ice. Subsequently, 300 µL Tris HCl EDTA containing the extracted nuclei were collected and mixed with 200 µL 1% low melting point (LMP) agarose in phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Two drops (100 µL) of the resulting suspension were then pipetted onto glass slides coated with 1% LMP (prepared the day before and air-dried overnight at room temperature), covered with glass slips, and solidified on ice.

The two different versions of SCGE were used, namely alkaline and neutral. In the case of alkaline SCGE, samples are exposed to pH ≥ 10.0 and DNA unwinding takes place, allowing visualization of SSBs and DNA-protein crosslinks (Ventura et al., 2013). With neutral SCGE, lysis and electrophoresis are carried out at pH 8.3, a condition that prevents DNA unwinding, and consequently only DSBs can be detected. For the alkaline SCGE, the glass slides containing isolated nuclei were subjected to electrophoresis in an alkaline buffer (0.3 M NaOH, 1 mM EDTA pH >13) at 25 V and 300 A, for 25 min in a cold room (4°C) after 20 min of nuclei denaturation in the same buffer. For neutral SCGE, the slides were electrophoresed in TBE (Tris-borate-EDTA) (89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA, pH 8.3) at 20 V and 10 mA, for 8 min in the cold room (4°C) as well. Following electrophoresis, the slides were washed twice with Tris HCl pH 7.5 for 5 min and rinsed in 70% ethanol (v/v) for 12 min. All the wash steps were performed in the cold room (4°C). After an overnight drying at room temperature, the slides were stained with 20 µL 4',6- diamidino-2-phenylindole (DAPI, mg mL^{-1} stock solution) (Sigma-Aldrich) and visualized at a fluorescence microscope (Olympus BX51, Olympus, Germany) with an excitation filter of 340-380 nm and a barrier filter of 400 nm. For each slide, about one hundred nuclei were scored. The visible nuclei are classified into 5 different classes according to the length of their tails, which reflects the entity of the damage (Collins, 2004). The results were expressed in arbitrary units

(a.u) calculated according to the formula proposed by Collins (2004): [Σ(Nc × c) × 100]/Ntot, where Nc = number of nuclei of each class, c = the class number (e.g., 0, 1, 2), and Ntot = total number of counted nuclei. All analyses were performed in triplicates.

3.2.4. DNA diffusion assay

The DNA diffusion assay was performed to evaluate cell death events and distinguish cells subjected to PCD or necrosis from viable cells as described by Macovei et al. (2018). Nuclei extraction was performed from radicles of 7-days old seedlings using the same methodology described for SCGE analyses. The glass slides containing nuclei embedded in 1% LMP agarose were prepared as described in paragraph 3.2.3. Once prepared, the slides containing nuclei were incubated in high salt lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl pH 7.5) for 20 min in a cold room (4°C) to disrupt the nuclear membrane and permit DNA diffusion. Subsequently, the slides were immersed in neutral TBE for 5 min for three consecutive times to remove lysis solution and rinsed in 70% ethanol for 5 min at 4°C. In the last steps, the slides were rinsed in 70% ethanol for 5 min and subsequently in absolute (99.8%) ethanol for an additional 5 min and air-dried overnight. After drying, the slides were stored at room temperature under dark conditions until observation. To evaluate the nuclei morphology, the prepared slides were stained with 20 µL DAPI and observed with an Olympus BX51 fluorescence microscope with a 100W mercury lamp. The removal of H_2O molecules from both DAPI and DNA determines the emission of blue visible using a fluorescence microscope with an excitation filter of 340-380 nm and a barrier filter of 400 nm. For each slide, about one hundred nuclei were scored and each experiment was performed in triplicates. The overall cell death level was scored in arbitrary unit (a.u.) while an additional analysis was used to represent the percentage of each class of nuclei (0-nuclei from viable cells, 1-nuclei from PCD cells; 2-nuclei from necrotic cells).

3.2.5. RNA Extraction, cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from treated and untreated *M. truncatula* seedlings as described (Oñate-Sanchez & Vicente-Carbajosa, 2008). Briefly, plant material frozen in liquid N2 was ground using sterile clean mortar and pestle. The obtained powder was transferred into cold Eppendorf tubes containing 550 µL Extraction Buffer (0.4 M LiCl, 0.2 M Tris pH 8.0, 25 mM EDTA, 1% SDS in DEPC water) and 550 µL chloroform. The solution was mixed by vortex for 10 s. Then, the samples were centrifuged at 10000 rpm for 3min at 4°C. The obtained supernatant was transferred into a new tube with 500 µL of water-saturated acidic phenol. The solution was mixed by vortex for 10 s and 200 µL of chloroform was added. A subsequent centrifuge step was performed at 10,000 rpm for 3 min at 4°C. The supernatant was transferred into a new tube and a 1/3 volume of 8 M LiCl was added. The tube was mixed by inverting, incubated at 4°C for 1 h, and centrifuged at 10,000

rpm for 30 min at 4°C. The resulting pellet was washed with 70% ethanol and air-dry for 15 min in ice. Finally, the pellet was suspended in 30 µL of DEPC (diethyl pyrocarbonate) water. All the buffers were prepared with DEPC water. An electrophoretic analysis was performed to test the RNA integrity, in a cold room. The RNA was treated with DNase as follows: 1 µg of RNA was incubated with 1 U RNase-free DNase I (Thermo Scientific) in 10 µL of enzyme buffer, as indicated by the manufacturer. The DNase digestion was performed at 37°C for 30 min, then the enzyme activity was stopped by adding 1 µL of EDTA and further incubating for 10 min at 65°C. Subsequently, the amount of RNA was quantified with a NanoDrop spectrophotometer (Biowave DNA, WPA, ThermoFisher Scientific). cDNAs were obtained using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's suggestions.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the Maxima SYBR Green qPCR Master Mix (2X) (ThermoFisher Scientific) according to supplier's indications, using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics Pty Ltd., Brisbane, Queensland Australia). Amplification conditions were as follows: denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. Oligonucleotide primers were designed using Primer3Plus (https://primer3plus.com/) and further validated through the online software Oligo Analyzer (https://eu.idtdna.com/calc/analyzer). The list of investigated genes along with their Phytozome accession numbers, forward and reverse primers are given in **Table 1**.

Table 1 List of oligonucleotide sequences used for the qRT-PCR reaction.

GENE	ACCESION NO.	FW PRIMER (5'-3')	RV PRIMER (3'-5')
REFERENCE GENES			
MtELF1α	Medtr6g021805	GACAAGCGTGTGATCGAG	TTTCACGCTCAGCCTTAA
MtACT	Medtr3g095530	TCAATGTGCCTGCCATGTATG	ACTCACACCGTCACCAGAATC
MtTub	Medtr7g089120	TTTGCTCCTCTTACATCCCGTG	GCAGCACACATCATGTTTTTGG
MtUbi	Medtr3g091400	GCAGATAGACACGCTGGGA	AACTCTTGGGCAGGCAATAA
MtPDF2	Medtr6g084690	GTGTTTTGCTTCCGCCGTT	CCAAATCTTGCTCCCTCATC
MtPPRrep	Medtr6g079830	GGAAAACTGGAGGATGCACG	CAAGCCCTCGACACAAAACC
MtGAPDH	Medtr3g085850	TGCCTACCGTCGATGTTTCAGT	TTGCCCTCTGATTCCTCCTTG
GENES OF INTEREST			
MtTOR	Medtr5g005380	TGATGTTACCGTACGCCACT	TAAAGCGGCAAATACTGCAC
MtTop1α	Medtr0172s0010	ATACACGTGGGCTATTGTCG	TCACTTGGATGAATGCGTT
MtTop2	Medtr3g031040	AGGATCCGTCGTGGGATTGTAAGGC	ACAACAGAGAGGCCAGCCATAG
MtTDP1α	Medtr7g050860	ACGAGTTGGGAGTGCTCTTT	GGGATTTATCCTTCGATTGTTT
MtTDP2α	Medtr4g132300	CAGATGTTCAGCAAGGAACG	CCCGTCTTGCAAAGGATATT
MtTDP1β	Medtr8g095490	GGTTGGTTTGAGCCATCTTT	GCAGGCACATTGTGATTTCT
MtH4	Medtr2g096100	CCGTAAGGTGCTTCGTGA	CAAACCGCTTATACGCTT
MtPARP1	Medtr1g088375	AAACCCACCCTCCTTCGT	GTCCCTCGGTCTCTTTCC
MtNBS1	*Medtr5g076180*	*TGCAAACCCGATTTCAATAA*	*GATGAAATAAGCACGCATGG*
MtRAD50	Medtr3g084300	GGCGAGAAAGTTGTTTGCCTTAG	GCCAATTTGCTTCATCTTGA
MtERCC1	Medtr1g082570	CGTTCGTCAAATCCTCAGAA	TGAAGCTGCAGGAGCATTAT
MtMRE11	Medtr2g081100	TCCAAAGTGGTGCTGATGA	ATGGATTCATTGTCCGAACTG
MtMUS81	Medtr3g022850	AAGAAGCCACTGGATTGTTCC	ATTTGGATGGCTTCTGGAAA
MtCDKA1	Medtr4g094430	CGTCTTGAGCAGGAAGAT	TCCTGTGCTGCATTTCTT
MtCycD2	Medtr5g032550	GGCTCTTGATTGGATTT	ACAAGTCACACCTTCTGGA
MtCycB1	Medtr5g088980	AACTCATGGCGAGCTTTC	AGCAACAGCACAACGATC
MtCycD3	Medtr3g102310	ACAGCGTTGAGCCTAGTTTAG	TTCATACCCTGACCACAG
MtACYLTR	Medtr2g089765	CGCCTCTTGATCTTCCTTCAC	GAATCTCGAACCAAACCCGC
MtAGO1A	Medtr6g477980	TGACAGTGGCTCAATGACAA	GGGGTCTAACAGCAGCATTA
MtATUBC2	Medtr4g108080	TACGATGTTGCTGCGATTCT	TCACGCTTGTTCTCACTGAA
MtE2FE-like	Medtr4g106540	CAGGCGCCTTTATGATATTGC	AGCCACCTGAATGCTGGTTT
MtDNAM	Medtr1g086590	TGCATGCTTTCGTTAGGTGG	AGTTGAGTTCACTGCTGCTT
MtRAD54-like	Medtr5g004720	CGTTGCCAAAACAATGATGGG	AGCCTGCAATCTCAGCAAATC
MtSOG1A	Medtr5g053430	TGGTGCGAAGGGACAGATAA	TCACACAAGGACAATGCGTC
MtSOG1B	Medtr1g093680	GGAAGCCGAAAGCGTAGAAA	TTCTGAAGCCCGTTCAAGAG

The relative quantification was carried out using the *MtAct* (actin-related protein 4A, Medtr3g095530) and *MtELF1α* (elongation factor 1*α*, Medtr6g021805) as reference genes as they resulted as the most stable under the tested conditions following the geNorm (https://genorm.cmgg.be/, Vandesompele et al., 2002) analysis. For each oligonucleotide set, no-template water control was used. The raw, background-subtracted fluorescence data provided by the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics) was used to estimate PCR efficiency (E) and threshold cycle number (C_t) for each transcript quantification. The Pfaffl method (Pfaffl, 2001) was used for the relative quantification of transcript accumulation. All reactions were performed in triplicate. The data is presented as fold change (FC), where values for each treatment were normalized to their corresponding DMSO control. The heatmaps were constructed using the shinyheatmap software (http://www.shinyheatmap.com/, Khomtchouk et al., 2017) freely available online.

3.2.6. MicroRNA expression analysis

After isolation of total RNA (Oñate-Sanchez & Vicente-Carbajosa, 2008) from treated and untreated *M. truncatula* seedlings, a two-tailed qRT-PCR technique was

performed (Androvic et al., 2017). The expression profiles of 6 miRNAs (mtr-miR156a, mtr-miR168, mtr-mir172c-5p, mtr-miR2600e, mtr-mir395e, mtr-miR5741) were analyzed in 7-days old untreated and treated seedlings. Sequences of the mature miRNA oligonucleotides were obtained from the miRBase database (http://www.mirbase.org/, Kozomara et al., 2019). Different sets of primers were used to perform reverse transcription (RT) and qRT-PCR for each mature miRNA, one to synthesize the cDNA and two for the SYBR qPCR amplification). cDNAs were obtained using the qScript® Flex cDNA Synthesis Kit (QIAGEN, Beverly, Massachussets). The RT primers (**Table 2**) were designed to have a two-tailed structure as shown in **Fig. 15**. Basically, a two-tailed primer has the following fundamental functions: (1) it promotes the specific reverse transcription of the target miRNA template; (2) it provides additional sequence to the cDNA making it long enough for PCR amplification; (3) it includes the sequence of the forward PCR primer. Specifically, the two-tailed primers are made of two hemiprobes connected by a hairpin folding sequence of about 37 nucleotides. Following specific rules, the hairpin folding sequence can be developed but some of them have been already described (**Fig. 16**). The two hemiprobes are complementary to separate regions of the microRNA of interest. When designing the primer, the first 10 nt of the miRNA are added in reverse complementary order to the 5'-terminus, and the last 5/6 nt of the miRNA are added in reverse complementary order to the 3'-terminus of the hairpin. The 5'-hemiprobe promotes the discrimination between highly similar miRNA sequences, especially when the differing nucleotides are in the center or close to this terminus. The short 3'-hemiprobe provides high discriminatory power to mismatches at the 3'-terminus. RNAfold WebServer (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) online tool was used to predict the stable secondary structure. This stable structure is characterized by a high final melting temperature (65-70°C). To obtain the cDNA, a forward primer specific for the designed region in the 5'-terminus of the two-tailed RT-primer and a reverse primer specific for the miRNA target sequence, were used. Subsequently, qRT-PCR was performed as described in paragraph 3.4.1, and the obtained values were expressed as fold change to each relative DMSO control. The sequence of oligonucleotide primers used for qRT-PCR are presented in **Table 3**.

Table 2. List of two-tailed primers used for the RT reaction. Accession numbers, relative to miRbase database are indicated.

miRNA	ACCESION NO.	RT PRIMER (5'-3')
mtr-miR156a	MIMAT0001654	TCTTCTGTCAGCTTGAGTCCTCGTAGAGTTGCTACGAGATATGATAATGTGCT
mtr-miR168a	MIMAT0011089	CACCAAGCAACAACGACCAGAGCTAGAGAACCTAGCTCACCCACTACTTCCC
mtr-miR172c-5p	MIMAT0021265	ATGATGCTACCGACGAATACTGCTAGAGTTGCTAGCAGAGCCCTTAATGTGA.
mtr-miR2600e	MIMAT0021331	CACAATGCTTCAACGACCAGAGCTAGAGAACCTAGCTCACCCACTACGCCAA
mtr-miR395e	MIMAT0003858	ACACTTCATCAACGACCAGAGCTAGAGAACCTAGCTCACCCACTACGAGTTC
mtr-miR5741a	MIMAT0023118	TTAGTCCCTATCAAGCTCTCCAGGTACAGTTGGTACCTGACTCCACGCAAACC

Table 3. List of oligonucleotide sequences used for the qRT-PCR reaction.

miRNA	ACCESION NO.	FW PRIMER (5'-3')	RV PRIMER (3'-5')
mtr-miR156a	MIMAT0001654	CGATGCTACCGACGAATACTG	GCCATCATCATCAAGATTCACA
mtr-miR168a	MIMAT0011089	GCCACCAAGCAACAACGAC	GATGGTGCTGGTCGGGAA
mtr-miR172c-5p	MIMAT0021265	ATGATGCTACCGACGAATACTG	GTAGCATCATCAAGATTCACA
mtr-miR2600e	MIMAT0021331	CACAATGCTTCAACGACCAGAG	AAGCATTGTGGCATTGTGATTGGC
mtr-miR395e	MIMAT0003858	ACACTTCATCAACGACCAGAG	ATGAAGTGTTTGGGGGAACTC
mtr-miR5741a	MIMAT0023118	TTAGTCCCTATCAAGCTCTCCAG	TAGGGACTAAATTGATGGTTT

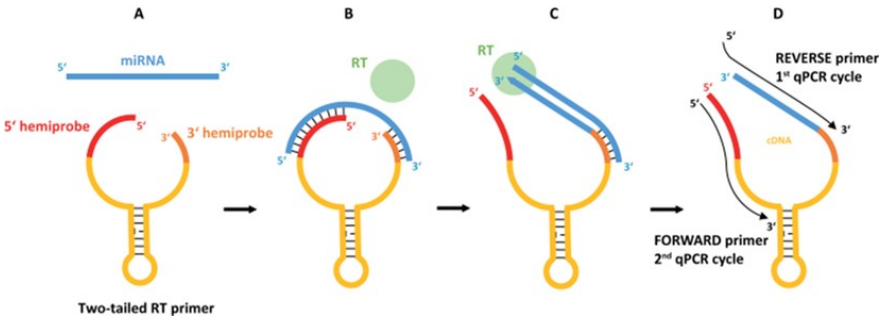

Fig. 15. Two-tailed qRT-PCR primer design. **(A)** Two-tailed RT primer is composed of two hemiprobes connected by a hairpin folding sequence. **(B)** The hemiprobes bind cooperatively, one at each end of the target miRNA, forming a stable complex. **(C)** Reverse transcriptase binds the 3′-end of the hybridized two-tailed RT primer and elongates it to form tailed cDNA. **(D)** The cDNA is amplified by qPCR using two target-specific primers (Androvic et al., 2017).

Fig. 16. Example of hairpin folding sequences useful as the backbone to design Two-tailed RT-primers

3.2.7. Experimental setup to test plant miRNAs *trans*-kingdom valence in human cells

To investigate miRNA trans-kingdom valence, *in vitro* experiments were set-up. *Malus domestica* cv. Golden Delicious together with human colorectal adenocarcinoma cell line HT-29, were chosen to gain insight on the ability of plant microRNAs to target human genes *in vitro*.

3.2.7.1. Identification of miRNAs abundantly expressed in *Malus domestica* cv. Golden Delicious

RNA was extracted from the *Malus domestica* cv. Golden Delicious and cv. Stark fruits and from a commercially available fruit mix using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, MA, USA), following the manufacture's instruction. Briefly, tissues were minced in small pieces using a scalpel. For each tissue, 1 gram of minced tissue was transferred into 15 mL conical centrifuge tubes containing 10 mL of Lysis/Binding Buffer (1:10 w/v), and the samples were further homogenized using Turrax homogenizator (Ika) until visible clumps were dispersed. Subsequently, miRNA homogenate additive was added to 1 mL of tissue lysate and the tubes were inverted and kept on ice for 10 min. The organic extraction was then performed by adding to the tubes a volume of acid-phenol:chloroform equal to the initial lysate volume. The solution was vortexed for 30-60 sec. A subsequent centrifuge step was performed at 10000 rpm at room temperature to separate the aqueous and organic phases. The aqueous (upper) phase was carefully removed without disturbing the lower phase and transferred into a fresh tube. The removed volumes have been noted and an enrichment procedure for small RNAs was carried out. This enrichment was accomplished by first immobilizing large RNAs on a filter cartridge (glass fiber filter). To do so, 1/3 volume of 100% ethanol was added to the aqueous phase recovered from the organic extraction and passed through the filter cartridge, thus immobilizing the large RNAs on it and collecting the flow-through containing mostly small RNA species. A centrifuge step was performed at 10000 rpm at room temperature to pass the mixtures through the filters that were then recovered and collected into new tubes. Then, 2/3 volume 100% ethanol was then added to these filtrates, and each mixture was passed through a new filter cartridge to immobilize small RNAs. A centrifuge step to favor the passage of the filtrates was performed at the above-mentioned conditions and the filtrates discarded. The filter was then washed a few times (with a specific wash solution supplied by the manufacturer), and the small-RNA enriched samples were eluted. Subsequently, the amount of miRNA was quantified with Epoch2 spectrophotometer (BioTek).

cDNAs were obtained using the TaqMan™ MicroRNA Reverse Transcription Kit (ThermoFisher Scientific) according to the manufacturer's suggestions. qRT-PCR was performed in 96-well optical reaction plates with the TaqMan™ Universal Master Mix II, NO UNG, and TaqMan™ MicroRNA Assay (ThermoFisher Scientific) using a Quantum5 machine (Thermo Fisher Scientific, MA, USA). All reactions were conducted in triplicate. Different sets of primers were used to perform reverse transcription (RT) and qRT-PCR for each mature miRNA, one to synthesize

the cDNA and two for the TaqMan qPCR amplification. The Assay name along with their Assay ID and the catalog number are illustrated in **Table 4**. Pre-validated TaqMan MicroRNA Assay was used for all the miRNAs investigated (ath-miR159a, ath-miR160a, ath-miR166a, ath-miR390a, ath-miR396b) except miR-482a-3p and miR-858 which were specifically designed using the Custom TaqMan Small RNA assay design tool (Thermo Fisher Scientific, MA, USA). Amplification conditions were as follows: polymerase activation at 95 °C for 10 min, and 40 cycles of 95 °C for 15s and 60 °C for 1 min. Quantitative normalization was performed using U6 as an internal control. Relative quantification was performed using the ΔΔCT method.

Table 4. Assays used for the TaqMan qPCR amplification.

Assay name	**Assay ID**	**Catalog No.**
ath-miR159a	000338	4427975
ath-miR160a	000341	4440886
ath-miR166a	000347	4427975
ath-miR390a	001409	4427975
ath-miR396b	000367	4440886
mdm-mir482a-3p	476846_mat	4440886
mdm-miR858	472525_mat	4440886

3.2.7.2. Artificial miRNAs delivery to human colorectal adenocarcinoma cells

The human colon cancer cell line, HT-29 cells (ATCC, MI, Italy) were grown in McCoy's added with 10% FBS and 1% Pen/Strep and kept at 37°C with 5% CO_2. Artificial microRNAs mimicking specific plant miRNAs (miRNA-mimic, Thermo Fisher Scientific, MA, USA) were transfected into the HT-29. Data relating to miRNA-mimics are shown in **Table 5.** MirVana™ miRNA Mimic, Negative Control #1, random sequence miRNA mimic molecule extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function, was used as a negative control. The miRNA-mimics were resuspended at the final concentration of 5 μM with Nuclease Free Water, before their use.

Table 5. Products used for transfection analyses.

Product	**miRBase ID**	**Assay ID**	**Catalog #**
mirVana™ miRNA Mimic, Negative Control #1			4464058
mirVana® miRNA mimic	mdm-miR858	MC25773	4464066
mirVana® miRNA mimic	mdm-miR482a-3p	MC25918	4464066

TransIT-X2 Dynamic Delivery System (Mirus Bio LLC) was used to transfect miRNA-mimics into HT-29 cells following the manufacturer's instructions.

Transfection was carried out on a 12-well multiwell plate, according to the scheme in **Table 6.**

Table 6. Experimental design of multiwell plates used for transfection.

HT29 +miRCTRL	HT29 +miRCTRL	HT29 +miRCTRL	Medium
HT29 +miR482	HT29 +miR482	HT29 +miR482	Medium
HT29 +miR858	HT29 +miR858	HT29 +miR858	Medium

Briefly, 100 µl Optimem Serum, 11 µl miRNA-mimic (5uM), and 3 µl TransIT-X2 were added into a 1.5 mL sterile Eppendorf tube, pipetted gently to mix completely the solution and incubated at room temperature for 15-30 min in dark conditions to obtain the TransIT-X2:miRNA complexes. Subsequently, 50000 cells were resuspended in 1 mL medium and added to each well. Thus, 1.1 mL of the TransIT-X2:miRNA complexes previously prepared was added to the well containing the suspended cells. An incubation step at 37°C, 5%CO_2 was performed for 72 h. After incubation, cells were harvested and assessed for knockdown of target gene expression.

RNA extraction was carried out using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's suggestion, to obtain both the fraction enriched with microRNA and the fraction of total RNA. To evaluate the transfection success TaqMan qRT-PCR was performed following the same procedure described in paragraph 3.2.6.1.

3.2.7.3. qRT-PCR of miRNA putative targets in HT-29 lines

Total RNA was isolated using MiRVana, following the manufacture's instruction. To avoid possible DNA contamination, RNA was treated with DNAase (Thermo Fisher Scientific, MA, USA). cDNA was synthesized by retro-transcribing 1 µg of total RNA in a total volume of 100 µl using High Capacity DNA Kit (Thermo Fisher Scientific, MA, USA) according to the manufacture's suggestions. The sequences of primers used to detect mRNA expression levels are listed in **Table 7.** These genes were identified as putatively targeted by *M. domestica* selected miRNAs through RNAhybrid using miRNA sequences retrieved from miRBase database and human 3'UTRome sequence dataset. qRT-PCR assays were performed in 96 well optical reaction plates using the QuantStudio5 machine (Thermo Fisher Scientific). The assays were conducted in duplicate wells for each sample. The following reaction mixture per well was used: 5 µl Power Sybr Green (Thermo Fisher

Scientific, MA, USA), 1.2 µl of primers at the final concentration of 150 nM, 0.8 µl RNase free water, 3 µl cDNA. Amplification conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s then at 60°C for 60 s. Quantitative normalization was performed using *Cyclophilin* and *GAPDH* as reference genes. Relative quantification was performed using the ΔΔCT method.

Table 7. List of oligonucleotide sequences used for the qRT-PCR reaction.

Gene	**Primer Sequence**
Cyclophilin	FW 5'TTTCATCTGCACTGCCAAGA3' RV 5'TTGCAAAACACCACATGCT3'
GAPDH	FW 5'CAACTTTGGTATCGTGGAAGGAC3' RV 5'ACAGTCTTCTGGGTGGCAGTG3'
RXRα	FW 5'GAGCCCAAGACCGAGACCTA3' RV 5'AGCTGTTTGTCGGCTGCTT3'
SMAD3	FW 5'CCTACCACTACCAGAGAGTAGAGACACC3' RV 5'ATCTCTGTGTGGCGTGGCA3'
IL4R	FW 5'CTG ACC ACG TCA TCC ATG AG3' RV 5'GTG GAA GAT GAA TGG TCC CA3'
PROM1	FW 5'CAT CCA AAT CTG TCC TAA GAA CG3' RV 5'TCC ATC AAG TGA AAC CTG CAA3'
ROCK2	FW 5'CTC GCC CAT AGA AAC CAT CAC3' RV 5' GGC ACG TGT ATG AAG ATG GAT3'

3.3. Statistical analyses

For statistical analysis, data were subjected to Analysis of Variance (ANOVA) and the statistical significance of mean differences was determined using the Student's *t*-test. All analyses were performed in triplicates.

4. Results

4.1. A bioinformatics workflow to assess *trans*-kingdom miRNAs and DDR players

Because of the high availability of information and advances in high-performance computing technologies, big data analyses are emerging as descriptive and predictive tools to be used on a massive amount of data to formulate intelligently informed hypotheses (Kashyap et al., 2016). This massive data can be put to use in multiple fields, ranging from personalized medicine (Alyass et al., 2015) to industrial microbiome applications (van den Bogert et al., 2019).

In recent times, much focus is being directed towards *trans*-kingdom investigations. As so, it worth mentioning the current pandemic crisis where viruses from animals are transmitted to humans resulting in situations difficult to control and manage also given the amount of big data generated worldwide. Besides this specific case, other examples concerning the plant kingdom can be cited. For instance, recent evidence of a trans-kingdom plant disease complex between a fungus *Verticillium dahliae*) and plant-parasitic nematodes belonging to the genus *Pratylenchus* has been presented using machine learning methods (Wheeler et al., 2019). Moreover, addressing the model system used in the present work of thesis, *Medicago truncatula*, an ambitious project has reported that engineering trans-kingdom signaling in plants can result in controlling the expression of genes in rhizosphere symbiotic bacteria (Geddes et al., 2019). The concept of cross-kingdom miRNA transfer, thoroughly presented in the Introduction part (section 1.5), can be as well included in these examples. To address these issues, complex systems biology approaches, advanced computerized methods, and trans-kingdom networks are needed to allow an appropriate identification of causal relationships between different taxonomic groups (Rodrigues et al., 2018). Additionally, bioinformatics tools can be also put to use to investigate the evolutionary relationships among different *filla* and kingdoms. In this case, phylogenetic trees (based upon multiple sequence alignments of proteins from different species) are regularly used to figure out evolutionary relationships between homologous sequences, thus providing insights into the evolution of a protein family and the functional specificity of the members of the family (Palidwor et al., 2006).

Based on these premises, the results presented in this part of the thesis using different bioinformatics approaches implemented to address questions relative to the potential of plant miRNAs to target genes in human cells, predictions of plant miRNAs possibly involved in DDR, and an evolutionary perspective of the key master-regulator of DDR in plants, namely the Suppressor of Gamma-ray 1 (SOG1).

4.1.1. *In silico* data mining and development of networks and alignment analyses between plants and humans

Starting from a list of 426 *M. truncatula* miRNA deposited in miRase, it was possible to predict their targets (using psRNATarget and RNAHybrid, respectively) as 3,468 *M. truncatula* transcripts (2,680 unique transcript and 2,083 unique genes) and 936 *H. sapiens* target transcripts (825 unique transcripts and 758 unique genes). Additionally, 2,297 *M. truncatula* target transcripts (1,739 unique transcripts and 1,376 unique genes) and 2,226 human target transcripts (1,754 unique transcripts and 1,549 unique genes) were investigated through the alignment strategy. The number of targets was tuned to obtain a balanced number of elements between the two species. The target genes could be associated with one or more than one miRNA (**Fig. 17**).

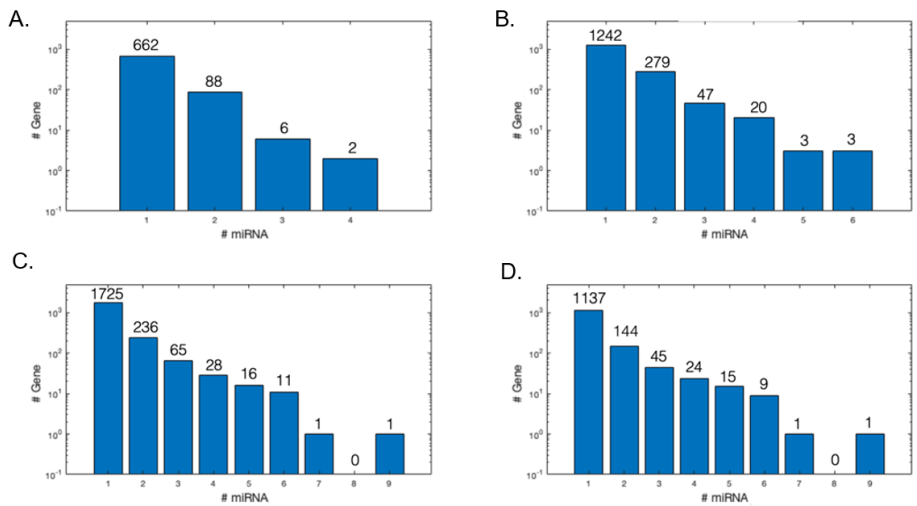

Fig. 17. *Homo sapiens* and *Medicago truncatula* miRNA targets found in the two bioinformatics pipelines. Bars represent the numbers of predicted genes that are targeted by one or more miRNAs. **(A)** Gene targets derived from *H. sapiens* network-based pipeline and **(B)** alignment-based pipeline. **(C)** Gene targets derived from the *M. truncatula* network-based pipeline and **(D)** alignment-based pipeline. The y-axis is shown in logarithmic scale to better visualize the bars with a low number of target genes.

The network-based approach was used to focus on the biological processes enriched among the genes targeted by the set of *M. truncatula* miRNAs. Because the GeneMania tool used to build networks does not have *M. truncatula* as an available organism, an additional step had to be carried out to map these genes to the *Arabidopsis thaliana* genome. Among the 50,894 *M. truncatula* genes and the 27,416 *A. thaliana* genes annotated in PhytoMine, 18.763 *M. truncatula* genes (37 % of the genome) were mapped in 12,537 *A. thaliana* genes (46% of the genome). As for humans, the construction of the network was straightforward with the GeneMania tool. The resulting gene networks obtained based on these inputs were composed of 704 nodes for *A. thaliana* and 753 nodes for *H. sapiens* (**Fig. 18**).

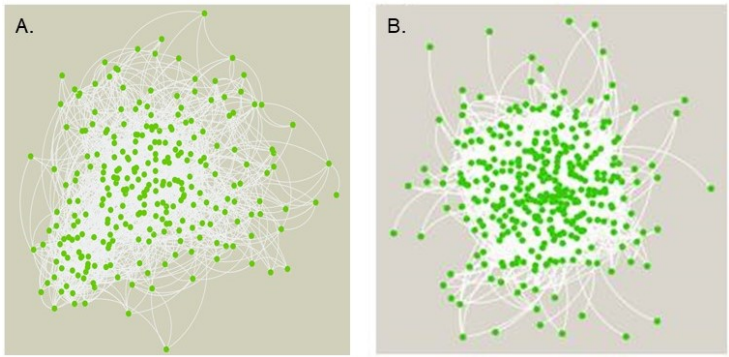

Fig. 18. Coexpression networks obtained from GeneMania tool for **(A)** *A. thaliana* and **(B)** *H. sapiens*.

Unlike the network-based pipeline in which over-represented biological processes were searched in the network of all the miRNA target genes, the alignment-based approach focused on sequence similarities among the targets of a given miRNA. In this approach, the analysis included alignments of every single targeted gene (and corresponding protein sequence) between *M. truncatula* and *H. sapiens*, resulting in a total number of 9,626 alignments. By applying a threshold *p*-value of 0.05 for nucleotide alignments, 2,735 sequences corresponding to 115 miRNAs, resulted significant. These miRNAs were predicted to target a total of 315 genes in *M. truncatula* and 801 genes in *H. sapiens*. When considering both the gene and protein sequences, 242 similarities between plant and human transcripts were found, accounting for 93 genes (targeted by 54 miRNAs) in *M. truncatula* and 149 in *H. sapiens*.

Giving the absence of *M. truncatula* organism in readily usable bioinformatics tools for network development and analysis, the construction of a new *M. truncatula* co-expression network (using publicly available gene expression microarray datasets) was pursued. An expression panel of 24,777 genes was obtained and used to build a genome-scale co-expression network with 62,857 undirected edges (**Fig. 19A**). Among the 2,083 predicted target genes, 1,251 were mapped in this network, resulting in a subnetwork of 6,081 nodes and 9,534 edges (**Fig. 19B**). The giant component of this sub-network included 5,943 nodes (of which 1,208 were target genes) and 9,405 edges (of which 3,102 were direct interactions among miRNA target nodes). The clustering procedure found 45 clusters which were analyzed via enrichment analysis (**Fig. 19C**).

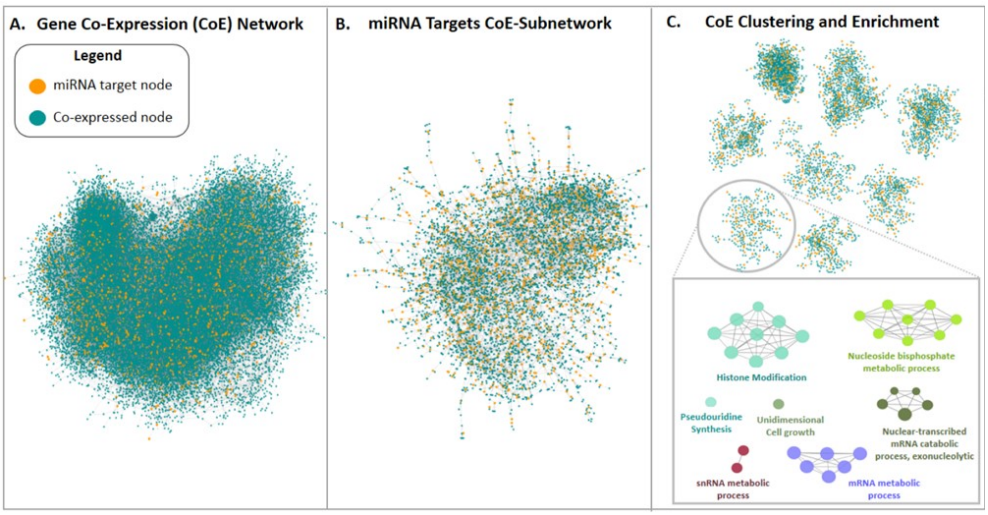

Fig. 19. Construction of the *M. truncatula* co-expression network using a customized network-based approach. **(A)** Genome-scale co-expression network and **(B)** miRNA targets network are shown, where blue nodes represent genes not found among miRNA targets, while orange nodes are miRNA targets. **(C)** A representative set of the clusters resulted from the miRNA targets network analysis. Each cluster was analyzed via enrichment analysis using ClueGO. (Bellato et al., 2019)

4.1.2. *M. truncatula* miRNAs putatively target common functions in plants and humans

The generated datasets were used to identify common functions putatively targeted by *M. truncatula* miRNAs in plant and human cells by comparing the Gene Ontology (GO) nominatives. When considering identically named GO terms between *A. thaliana* and *H. sapiens*, the common biological functions included 'vesicle docking involved in exocytosis' (GO:0006904), 'modulation by virus of host morphology or physiology' (GO:0019048), 'cellular response to virus' (GO:0098586), 'positive regulation of posttranscriptional gene silencing' (GO:0060148), and 'branched-chain amino acid metabolic process' (GO:0009081) (**Table 8**). The identification of identical terminology underlines the evolutionarily conserved functions between distant species. Besides the identical GO terminologies, other common processes were present in both networks (e.g. nucleic acid and amino acid metabolism, response to stress, signaling).

When comparing the obtained biological processes between *M. truncatula* and *H. sapiens* from the point of view of the *de novo* network reconstruction, these are shown to be related to exocytosis, DNA replication, transcription, and modifications, amino acid activation and transport, RNA related processes, histone modification, and protein modifications (**Table 9**).

On the other hand, when analyzing the alignment-based approach, the genes involved in similar functions between *M. truncatula* and *H. sapiens* were mainly related to transcription factors, hormone-responsive elements, and cell division (**Table 10**).

By comparing the network-based and alignment-based approaches (**Table 8** vs. **Table 9**), it is possible to observe that the results do not always overlap. To focus on one specific example, mtr-miR168a (part of a highly conserved plant miRNA family, abundantly found in plant tissues and extensively studied from the point of view of trans-kingdom approach) targets are shown in **Table 11** using both approaches. However, drawing attention to the 'response to virus' function, it is possible to observe that this GO term was identified in both approaches, as demonstrated by the common predicted target gene PVR (Poliovirus Receptor). Therefore, the results obtained show the network-based and the alignments approaches are complementary rather than equivalent.

Table 8. Common biological processes shared between *A. thaliana* and *H. sapiens* resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	GO ID	*A. thaliana*		*H. sapiens*	
		Gene	miRNA	Gene	miRNA
vesicle docking involved in exocytosis	GO:0006904	EXO70B1 EXO70D1 EXO70H7 KEU SEC5A SEC8	mtr-miR5244 mtr-miR2653a mtr-miR397-5p mtr-miR5559-3p mtr-miR7698-5p mtr-miR2679a	SNPH	mtr-miR399t-5p
modulation by virus of host morphology or physiology	GO:0019048	AGO2 DCP2	mtr-miR2673a mtr-miR5238 mtr-miR2655b	BCL2L11 KPNA4	mtr-miR5273 mtr-miR169k
cellular response to virus	GO:0098586	AGO1 SDE3	mtr-miR168a mtr-miR168c-5p mtr-miR2592a-3p mtr-miR2592bm-3p	BCL2L11 PUM2 RIOK3	mtr-miR5273 mtr-miR160c mtr-miR160a
positive regulation of posttranscriptional gene silencing	GO:0060148	DRD1	mtr-miR2650	FXR1 PUM2	mtr-miR482-3p mtr-miR160c
branched-chain amino acid metabolic process	GO:0009081	BCAT3 CSR1	mtr-miR5212-3p mtr-miR2660	BCKDK IVD	mtr-miR5273 mtr-miR2640

Table 9. Common biological processes shared between *M. truncatula* and *H. sapiens* resulted from the de novo reconstruction of the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*M. truncatula*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
Exocytosis	GO:0006887	Medtr4g102120 Medtr8g023330	mtr-miR5559-3p mtr-miR5558-3p	GO:0006887 GO:0006904	SNPH RIMS3 SYT1 SYT2 NOTCH1 RAB3GAP1 RPH3AL SYT15	mtr-miR399t-5p mtr-miR482-5p mtr-miR5211 mtr-miR2640 mtr-miR5266 mtr-miR5209 mtr-miR2589 mtr-miR166d
DNA replication, transcription, and modifications	GO:0006261 GO:0090329	Medtr4g106540	mtr-miR5741a	GO:0090329 GO:2000104 GO:0006268 GO:0044030 GO:2000678 GO:0032786	INO80 LIG3 HMGA1 GRHL2 PER2 SIN3A BRD4	mtr-miR399t-5p mtr-miR5294a mtr-miR5276 mtr-miR2589 mtr-miR169k mtr-miR156b-3p mtr-miR5266
Amino acid activation and transport	GO:0043038 GO:0043039 GO:0006418	Medtr7g083030	mtr-miR2657	GO:0009081 GO:0009083 GO:0051955 GO:0051957 GO:0009065	BCKDK IVD PER2 RAB3GAP1 NTSR1 TINAGL1 NANOS2 PRODH	mtr-miR5273 mtr-miR2640 mtr-miR169k mtr-miR5209 mtr-miR408-3p mtr-miR166d mtr-miR160c mtr-miR169d-3p
RNA related processes	GO:0016071 GO:0006397 GO:0008380 GO:0000375 GO:0000377 GO:0000398	Medtr3g077320	mtr-miR2629f	GO:0050686 GO:0006376 GO:0061014 GO:0061157 GO:0050686	CELF1 CELF2 GIGYF2 TNRC6B KHSRP MEX3D RNPS1 SUPT5H	mtr-miR2670f mtr-miR399t-5p mtr-miR166d mtr-miR5211 mtr-miR398b mtr-miR2673a mtr-miR398b
Histone modification	GO:0016570 GO:0016573	Medtr1g086590 Medtr4g108080	mtr-miR395e mtr-miR156a	GO:0043981 GO:0043982	KANSL1	mtr-miR482-5p
Protein modifications	GO:0043543 GO:0006473 GO:0006475 GO:0018394 GO:0018393	Medtr1g086590	mtr-miR395e	GO:0018345 GO:0006517 GO:0036507 GO:0036508 GO:0042532	CLIP3 ZDHHC18 MARCH6 UGGT1 NF2	mtr-miR527 mtr-miR168b mtr-miR390 mtr-miR5270a mtr-miR5206b

Table 10. *M. truncatula* miRNAs and their putative target genes are related to similar functions in *M. truncatula* and *H. sapiens* as revealed by the alignment-based approach. The genes and their respective accessions are provided for each organism.

mtr-miRNA	*M. truncatula*		*H. sapiens*	
	Accession	Gene	Accession	Gene
mtr-miR166d	Medtr2g086390	ABA response element-binding factor	NM_006484	DYRK1B
mtr-miR160a	Medtr5g061220	auxin response factor	NM_175914	HNF4A
mtr-miR2673a	Medtr2g014260	zinc finger C-x8-C-x5-C-x3-H type protein	NM_001170538	DZIP1L
mtr-miR2673a	Medtr4g082580	WRKY transcription factor 3	NM_021973	HAND2
			NM_032772	ZFN503
mtr-miR164b	Medtr2g078700	CUP-shaped cotyledon protein, putative	NM_001099694	ZNF578
	Medtr4g108760		NM_001040653	ZXDC
mtr-miR164d	Medtr3g435150	NAC transcription factor-like protein	NM_001018052	POLR3H
mtr-miR5287b	Medtr7g088980	cell division cycle protein-like/CDC48 protein	NM_001277742	CYP26B1

Table 11. Predicted genes targeted by mtr-miR168 in human cells as revealed by the network-based and alignment-based approaches.

Network-based approach			Alignment-based approach	
Predicted target	Gene description	GO Term	Predicted target	Gene description
ST8SIA1	Sialyltransferase 8A	sphingolipid biosynthetic process	CDAN1	Codanin 1
RGS6	Regulator of G Protein Signaling 6	regulation of G-protein coupled receptor protein signaling pathway	NISCH	Nischarin
IL18RAP	Interleukin 18 Receptor Accessory Protein	positive regulation of natural killer cell mediated immunity	CEMP1	Cementum Protein 1
PVR	Poliovirus Receptor	positive regulation of natural killer cell mediated immunity	PVR	Poliovirus Receptor
SYN2	Synapsin 2	neurotransmitter secretion	HTRA3	HtrA Serine Peptidase 3
PPFIA1	Protein Tyrosine Phosphatase Receptor Type F Polypeptide-Interacting Protein Alpha-1	regulation of actin filament bundle assembly	ZNF710	Zinc Finger Protein 710
ZDHHC18	Zinc Finger DHHC Domain-Containing Protein 18	protein palmitoylation		
B3GAT1	Beta-1,3-Glucuronyltransferase 1	glycosaminoglycan biosynthetic process		

4.1.3. Putative microRNAs signatures in DDR from a *trans*-kingdom perspective

DDR-associated functions were investigated considering that information relative to miRNAs targeting this essential process is still scarce, especially in plants. Each approach provided different sets of information some of which were used in subsequent analysis. Some examples are reported in **Table 12** which summarize a series of processes related to the DDR pathway and downstream processes putatively targeted by *M. truncatula* miRNAs in both plant and animal kingdoms. This data shows that some genes that are involved in DNA repair are also involved in chromatin remodeling, emphasizing the connection between the two processes. For example, the network-based approach showed that *A. thaliana* DME (Demeter) and DML1 (Demeter-like 1) are associated with both DNA repair (BER-base excision repair, GO:0006284) and chromatin modification-related functions (GO:0006306, GO:0044728). In human cells, PPP4C (Protein Phosphatase 4 Catalytic Subunit) is known for its involvement in different processes among which DNA damage checkpoint signaling and regulation of histone acetylation (Zhou et al., 2002; Lee et al., 2010). Other genes and respective miRNAs and GO Terms are related to functions such as cell cycle and senescence in plants (or aging in animals). In plants, genes identified as involved in such functions are ASF1B (Anti-Silencing Function 1B, histone chaperone) and KU80 known for their role in the S-phase replication-dependent chromatin assembly (Zhu et al. 2011) and maintenance of genome integrity (West et al., 2002), respectively. In human cells, the SIN3A (Histone Deacetylase Complex Subunit Sin3a) and HMGA1 (High Mobility Group Protein A1) genes, have roles associated with chromatin regulation and cell cycle progression (Silverstein & Ekwall, 2004; Pierantoni et al., 2015).

Within the alignment-based approach, mtr-miR2589 was predicted to target the *M. truncatula* Medtr6g047800 (tRNA methyltransferase complex GCD14 subunit) and the *H. sapiens* SETD1A (SET Domain Containing 1A, Histone Lysine Methyltransferase), functions involved in chromatin organization in both organisms (**Table 13**). The alignment-approach also led to the identification of a conserved miRNA (mtr-miR319d-5p) predicted to target genes associated with cell death functions in both *M. truncatula* (DCD-development and cell death domain protein) and *H. sapiens* (MESD, PRR5L). Another interesting finding is represented by mtr-miR2600e. This miRNA is predicted to target an anthocyanin acyltransferase (Medtr2g089765) in *M. truncatula* and the UVSSA (UV Stimulated Scaffold Protein A) gene in *H. sapiens* and so is potentially related to antioxidant functions both in plants and humans (response to UV irradiation in plants and transcription-coupled nucleotide excision repair in humans).

Table 12. Biological processes related to DNA repair, chromatin remodeling, cell cycle, and cellular senescence common to *A. thaliana* and *H. sapiens* resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*A. thaliana*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
DNA repair	GO:0006284 GO:0045003 GO:0000724	DME DML1 AT1G75230 RAD54 RECA1 ASF1B GMI1 KU80	mtr-miR2086-3p mtr-miR2651 mtr-miR5240 mtr-miR172c-5p mtr-miR5558-3p mtr-miR1509a-3p mtr-miR169l-3p mtr-miR5272f	GO:2000779	FOXM1 PPP4C	mtr-miR169d-3p mtr-miR169k
Chromatin remodeling	GO:0006306 GO:0044728 GO:0006305 GO:0006304 GO:0031056 GO:0031058 GO:0031060 GO:0031062 GO:1905269 GO:1902275	DME DML1 DRD1 EMB2770 SDG14	mtr-miR2086-3p mtr-miR2651 mtr-miR2650 mtr-miR7696c-5p mtr-miR2650 mtr-miR2086-3p mtr-miR7696c-5p	GO:0043981 GO:0043982 GO:0070828 GO:0031507 GO:0031936 GO:0006268 GO:0044030 GO:0031935 GO:0031937	KANSL1 HMGA1 TNRC18 GRHL2 PHF2 SIN3A ZNF304	mtr-miR482-5p mtr-miR5276 mtr-miR2589 mtr-miR2589 mtr-miR160c mtr-miR156b-3p mtr-miR166e-5p
Cell cycle	GO:0000075 GO:0045930 GO:0007093	ASF1B RAD9	mtr-miR1509a-3p mtr-miR2638b	GO:1901989 GO:1901992 GO:1902751 GO:0010971 GO:0071157	BRD4 EIF4G1 PHB2 SIN3A MDM2 MDM4	mtr-miR5266 mtr-miR166d mtr-miR5266 mtr-miR156b-3p mtr-miR169k mtr-miR5266
Cellular senescence	GO:0000723	KU80 TRB1	mtr-miR5272f mtr-miR5558-5p	GO:2000772	ABL1 HMGA1 VASH1	mtr-miR5276 mtr-miR5276 mtr-miR160c

Table 13. Examples of mtr-miRNAs and their putative target genes in *M. truncatula* and *H. sapiens* as revealed by the alignment-based approach. The genes and their respective accessions are provided for each organism.

mtr-miRNA	*M. truncatula*		*H. sapiens*	
	Accession	Gene	Accession	Gene
mtr-miR2600e	Medtr2g089765	anthocyanin 5-aromatic acyltransferase	NM_020894	UVSSA
mtr-miR5285b	Medtr8g105290	nuclear pore complex Nup155-like protein	NM_000370	TTPA
mtr-miR319d-5p	Medtr4g134770	translation elongation factor EF1B, gamma chain	NM_015154	MESD
	Medtr4g084080	DCD (development and cell death) domain protein	NM_001160169	PRR5L
mtr-miR2589	Medtr1g103100	40S ribosomal protein S3a-1	NM_003565	ULK1
	Medtr6g047800	tRNA methyltransferase complex GCD14 subunit	NM_014712	SETD1A
mtr-miR482-5p	Medtr5g079860	23S rRNA m2A2503 methyltransferase	NM_014747	RIMS3
			NM_012318	LETM1
mtr-miR5286b	Medtr4g038400	ribosomal protein S12/S23 family protein	NM_001010858	RNF187

The results obtained through the three developed bioinformatic approaches were used as a starting point to identify and investigate conserved and function-specific miRNAs and their putative targeted genes such as those involved in DDR-related processes like DNA repair and chromatin remodeling. Specifically, the following miRNAs and genes were taken into account for further analyses:

1) mtr-miR156a, identified as putatively targeting *MtATUBC2* (ubiquitin-conjugating enzyme, Medtr4g108080), involved in histone modification processes;
2) mtr-mir172c-5p, putatively targeting *MtRAD54-like* (DNA repair and recombination RAD54-like protein, Medtr5g004720), involved in DSBs repair;
3) mtr-miR2600e, putatively targeting *MtACYLTR* (anthocyanin 5-aromatic acyltransferase, Medtr2g089765), involved in antioxidant defence;
4) mtr-mir395e, putatively targeting *MtDNAM* (DNA methyltransferase 1-associated protein, Medtr1g086590), associated with histone modifications;
5) mtr-miR5741a, putatively targeting *MtE2FE-like* (E2F transcription factor-E2FE-like protein, Medtr4g106540), involved in DNA-dependent DNA replication;
6) mtr-miR168, targeting *MtAGO1A* (argonaute protein 1, Medtr6g477980), involved in the cellular response to virus both in plant and human, and use as a control since the relation between this miRNA and target bene are already experimentally validated.

Hence, together with the identification of microRNAs putatively targeting common functions in plants and humans, the *in silico* analysis paved the way for subsequent molecular analysis of miRNAs signatures in plant DDR.

4.2. Bioinformatic investigation of the *SOG1* gene family in plants

The SOG1 transcription factor has been widely investigated in the model plant *A. thaliana*. The knowledge is still scanty in agricultural species and other plant models, including *M. truncatula*. Considering the central role played by SOG1 as DDR effector and the importance of legumes as an essential food and feed resources, *in silico* investigation was performed to identify and characterize SOG1 putative homologs in *M. truncatula* model legume. Subsequently, the search for putative SOG1 homologs in other species was performed as well. A phylogenetic tree was constructed to analyze the relationship between different taxa.

4.2.1. Identification of SOG1 homologs in *M. truncatula*

The search performed in Phytozome allowed the retrieval of two *MtSOG1* genes in *M. truncatula*, identified as the accessions Medtr5g053430 and Medtr1g093680. The former shows a higher percentage of similarity (70.2%) to *AtSOG1* than the latter (63.9 %). For this reason, Medtr5g053430 was labeled as *MtSOG1A* and Medtr1g093680 as *MtSOG1B*. The *MtSOG1A* is localized on chromosome 5 while the *MtSOG1B* on chromosome 1. The genomic sequence of *MtSOG1A* is 4743 bp

long while the length of its transcript, coding, and peptide sequence is 1744 bp, 1329 bp, and 442 aa, respectively. Concerning *MtSOG1B*, its genomic sequence is 5664 bp long whereas the length of its transcript sequence 1889 bp, and its coding and peptide sequences are 1446 and 481 aa long. **Fig. 20** shows the transcript organization and chromosome location for the two *MtSOG1* genes in comparison with the well-known *A. thaliana AtSOG1*. It is thus possible to observe that all three genes have six functional exons (orange boxes). Since two SOG1 homologs were identified in *M. truncatula*, several bioinformatic tools were used to investigate their degree of similarity among themselves. The alignment of the MtSOG1A and MtSOG1B amino acid sequences shows that the two sequences have a high percentage of similarity, namely 74.8%. A schematic representation of the alignment between the two sequences is shown in **Fig. 21,** along with evidencing the presence of conserved protein domains. The NAM (No Apical Meristem) domain and the serine-glutamine (SQ) motifs on the C-terminal region are shown in green and orange boxes, respectively. In the MtSOG1A (445 aa) and MtSOG1B (481 aa) sequences, the NAM domain is located starting from aa 59 to aa 197, respectively. From literature, it is known that the C-terminal region of AtSOG1 protein sequence contains five SQ motifs which are located at positions 350, 356, 372, 430, 436, respectively (Yoshiyama et al., 2013). These motifs are the preferred target for phosphorylation by human ATM and ATR kinases. The alignment of the MtSOG1A and MtSOG1B peptide sequences with AtSOG1 showed that these SQ motifs also present in *M. truncatula*. The SQ motifs are located at positions 342, 348, 364, 425, and 431 at the C-terminal region of MtSOG1A.

Fig. 20. Schematic representation of gene organization as evidenced in the Phytozome (https://phytozome.jgi.doe.gov/) genome browser. (**A**) *AtSOG1*. (**B**) *MtSOG1A*. (**C**) *MtSOG1B*. Exons are presented as orange boxes.

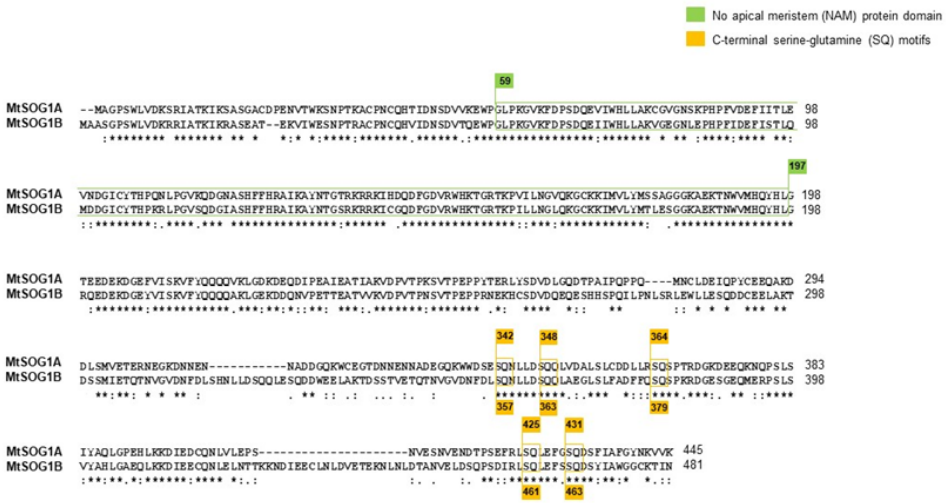

Fig. 21. Alignment of MtSOG1A and MtSOG1B protein sequences performed using ClustalW web tool. The presence of NAM (No Apical Meristem) and SQ (Serine, Glutamine) motifs is evidenced in green and orange boxes, respectively.

4.2.2. Putative protein-protein interaction networks associate MtSOG1A and MtSOG1B with DDR

To investigate the putative function of MtSOG1A and MtSOG1B, putative protein-protein interaction networks were performed using STRING. Together with the two proteins identified in *M. truncatula*, the well-characterized *Arabidopsis* AtSOG1 sequence was also used for this search. The results of this analysis are shown in **Fig**. **22A** and **22B**, for *A. thaliana* and *M. truncatula*, respectively. In the case of *M. truncatula*, it is important to underline the fact that both sequences led to the generation of the same network (**Fig. 22B**), thus corroborating the high sequence similarity. The ATR (Rad3-related protein), ERCC1 (Excision Repair 1), GTF2HA (General Transcription Factor 2HA) and WEE1 (G2 Checkpoint Kinase) proteins appear both in the AtSOG1 and MtSOG1A/B-derived protein-protein interaction networks. Nevertheless, some proteins are present in one network and not in the other. This is the case of FEN1 (Flap endonuclease 1). This structure-specific nuclease has a 5'-flap endonuclease and a 5'-3' exonuclease activity, being involved in DNA replication and repair. During DNA replication, it cleaves the 5'-overhanging flap structure that is generated by displacement synthesis when DNA polymerase encounters the 5'-end of a downstream Okazaki fragment. It enters the flap from the 5'-end and then tracks to cleave the flap base, leaving a nick for ligation. Also involved in the long patch base excision repair (LP-BER) pathway, by cleaving within the apurinic/apyrimidinic (AP) site-terminated flap (Shen et al., 1996). The presence of putative protein interactors linked to the transduction of DNA damage signals (ATR), DNA repair pathways (FEN1), and cell cycle checkpoint

regulation (WEE1), suggest that the *in silico* identified MtSOG1 proteins have a role in DDR.

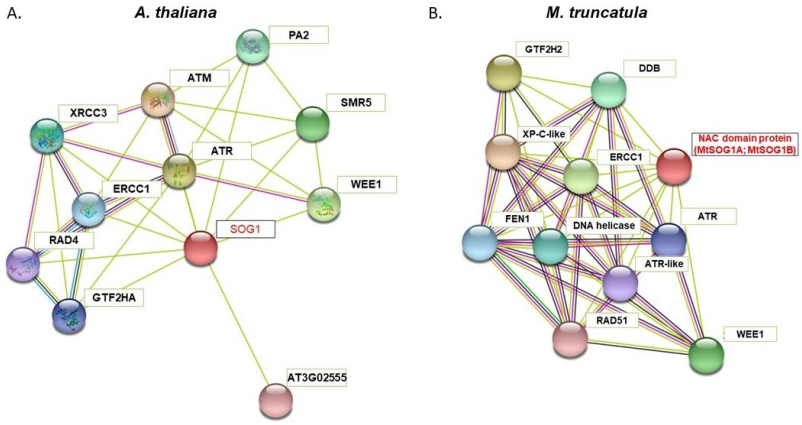

Fig. 22. Putative protein-protein interaction networks generated by STRING for (**A**) AtSOG1 and (**B**) MtSOG1A/B proteins.

4.2.3. Phylogenetic tree of SOG1 homologs across different plant taxa

The BLASTP alignment of AtSOG1 peptide sequence against the sequences of all the species available on Phytozome v.12 allowed the retrieval of 72 SOG1 putative homologs from 49 species. The subsequent alignment of the retrieved peptide sequences through MAFFT led to the selection of 69 gene models. Some accessions were discarded because of the low similarity to AtSOG1 and/or the lack of SQ motifs at the C-terminal end. The retained sequences were used to generate a phylogenetic tree by employing the MEGAX software. This phylogenetic tree, presented in **Fig. 23**, shows the relationship between different plant taxa based on the sequence similarities of SOG1 homologs. According to the phylogenetic tree, most of the Phytozome database classification, based on plant evolutionary phylogeny, is maintained as concerns with the distribution and features of the SOG1 sequences. For example, all the members of *Brassicaceae* family (*A. helleri*, *A. lyrata*, *A. thaliana*, *B. stricta*, *B. oleracea capitata*, *B. rapa*, *C. grandiflora*, *C. rubella*, *E. salsugineum*) are clustered together. The same it is evidenced for the *Fabidae* family (*F. vesca*, *G. max*, *M. domestica*, *M. truncatula*, *P. Persica, P. vulgaris*), except for *C. sativus*. In this particular case, it is also necessary to underline the fact that two clusters are observed in the *Fabidae* clade: one of the legumes (*G. max*, *M. truncatula* and *P. vulgaris)* and the other one for fruit trees and plants (*F. vesca, P. Persica, P. vulgaris*). Nevertheless, the clades generated based on SOG1 sequences are highly similar to what is expected from taxonomy. To this purpose, it is worth mentioning that the *Fabidae* Group generally includes: (1) the nitrogen-fixing clade (Rosales, Fabales, Cucurbitales, and Fagales); (2) Zygophyllales; and (3) a weakly supported clade of Celastrales, Oxalidales, and Malpighiales (Endress & Matthews, 2006).

For the majority of species, it is possible to observe the presence of only a single SOG1 homolog (gene model), except for *B. rapa*, *G. max*, *K. fedtschenkoi*, *K. laxiflora*, *L. usitatissimum*, *M. domestica*, *M. esculenta*, *M. truncatula*, *P. thricocarpa*, *P. virgatum*, *S. italica*, *S. purpurea*, *S. viridis*, which presented multiple homologs. Among these, *G. max*, *K. laxiflora*, *P. virgatum* are the species with the highest number of putative SOG1 sequences (4 gene models).

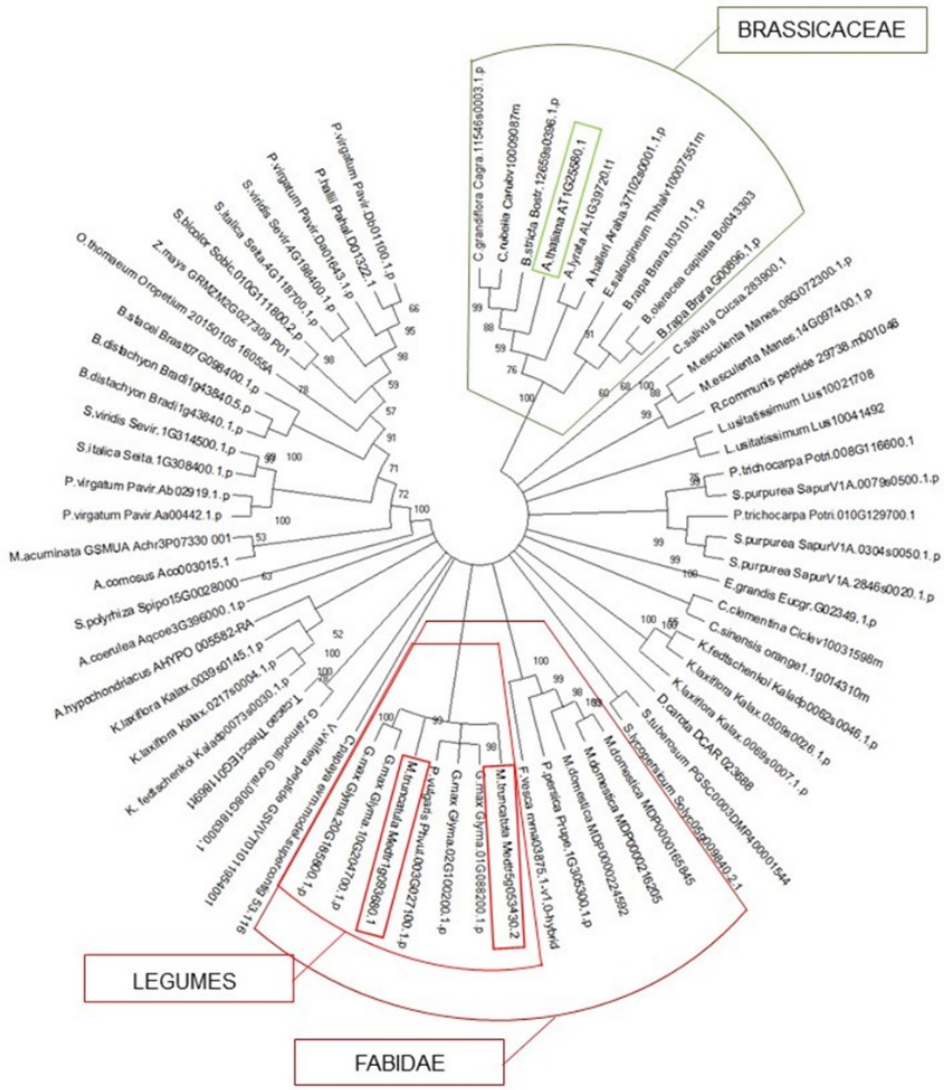

Fig. 23. Phylogenetic analysis of SOG1 proteins in 49 plant species present within the Phytozome database. The phylogenetic tree was constructed using MEGAX using the Neighbor-Joining (NJ) method. The *Brassicaceae* and *Fabidae* families are evidenced in green and red, respectively.

4.3. *In planta* experimental setting

In the previous chapter, the proposed bioinformatics approaches have led to the identification of *M. truncatula* miRNAs putatively targeting genes involved in DDR-associated pathways. To evaluate if these miRNAs may be involved in the regulation of DDR, the first step consisted of setting up an original experimental system. Moreover, the fact that *M. truncatula* possesses multiple SOG1 homologs, possibly representing master-regulators of DDR, allows their use as indicators for the efficiency of the designed experimental system. Hence, the next subchapters of the results will present how this novel system was developed and all the analyses performed to prove that the types of conditions hereby used affect the level of the DDR pathway.

Due to the crucial role that DDR plays in maintaining genome stability, DNA damage dynamics, and DNA repair pathways, it represents a promising field of research to study seed quality. Within the context of seed germination, where active cell proliferation is determinant for the development of healthy seedlings, DNA damage must be repaired before the start of cell division to ensure the generation of robust plants. Although considerable progress has been made in recent years, the role of DDR and DDR-associated pathways during seed germination is still poorly understood, reason why we decided to focus on this fundamental aspect of plant development. To implement an experimental setting that induces genotoxic stress and subsequent DDR activation, two chemical agents were taken into consideration: camptothecin (CPT), an inhibitor of Top1 enzyme, and NSC120686, an inhibitor of the human TDP1 enzyme.

4.3.1. Development of the experimental setup

The CPT and NSC120686 inhibitors require to be dissolved in DMSO, which, at certain concentrations, can impair plant development (Zhang et al., 2016c). Thus, it was necessary to first identify the CPT concentration at which minimal or null DMSO effects are evident at the phenotypic level. In view of this, three different concentrations, namely CPT 25 μM (CPT_1), 50 μM (CPT_2), and 100 μM (CPT_3), along with their corresponding DMSO concentrations (DMSO 0.29%, DMSO_1, 0.58%, DMSO_2, and 1.16%, DMSO_3) were tested during *M. truncatula* seed germination. Seeds are considered germinated when radicle protrusion reaches at least 1 mm. Biometrical analyses, consisting of measuring the seed germination percentage (%), seedlings length, and fresh weight (FW), were used to establish the phenotypic effect of CPT and DMSO after 7 days of treatment (**Fig. 24**). No significant differences ($P > 0.05$) between NT and treatments were observed regarding germination percentage (**Fig. 24A**), as germination rates varied between 44.4±31.68% (DMSO_3) and 82.2±8.39% (NT). This indicates that treatments at the imposed DMSO and CPT concentrations do not affect germination percentage. Nonetheless, growth inhibition was observed in seedlings at the end of the seventh 7th day. A significant ($P < 0.05$) decrease in seedling length is evident when comparing NT (56.27±1.35 mm) with CTP_1 (13.73±0.19 mm), CTP_2

(12.87±0.46 mm), and CTP_3 (12±0.17 mm) treatments (**Fig. 24B**). Among the tested DMSO concentrations, only DMSO_3 (36.53±8.24 mm) appeared to negatively affect seedling growth in a significant manner. When considering the seedling fresh weight (FW), no significant differences were observed between NT and treatments (DMSO, CTP), as all samples weighted between 0.12 - 0.22 g (**Fig. 24C**). These analyses allowed the identification of an optimal CPT concentration, able to cause an evident phenotypic effect, considering also its corresponding DMSO concentration in such a way to not cause any phenotypic effect on seedling growth. Hence, the concentration of CPT that met these requirements was 25 µM dissolved in 0.29% of DMSO. The subsequent experiments were carried out using this concentration.

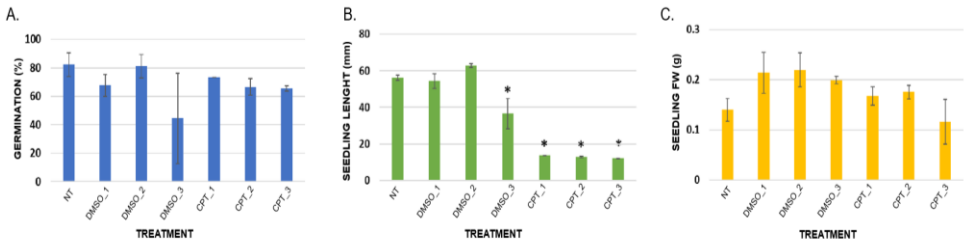

Fig. 24. Biometric analyses to evaluate the phenotypic effect of camptothecin (CPT) and dimethyl sulfoxide (DMSO) treatments on 7-day-old *Medicago truncatula* seedlings. **(A)** Germination percentage (%). **(B)** Seedling length (mm). **(C)** Seedling fresh weight, FW (g). Data are represented as mean ± standard deviation of three independent replicates. Statistically significant ($P < 0.05$) differences between treatments and non-treated (NT) samples are represented with an asterisk (*). CPT, camptothecin; DMSO, dimethyl sulfoxide.

Regarding the selection of the NSC120686 concentration, this was performed based on previous results obtained using the agent on *M. truncatula* calli (Macovei et al., 2018) and still considering the minimal amount of DMSO used for the dissolution of the agent. Consequently, the selected concentration of NSC120686 was 25 µM (NSC) dissolved in 0.17% DMSO.

The last treatment within the proposed experimental design consists of synergistically exposing *M. truncatula* seeds to 25 µM CPT and 25 µM NSC120686 (denominated as CPT+NSC), dissolved in 0.23% DMSO. As described in "Materials and methods, each corresponding DMSO concentrations, hereby denominated DMSO_N, DMSO_C, and DMSO_CN, were also tested together with the non-treated (water) control (NT).

4.3.2 CPT and NSC treatments have no effects on seed germination but affect the development of *M. truncatula* seedlings

To verify whether CPT and NSC influence seed germination, a phenotypic characterization was performed by evaluating the germination percentage (%), seedling length (mm), and fresh weight (g) after 7 days of treatment (**Fig. 25**).

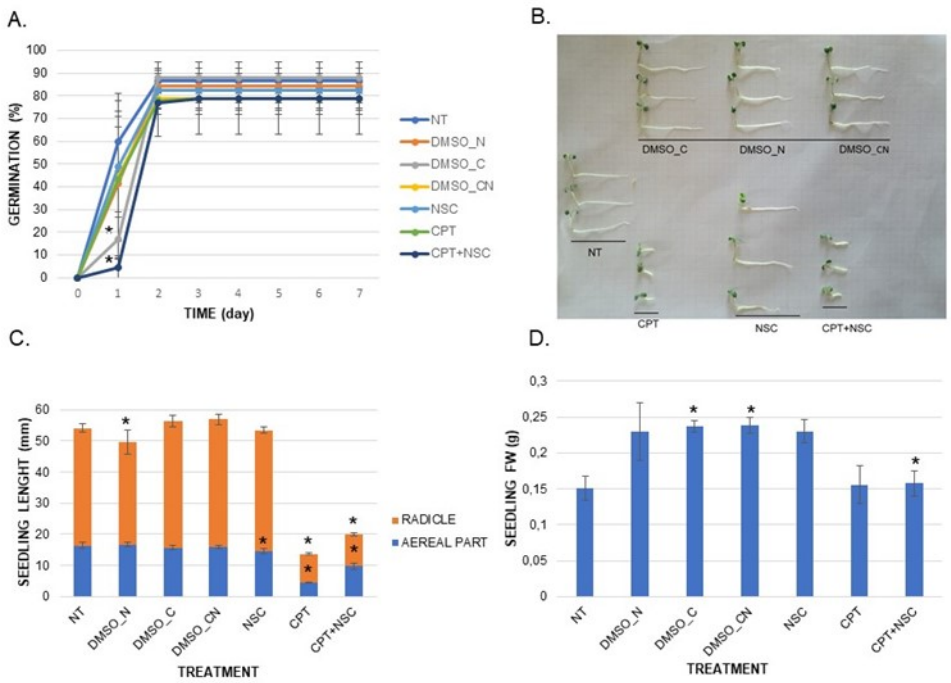

Fig. 25. Biometric analyses to evaluate the phenotypic effect of CPT, NSC, and CPT+NSC treatments and corresponding DMSO concentrations (DMSO_N, DMSO_C, DMSO_CN) on *Medicago truncatula* seed germination. **(A)** Germination percentage (%). **(B)** Representative image of 7-days old seedlings. **(C)** Seedling length (mm). **(D)** Fresh weight, FW (g). Data are represented as mean ± standard deviation of three independent replicates. Statistically significant ($P < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; DMSO, dimethyl sulfoxide.

When comparing the non-treated (NT, 60.00±13.33%) to the treated samples, a significant decrease in the germination rate was observed only on the first day for the DMSO_C (16.66±12.01%) and CPT+NSC (4.44±5.09%) treatments. The high standard deviations in the germination percentage bars are indicative of non-uniform seed germination. No significant differences were observed during the following days until the end of the experiment. The maximum percentage of germinated seed (plateau) was reached between the second and third day with a mean germination rate spanning from 78.8±5.09% (CPT) to 87.77±6.94% (DMSO_C) **(Fig. 25A)**. These analyses suggest that imposed treatments do not influence the germination process per se. **Fig. 25B** shows the morphology of the 7-days old seedlings, grown in the presence of CPT, NSC, CPT+NSC, and their corresponding DMSO controls (DMSO_N, DMSO_C, DMSO_CN). Treatment with the NSC inhibitor did not result in a visible change in seedling morphology while seedlings treated with CPT and CPT+NSC appear shorter and stockier than control seedlings. Significant differences are registered when measuring the seedling length and fresh weight (FW) (**Fig. 25C** and **25D**). Both parameters show a similar trend in terms of reduced seedling length and fresh weight caused by CPT and the combination CPT+NSC.

The comparison between NT and these treatments indicated that radicles were more severely affected than the aerial parts (**Fig. 25C**). Regarding seedling development, the average of the aerial part and the radicle length was reduced also in NSC and DMSO_N treated samples, respectively. Significant differences relative to the fresh weight (FW) of the seedlings were detected for DMSO_C and DMSO_N in terms of increased weight, as well as for the NSC+CPT-treated samples in terms of decreased weight (**Fig. 25D**).

In conclusion, the imposed treatments do not affect germination percentage but have a major effect on seedling development. Indeed, significant differences were revealed for seedling length and FW parameters, showing similar results regarding the impairment of seedlings growth caused by CPT and CPT+NSC treatments. It may be hypothesized that CPT contributed the most to the impairment of the seedling growth since a lesser effect was observed when the NSC agent was delivered alone.

4.3.3. The imposed treatments induce different cell death profiles

A DNA diffusion assay was performed to evaluate cell mortality in 7-day-old *M. truncatula* seedlings subjected to CPT and NSC treatments. **Fig. 26A** shows representative images of nucleus morphology characteristic for viable (class 0), PCD (class 1), and necrotic (class 2) events. For each treatment, the results of the diffusion assay were expressed both as percentage of nuclei per class (**Fig. 26B**), and arbitrary units (a.u.) to express the overall level of mortality (**Fig. 26C**).

The data show that the NT and DMSO_N samples are both characterized by high percentage of viable nuclei (86.36 ± 6.00%, NT; 83.63 ± 3.16%, DMSO_N) and low percentage of PCD (11.36 ± 2.00%, NT; 13.70 ± 3.65%, DMSO_N) and necrosis (2.27 ± 1.00% ; 2.68 ±0.00%, DMSO_N). Seedlings treated with DMSO_C and DMSO_CN show a decrease in viable nuclei (47.60 ± 3.40%, DMSO_C; 55.74 ± 4.74%, DMSO_CN) towards PCD, while the nuclei classified as belonging to necrotic cells (class 2) are not present. Nuclei classified as class 2 are mostly present in CPT and CPT+NSC samples, while the NSC treatments evidence the presence of class 1 nuclei characteristic for PCD events (**Fig. 26B**). Concerning the NSC- and CPT+NSC-treated samples, there is a more marked decrease in viable nuclei (27.18 ± 6.76% NSC; 31.52 ± 11.18 %, CPT+NSC) and an increase in nuclei subjected to PCD (52.12 ± 5.49%, NSC; 46.53 ± 12.77 %, CPT+NSC) and necrosis (27.38 ± 6.20 % 21.96 ± 6.20 %). Similarly, a reduction in viable nuclei is observed for CPT-treated samples (21.05±2.91), where the most represented nuclei belong to class 2 (57.13 ± 6.82 %), characteristic for the presence of necrotic events.

When considering the overall level of mortality, an increasing trend is noted passing from NT to CPT (**Fig. 26C**). Mortality progressively increases in samples treated with DMSO_C (FC=2.78), DMSO_CN (FC=3.29), NSC (FC=5.10), CPT (FC=8.56). The highest level of mortality was observed in seedling treated with CPT (136.08±6.11 a.u.).

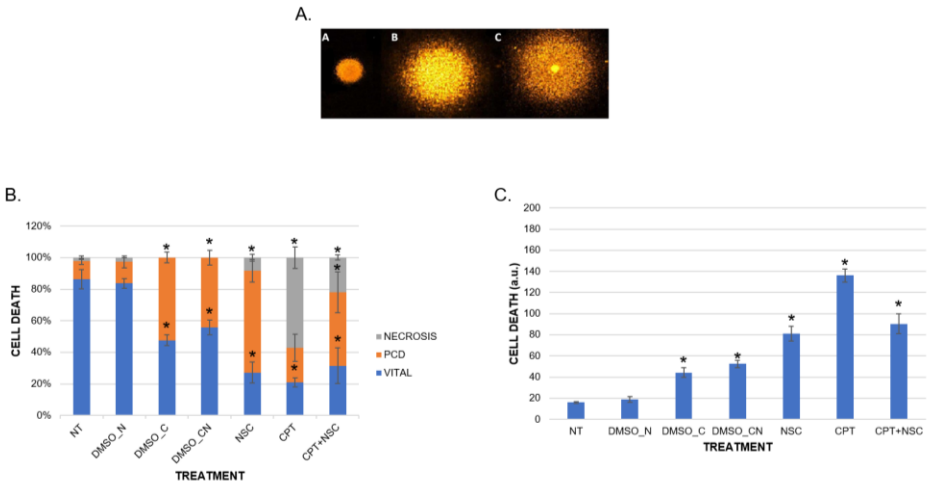

Fig. 26. The level of cell death induced by the imposed treatments in *M. truncatula* 7-days old seedlings. **(A)** Nucleus morphology and its related class identification number. **(B)** Cell mortality represented as percentage of nuclei per class. **(C)** Cell mortality scores represented as arbitrary units (a.u.). Values are expressed as mean ± standard deviation of three replicates. Statistical significance, as per the Students t-test, is shown with an asterisk (*, $P < 0.05$). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; DMSO, dimethyl sulfoxide.

Briefly, the NSC, CPT, and CPT+NSC treatments decrease cell vitality and induce different cell death events. The most severe effects are observed in camptothecin treatment characterized by a high level of necrosis. This finding represents a step towards understanding the aberrant phenotype of seedlings treated with the CPT inhibitor.

4.3.4. Comet assay reveals the presence of different types of DNA damage

To quantitatively measure DNA damage, SCGE was performed using both the alkaline and neutral versions of the assay. Within the neutral version (performed at a neutral pH), the DNA is kept as double strands so that the assay is used to detect double-stranded DNA breaks (DSBs). In contrast, the alkaline version is carried out at pH ≥ 10.0 and a denaturing step is included so that different types of breaks can be revealed, namely, SSBs are formed from alkali-labile sites, DNA-DNA or DNA-protein cross-links (Ventura et al., 2013). The results of these analyses are shown in **Fig. 27**. Representative images for each comet class (0 to 4) are provided **(Fig. 27A).** Concerning the NT (13.68±0.00 a.u and 10.86±4.49 a.u under alkaline and neutral conditions, respectively), the NSC treated samples showed a 7.22-fold increase in the level of DNA damage in alkaline condition while only a 1.99-fold increase was observed in neutral conditions. A 5.86- and 5.79-fold increase in the level of DNA damage was observed in CPT treated samples in alkaline and neutral conditions, respectively. The CPT+ NSC treated samples showed a 13.7-fold increase in the

level of DNA damage in alkaline conditions while an 8.4-fold increase was detected under neutral condition.

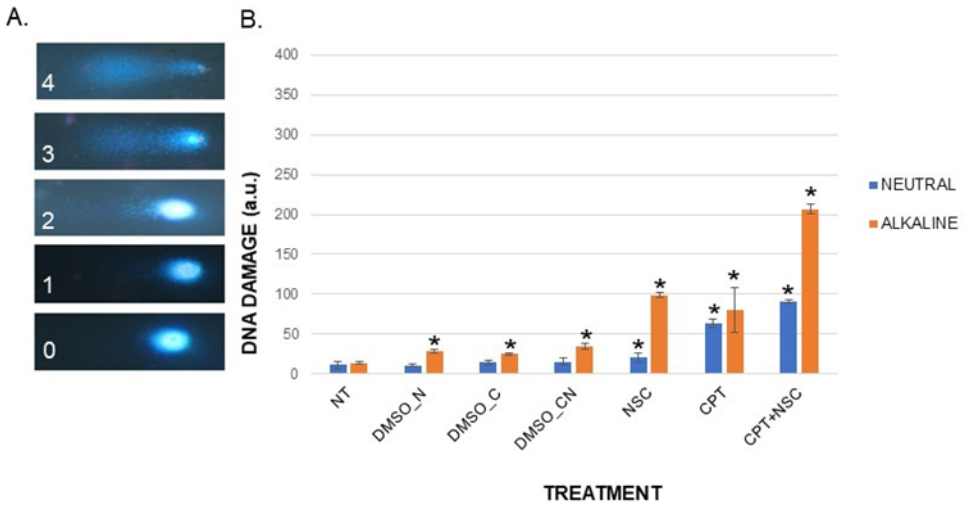

Fig. 27. DNA damage induced by the imposed treatments in *M. truncatula* 7-days old seedlings. **(A)** Nucleus morphology and its related class identification number. (B) DNA damage scores represented as arbitrary units (a.u.). Values are expressed as mean ± SD of three replicates. Statistical significance, as per the Students t-test, is shown with an asterisk (*, $P < 0.05$). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; DMSO, dimethyl sulfoxide.

Considering the DMSO controls, no significant differences in the accumulation of DNA damage as DSBs are evident under neutral conditions. However, a small but significant increase in the levels of DNA damage was registered under alkaline conditions. This may suggest that DMSO, could generate SSBs, alkali-labile sites, incomplete excision repair sites, DNA-DNA/DNA-protein crosslinks rather than more extensive damage like DSBs. As regards the NSC and CPT+ NSC treatments, both revealed a major accumulation of SSBs formed from alkali-labile sites, DNA-DNA, or DNA-protein cross-links. The non-significant differences in DNA damage found by comet assay performed in alkaline and neutral conditions in CPT-treated samples suggest the occurrence of similar SSBs and DSBs levels in the sample treated with this inhibitor.

Overall, the observed results indicate that the administration of CPT/NSC agents cause an accumulation of both SSBs and DSBs, but at different levels depending on the type of treatment. While in the case of NSC, SSBs and associated damage types are prevalent, for the CPT treatments an additional increase in the presence of DSBs is observed. The combination of the two agents (CPT+NSC) resulted in the highest level of DNA damage, combining both DSBs and SSBs, the latter being prevalent.

4.3.5. Identification of suitable reference genes for qRT-PCR analyses

Given that CPT/NSC treatments resulted in reduced seedling growth, increased cell mortality, and accumulation of DNA damage, the next step consisted in the analyses of expression profiles of DDR-related genes using qRT-PCR. Before starting the experiment, it was necessary to identify the most stable reference genes under the imposed conditions to be used for the relative quantification of the transcripts. To this purpose, a geNorm analysis was carried out to evaluate the stability of the following endogenous genes: *MtPDF2* (protodermal factor 2), *MtGAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *MtPPRep* (prolyl endopeptidase), *MtTub* (tubulin), *MtUbi* (ubiquitin), *MtAct* (actin), and *MtElf1α* (ETS-related transcription factor). The expression level of these six internal control genes was evaluated in the seven different samples derived from non-treated seedlings, DMSO-treated, and CPT/NSC-treated samples. GeNorm is a popular algorithm used to determine the most stable reference genes from a set of tested candidate reference. The program enables the elimination of the worst-scoring housekeeping gene (the one with the highest M value) and recalculation of new M values for the remaining genes to determine the most stable gene (the one with the lower M value) (Vandesompele et al., 2002). The results of this analysis showed that *MtAct* and *MtElf1α* had the most stable expression under the imposed conditions (**Fig. 28**).

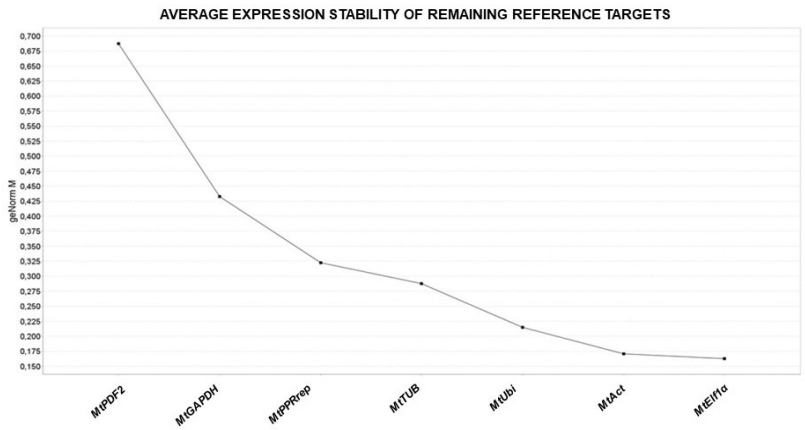

Fig. 28. GeNorm analysis for the selection of reference genes. The gene expression normalization factor (geNorm M) was calculated for each sample based on the geometric mean of the reference genes. The cDNA extracted from treated and non-treated 7-day-old *M. truncatula* seedlings was used for this analysis.

After the selection of reference genes, it was possible to proceed with the investigation of the following genes:
(1) the first group of genes is constituted by the *in silico* identified *MtSOG1A* and *MtSOG1B* genes. Similar to what reported for *AtSOG1*, we hypothesize that these genes may have a crucial role in the regulation of DDR downstream pathways;

(2) the second group is composed of *MtTDP1α*, *MtTDP1β*, *MtTDP2α*, *MtTop1α*, *MtTop2*; these are the genes that encode proteins directly affected by the CPT and NSC inhibitors;
(3) the third group including *MtMRE11*, *MtRAD50*, *MtNBS1*, *MtPARP1*, *MtERCC1,* and *MtMUS81*; these genes are involved in several DNA repair pathways, considered as alternative to the function of *TDP1* genes;
(4) the fourth group is made up of *MtTOR*, *MtCDKA1*, *MtCycB1*, *MtCycD2*, *MtCycD3*, *MtH4* genes, known to regulate the cell cycle;
(5) the fifth group of genes is constituted by *MtACYLTR*, *MtAGO1A*, *MtATUBC2*, *MtDNAM*, *MtE2FE-like,* and *MtRAD54-like* genes identified through the bioinformatics pipeline as involved in DDR-related processes and putatively targeted by specific *M. truncatula* microRNAs.

4.3.6. *MtSOG1* genes are upregulated in response to CPT/NSC treatments

Given the importance of SOG1 protein as DDR effector, the expression profiles of *MtSOG1A* and *MtSOG1B* genes have been investigated as they may reflect the effective impact of CPT/NSC treatments on DDR.

In the previously shown analyses, the used DMSO concentrations did not influence seed germination and seedling development, but a minimal negative effect on cell viability and DNA damage accumulation was observed. Thus, it was necessary to consider a possible effect of DMSO on the expression of the selected genes. **Fig. 29A** represents the data relative to mean values ± SD of the expression levels in water-treated samples (NT) and the three used DMSO concentrations (DMSO_N, DMSO_C, DMSO_CN). The figure clearly shows changes in the expression profiles of *MtSOG1A* and *MtSOG1B* genes in the DMSO samples compared to NT. For example, *MtSOG1A* seems to be downregulated by DMSO_N and upregulated by DMSO_CN, while *MtSOG1B* seems to be downregulated by all the DMSO concentrations used to dissolve the NSC and CPT compounds. It is also important to note that in the non-treated control (NT) samples, *MtSOG1B* is more expressed than *MtSOG1A*.

To investigate the effect of CPT/NSC treatments, the relative expression values obtained for *MtSOG1* genes are presented as fold-change (FC) to each correspondent DMSO concentration (DMSO_N for NSC, DMSO_C for CPT, and DMSO_CN for CPT+NSC) (**Fig. 29B**). The results show that both *MtSOG1A* and *MtSOG1B* genes are upregulated in response to the imposed treatments. Nonetheless, the degree of upregulation is different between the two genes during the treatments. While the *MtSOG1A* gene expression is highly induced by the NSC treatments, the *MtSOG1B* gene expression is highly triggered by the CPT treatment. The expression levels observed for *MtSOG1B* were compared to the expression levels obtained for *MtSOG1A* (FC to MtSOG1A). Specifically, the level of *MtSOG1B* transcript is 2.64-fold higher than *MtSOG1A* in seedlings treated with CPT inhibitor.

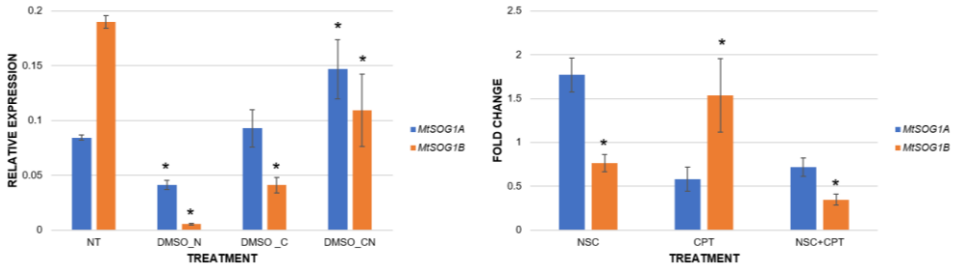

Fig. 29. Expression profiles of *MtSOG1A* and *MtSOG1B* genes in 7-days-old *M. truncatula* seedlings. (A) Relative gene expression levels in non-treated (NT) and DMSO-treated samples. **(B)** Gene expression profiles reported as fold-change to DMSO controls, during CPT, NSC, and CPT+NSC treatments. Values are expressed as mean ± SD of three replicates. Statistical significance, as per the Students *t*-test, is shown with an asterisk (*, $P < 0.05$). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; DMSO, dimethyl sulfoxide.

The concomitant exposure to NSC+CPT had a reduced effect on gene expression than the individual treatments; the genes are still upregulated compared to their DMSO controls, but at lower levels: 0.72±0.10 FC and 0.35±0.06 FC for MtSOG1A and MtSOG1B, respectively.

The observed expression patterns of the two *MtSOG1* genes indicate that the imposed treatments have a significant effect on the DDR pathway. Furthermore, the opposite behavior of *MtSOG1A* and *MtSOG1B* genes in response to different treatments suggests that they can be selectively activated in relation to specific stimuli.

4.3.7. CPT/NSC treatments induce differential expression of genes involved in DDR downstream pathways

Subsequently, genes involved in specific DDR downstream pathways (e.g. DNA damage, cell cycle) were taken into consideration to evaluate their expression patterns in response to CPT/NSC treatments. As summarized above, these included *MtTDP1α*, *MtTDP1β*, *MtTDP2α*, *MtTop1α*, and *MtTop2*, as genes that encode proteins directly affected by the CPT and NSC inhibitors, several genes specifically involved in MtTDP1-alternative DNA repair pathways (*MtMRE11*, *MtRAD50*, *MtNBS1*, *MtPARP1*, *MtERCC1,* and *MtMUS81*) and genes involved in cell cycle regulation (*MtTOR*, *MtCDKA1*, *MtCycB1*, *MtCycD2*, *MtCycD3*, *MtH4*).

Also, in this case, since the expression of the tested genes was affected in presence of DMSO (**Fig. 30**), results are presented as fold-change (FC) to each respective DMSO control so that it would be possible evaluate the effect that CPT/NSC treatments.

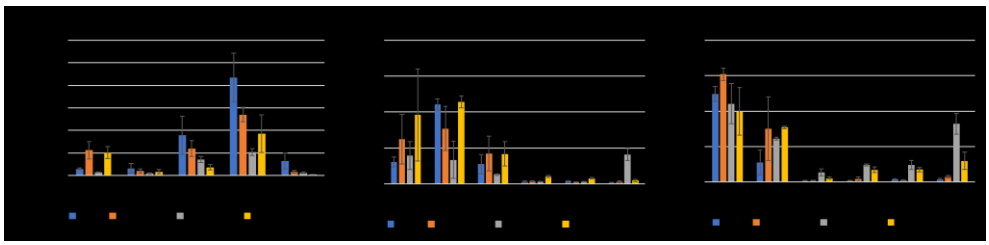

Fig. 30. Expression profiles in untreated (Ctrl) and DMSO-treated samples. (A) TDPs and topoisomerases genes. (B) Genes involved in TDP1-alternative repair pathways. (C) Genes involved in cell cycle regulation. Data are represented as mean ± standard deviation of technical replicates.

The FC values were subsequently used to generate a heatmap comprising all the afore-mentioned genes and shown in **Fig. 31** where blue color indicates down-regulated genes while red color indicates upregulated genes. Based on FC values, samples treated with NSC and CPT are distributed according to opposite color gradients.

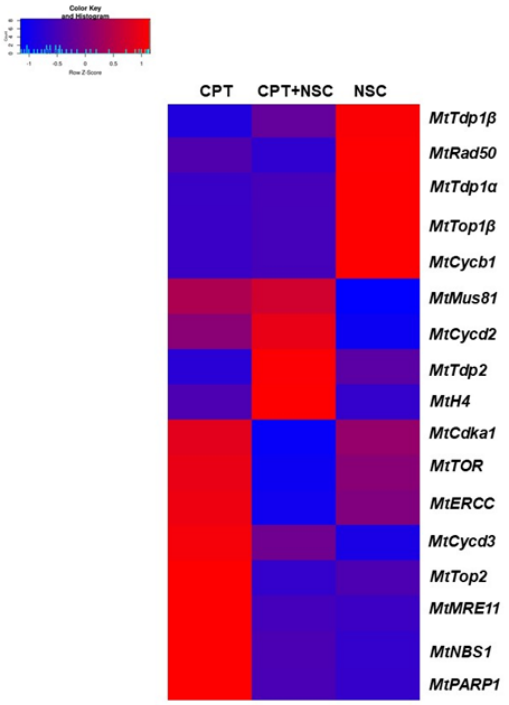

Fig. 31. Heatmap representing fold changes (FC) in genes expression values in response to CPT, NSC, and CPT+NSC treatments in 7-day-old *Medicago truncatula* seedlings. For each treatment, the values were normalized to their corresponding DMSO controls. The heatmap was constructed using the Shinyheatmap software.

MtTDP1α, *MtTDP1β*, *MtTop1β*, *MtCycB1,* and *MtRAD50*, upregulated in NSC-treated samples, are downregulated in CPT-treated samples, while *MtTop2*, *MtCycD3*, *MtMRE11*, *MtNBS1*, *MtPARP1* show an opposite trend being upregulated

in CPT-treated samples and downregulated in NSC-treated samples. *MtTOR*, *MtCDKA1, MtERCC1* expression levels did not change significantly compared to the relative DMSO_N control in the seedling treated with NSC. Instead, they are upregulated in CPT treatment. When considering the CPT+NSC treatment, the only upregulated genes are *MtTDP2α*, *MtCycD2*, *MtH4,* and *MtMUS81*. The same genes are mostly downregulated in the other two treatments. Briefly, the selected genes are differentially expressed according to the imposed treatments. Most genes show opposite expression profiles when comparing NSC and CPT treatments.

When looking into the group of functions, samples treated with NSC revealed a general upregulation of the *MtTDP1* (*MtTDP1α*, *MtTDP1β*) and *MtTop1β* genes, but not for *MtTDP2α* and *MtTop2*. An opposing effect was observed in samples treated with CPT where an overall downregulation of *MtTDP1α*, *MtTDP1β*, and *MtTop1β* genes is encountered while *MtTop2* is upregulated. The combination of the two treatments (CPT+NSC) show a different profile, where *MtTDP2α* is the only upregulated gene of this group. With regards to the genes belonging to alternative DNA repair pathways, these are upregulated only in CPT treated samples, except for *MtRAD50* and *MtMUS81* which are upregulated in NSC and CPT+NSC treatment, respectively. Concerning the group of genes involved in cell cycle regulation, the NSC treatment resulted in the upregulation of *MtCycB1* while *MtCycD2*, *MtCycD3,* and *MtH4* are downregulated. In the same treatment, *MtTOR* and *MtCDKA1* do not change their expression relative to DMSO_N control. In the case of CPT treatment, this group of genes is upregulated, except for *MtCycB1* and *MtH4* which are downregulated. The *MtCycD2* gene does not change its expression levels relative to DMSO_C control. The CPT+NSC treatment resulted in the upregulation of *MtCycD2* and *MtH4* while all the other genes belonging to the group of genes involved in cell cycle regulation are downregulated.

Overall, the present investigation revealed the contrasting effect of a single administration of NSC and CPT treatments on *MtTDP1α*, *MtTDP1β,* and *MtTop1β* and *MtTop2* genes involved in alternative DNA repair pathways and cell cycle regulation and a different response in case of concomitant administration of CPT+NSC. The combined approach revealed that the most affected genes were *MtTDP2α*, *MtH4*, *MtMUS81*, *MtCyCD2*, showing upregulation, and *MtERCC1*, *MtTOR,* and *MtCDKA1* genes showing downregulation in response to the CPT+NSC treatment.

4.3.8 Expression analyses of selected microRNAs and their putative targets identified from the *in silico* approach as being involved in DDR

Since one of the main goals of this work was to identify miRNAs able to regulate DDR-associated processes, the following qRT-PCR analyses were conducted to evaluate the changes in expression patterns of miRNAs and putative target genes previously identified from the bioinformatic pipeline.

To this purpose, the following miRNAs were selected: mtr-miR168, mtr-miR156a, mtr-miR2600e, mtr-miR395, mtr-miR5741a, mtr-miR172c-5p and their expression evaluated in 7-days-old *M. truncatula* treated and untreated seedlings. For these

miRNAs, the *in silico* analysis suggested the involvement in different processes such as cellular response to virus both in plant and human (mtr-miR168a), histone modification (mtr-miR156a, mtr-miR395e), antioxidant defence (mtr-miR2600e), DNA-dependent DNA replication (mtr-miR5741a), and DSB repair (mtr-miR172c-5p). The putative targets of the chosen miRNAs, *MtAGO1A*, *MtATUBC2*, *MtACYLTR*, *MtDNAM, MtE2Fe-like*, and *MtRAD54-like* were also investigated through qRT-PCR as mentioned above.

The expression profiles of miRNAs and putative target genes are shown in **Fig. 32**. First, their expression in non-treated (NT) samples were monitored to evaluate their behaviors in physiological conditions. As shown in **Fig. 32A**, while all the tested miRNAs are highly expressed (except for mtr-miR395e) the expression of their putative target gene is significantly reduced, thus corroborating the expected trend where miRNAs activity inhibit the targeted gene expression. The ability of miR168 to target *AGO1A* gene is a well-known fact to the scientific community (Vaucheret et al., 2006), therefore, this miRNA was chosen as quality control for function/target validation. Indeed, a low level of *MtAGO1A* expression corresponds to a high level of miR168a expression in NT samples (**Fig. 32A**). Looking into the expression of this specific miRNA and its target gene during the imposed treatments, it is evidenced that while the expression of miR168 is low, the expression of *AGO1A* is high, especially in the NSC- and NSC+CPT-treated samples (**Fig. 32B**).

Since we have previously reported that gene expression is influenced by DMSO treatments, also in this case we decided to represent the data as FC to DMSO. The effect of CPT, NSC, and NSC+CPT treatments on miRNAs and target gene expression is thus shown as a heatmap in **Fig. 32C**, where blue color represents downregulation and the red color represents upregulation. Overall, the heatmap shows that when a miRNA is upregulated the corresponding candidate target gene is downregulated. Although not always evident, this is observed for all three treatments that were imposed. This is visible for the couple mtr-miR5741a/*MtE2FE*. Looking at the miRNAs expression according to each treatment, in the CPT-treated samples, they do not change their expression relative to DMSO_C, except for mtr-miR156a which is upregulated. As regards the NSC treatment, all miRNAs are downregulated except for mtr-miR172c-5p which is upregulated. The combined treatment reveals that the most affected miRNAs are mtr-miR395e and mtr-miR5741a, being highly upregulated. Although the expression profile of mtr-miR156a in NSC and CPT+NSC seems unchanged, its putative target *MtATUBC2* is markedly expressed in the combined treatment but not in the NSC treatment. The high variability of miRNAs expression profiles between samples subjected to the same treatment and different treatments gives a more nuanced picture of the role of these small molecules in the studied system. Anyway, these data support the hypothesis that the selected miRNAs target the predicted genes identified by bioinformatic approaches.

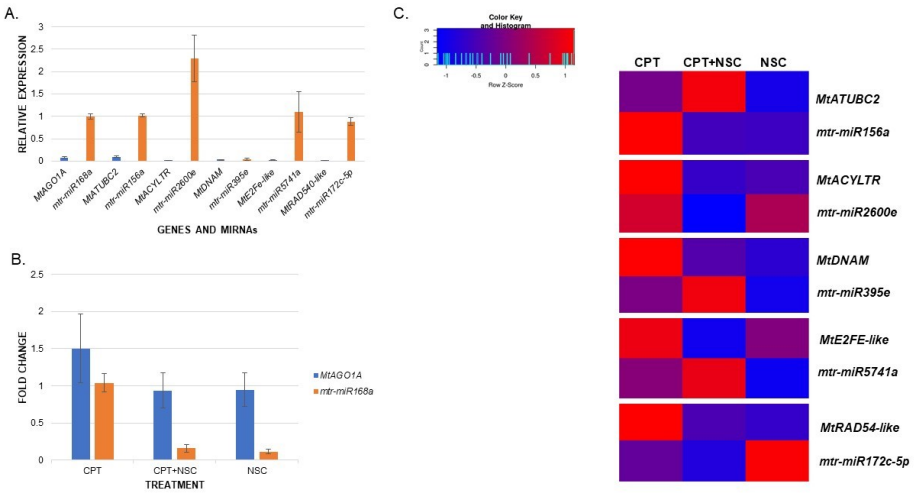

Fig. 32. Expression profiles of selected miRNAs and their putative targeted genes in 7-day-old *Medicago truncatula* seedlings **(A)** Relative expression of miRNAs/genes couples in non-treated (NT) samples. **(B)** Expression levels of MtAGO1A and mtr-miR168a in CPT-, NSC- and CPT+NSC-treated seedlings. **(C)** Heatmap representing fold changes (FC) to each corresponding DMSO of miRNAs and putative targeted genes in response to CPT, NSC, and CPT+NSC treatments. The heatmap was constructed using the Shinyheatmap software (http://www.shinyheatmap.com/). Statistical significance, as per the Students t-test, is shown with an asterisk (*, $P < 0.05$).

4.4. Proof of concept of the potential role of plant miRNAs in human cells

Based on the bioinformatics pipeline developed to predict miRNA target genes with potential action on human genes, 3 miRNAs (mdm-miR160, mdm-miR166, and mdm-miR390) were selected whose sequences are conserved in different plant species, including *M. truncatula*, *A. thaliana* and *M. domestica*. These miRNAs were used to predict the probabilities of pairing to human genes (using the human genome deposited in NCBI as a reference) based on the alignment of sequence and hybridization energy (MFE, minimum free energy). Besides these 3 miRNAs, 4 different miRNAs (mdm-miR159, mdm-miR396, mdm-miR482a-3p, mdm-miR858) were selected as they are reported in the scientific literature as prominently expressed in *M. domestica* fruit and peel (Xia et al., 2012; Ma et al., 2014). Considering the availability of genome and miRNA database for *M. domestica* cv. Golden Delicious, the species was selected to investigate the *trans*-kingdom potential of plant miRNAs, alongside with cv. Stark. **Fig. 33** shows the relative expression profiles of the *M. domestica* miRNAs analyzed compared to a mix of fruits used as control. All the analyzed miRNAs are highly expressed in *M. domestica* cv Golden Delicious, except for mdm-miR390 and mdm-miR396. The same miRNAs show a similar pattern in *M. domestica* cv Stark, except for mdm-miR160. Among the studied miRNAs, mdm-miR482a-3p and mdm-miR858 show the highest level of expression and hence were selected for subsequent analyses.

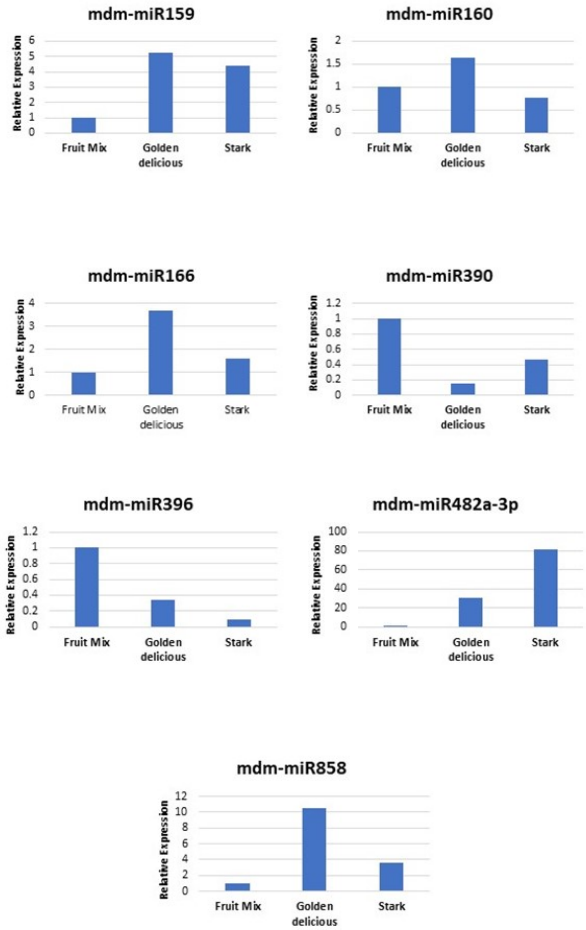

Fig. 33. Expression profiles of selected miRNAs in *Malus domestica* cv Golden Delicious and Stark. Control is represented by a mix of fruit.

Fig 34 shows expression levels of these two miRNAs in peeled apple (PA) and apple with peel (AWP). The observed trends show no major fluctuations when one or the other condition (PA or AWP) is considered. However, in view of future studies, it should be considered that the apple is frequently consumed without the peel.

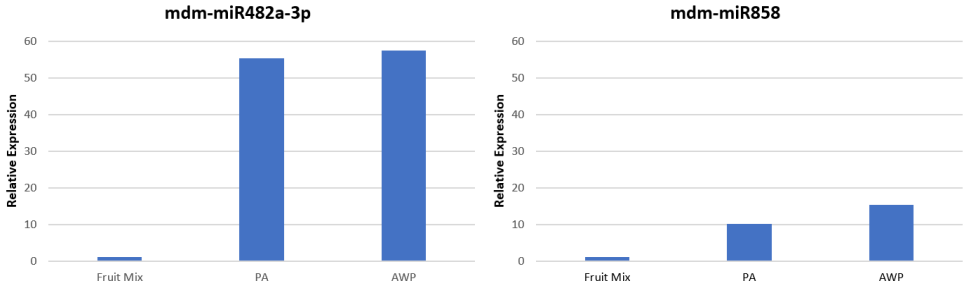

Fig. 34. Expression profiles of mdm-miR482a-3p and mdm-miR858 in peeled (PA) and unpeeled (AWP) fruits of *Malus domestica* cv Golden Delicious.

MiRNAs mimicking mdm-miR482 and mdm-miR858 were subsequently employed for transfection assay in HT-29 human cells. After 72 h these amiRNAs are detectable inside the cells (**Fig. 35**). Subsequent expression analyses were performed to evaluate the ability of these amiRNAs to regulate the activity of human genes *in vitro* (**Fig. 36**). To this purpose, the following human genes were tested:

- *RXRα* (Retinoid X Receptor Alpha), involved in the attenuation of the innate immune system in response to viral infections,
- *IL4R* (Interleukin 4 Receptor), involved in promoting Th2 differentiation,
- *SMAD3* (SMAD Family Member 3), functioning as a tumor suppressor,
- *PROM1* (Prominin 1) involved in stem cell maintenance,
- *ROCK2* (Rho Associated Coiled-Coil Containing Protein Kinase 2), involved in the modulation of processes related to cytoskeletal rearrangements such as focal adhesion formation, cell motility, and tumor cell invasion.

RXRα, *IL4R,* and *SMAD3* are the predicted targets of mdm-miR482a while *PROM1* and *ROCK2* are putatively targeted by mdm-miR858. The transfection into HT-29 cells of mdm-miR858 miRNA mimic doesn't show a clear fluctuation of the putative targets. When considering mdm-miR482 putative targets, a downregulation in mRNA levels is observed for *RXRα* and *IL4R* genes. However, these results obtained from one-shot experiments need to be further validated.

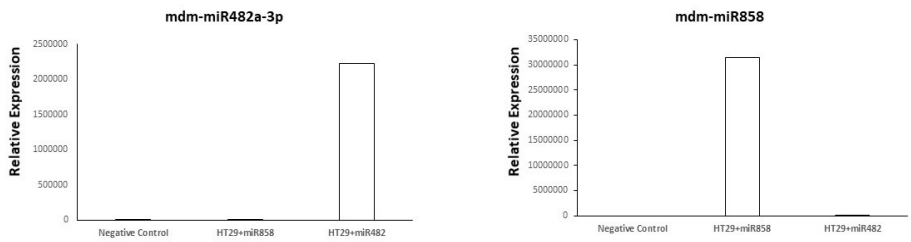

Fig. 35. Expression levels of amiRNAs mimicking mdm-miR482a-3p and mdm-miR858 from *Malus domestica* cv Golden Delicious, following transfection of human colorectal adenocarcinoma cells, HT-29.

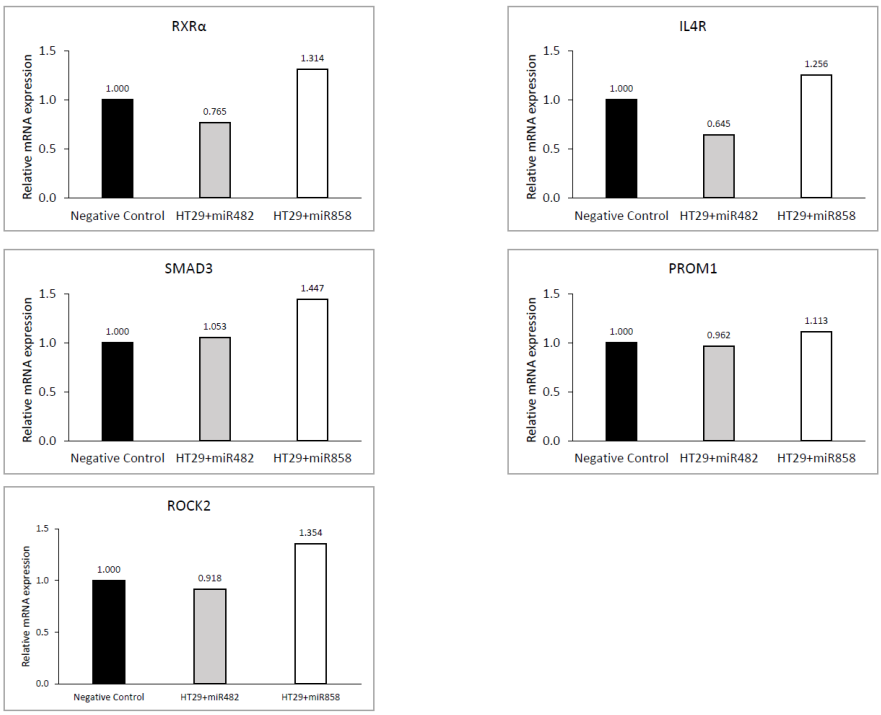

Fig. 36. Expression profiles of human genes putative targeted by mdm-miR482a-3p and mdm-miR858, conducted in the human colorectal adenocarcinoma cells HT-29 following transfection with the designated amiRNAs.

5. Discussion and conclusions

As already evidenced, plenary of information dealing with the effects of genotoxic stresses in plants and ways evolved to cope with these conditions are currently available. Nonetheless, the unexplored and unexplained variables are abounding as well. In this context, the present work of thesis focused on shedding light on the possible involvement of miRNAs in the regulation of DDR-associated processes, alongside investigating the trans-kingdom valence of plant miRNAs. This work aimed to look into these complex issues by tackling different approaches, both bioinformatics and experimental.

In silico investigations significantly contributes to the ongoing efforts to gain insight into miRNA properties and functions and provide the basis for further experimental validation. Model organisms, like *A. thaliana*, are used as driving systems to examine big data-driven questions connected to putative cross-species miRNA targets (Zhang et al., 2016a). The great interest in this field promoted the development of databases capable of predicting the functional impact of food-borne miRNAs in humans (Chiang et al., 2015; Shu et al., 2015). However, the existing databases cover only a small number of edible plant species (Chiang et al., 2015), hence there is a need to significantly expand the information and include alternative species with a potential impact on food safety. Alongside the fascinating trans-kingdom potential, miRNAs may contribute to the fine-tuning of DDR genes and this may affect the adaptive response of the plant to the environment, therefore its vitality, biomass production, and consequently the agricultural yields.

Within this framework, the multifaced bioinformatics approach pipeline hereby developed aimed at identifying plant miRNAs with their endogenous and cross-kingdom targets to look for conserved pathways between evolutionarily distant species and eventually obtain new insights into miRNAs associated with DDR. Starting from a list of publicly available *M. truncatula* miRNAs, it was assumed that any miRNA may potentially target genes in both plants and humans. Based on the presence of miRNAs belonging to conserved families, this pipeline will further aid translational research covering other economically relevant plant species and potential human target genes. Regarding specifically this aspect, as illustrated in **Fig. 37**, miR164, miR166, and miR390 share 100% sequence similarity in *M. truncatula* and other dicot plant species such as tomato and apple; miR166 was also previously found to be abundant in different human body fluids and tissues (Lukasik et al., 2018; Zhao et al., 2018). So, the putative human targets identified through the different bioinformatics approaches may serve as potential candidates to aid medical interventions. To give one example, inhibition of the AOC3 (Amine Oxidase Copper Containing 3), acting in adipogenesis and putatively targeted by miR166, might result in decreased fat deposition (Carpéné et al., 2007; Shen et al., 2012), thus addressing the big issues related to obesity and the many obesity-associated diseases.

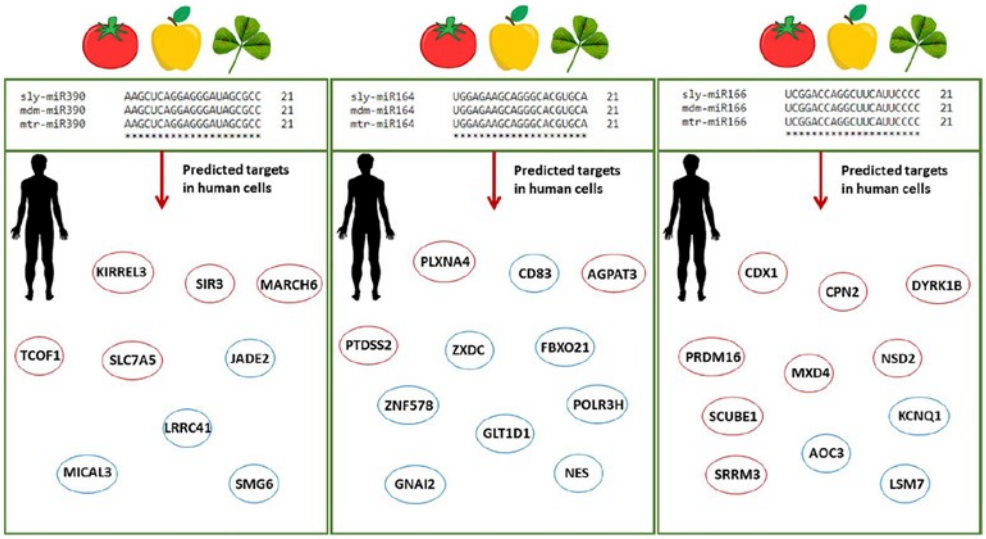

Fig. 37. Schematic representation of conserved plant miRNAs potentially targeting human genes. Alignments between three conserved miRNAs (miR390, miR164, miR166) from different plant species, namely *Solanum lycopersicum* (sly), *Malus domestica* (mdm), and *Medicago truncatula* (mtr), show 100% sequence similarity. The predicted human target genes found in the enriched biological processes of the network-based approach and among the genes with significant sequence similarity in the alignment-based approach are shown in red and blue circles, respectively (Bellato et al., 2019).

It is essential to underline that the design of these bioinformatics approaches was conceived in such a way to empower the identification of conserved pathways/players between evolutionarily distant species. In view of this, three different bioinformatics pipelines were developed (two network-based approaches, considering *A. thaliana* and *M. truncatula* model species, and one alignment-based approach) to confront plant and human targeted biological processes. From a biological point of view, the employed strategies led to both complementary and divergent observations. For example, 'exocytosis' was a common denominator in all three investigated species (*M. truncatula*, *A. thaliana*, and *H. sapiens*) when using the network-based approaches. In fact, the generated networks illustrated conserved biological processes (e.g., same function vs. same/different miRNAs). On the other hand, the alignment-based approach led to more direct identification of miRNAs targeting genes in *M. truncatula* and *H. sapiens* (e.g., same miRNA vs. similar/different functions). Given that evidence of miRNAs involvement in the regulation of DDR-related pathways is still limited in plants and considering the conservation of some DDR functions between plants and animals (Yoshiyama et al., 2013; Nikitaki et al., 2018), a dedicated focus was given to these specific pathways. In view of this, miRNAs predicted to target genes involved in DNA repair, recombination, and replication, chromatin remodeling, cell cycle, and cell death

were pinpointed in plants. To cite some examples, mtr-miR172c-5p, mtr-miR2638b, mtr-miR5272f, and mtr-miR2086-3p, were predicted to target the *Arabidopsis* RAD54, RAD9, KU80, and DME genes. In *M. truncatula* the 'DNA-dependent DNA replication' (GO:0006261) process is represented by Medtr4g106540 (E2F transcription factor-E2FE-like protein) as a predicted target of mtr-miR5741a. Within the alignment-based approach, mtr-miR2589, mtr-miR482-5p, mtr-miR5287b, and mtr-miR319d-5p were predicted to target two methyltransferases (Medtr6g047800, Medtr5g079860), the CDC48 (Medtr7g088980), and DCD (Medtr4g084080) genes. All these results have a relevant impact on plant research since they associate specific, previously unknown, miRNAs to the regulation of DDR functions. The bioinformatics analyses additionally uncovered mtr-miRNAs predicted to target human genes with roles in DNA repair and related processes. For example, PPP4C (putative target of mtr-miR169d-3p) catalyzes the dephosphorylation of RPA2 in response to DNA damage, thus permitting the recruitment of RAD51 to the damaged site (Lee et al., 2010b). FOXM1 (putative target of mtr-miR169k) is among the most overexpressed oncoproteins in many types of cancer and therapeutic interventions to suppress its function are of great interest (Halasi et al., 2018). Consequently, aside from the implications of plant science itself and considering the implications that some of these putative interactions could have for the biomedical sector, the obtained results from bioinformatics approaches offer novel hypotheses for future experimental validations.

Considering the main focus of this work of thesis on plant DDR related aspects, SOG1, the master-regulator of DDR, was also investigated from an *in silico* point of view. The presented data show the existence of multiple variants of this factor in different plant species. Specifically, in *M. truncatula* two SOG1 homologs, namely MtSOG1A and MtSOG1B, were identified, showing high sequence similarity (70.2% and 63.9 %, respectively) with the *Arabidopsis* AtSOG1, present in a single form. Its structure and role have been widely studied in the model plant *A. thaliana* (Preuss & Britt, 2003; Yoshyama et al., 2009, 2013, 2014, 2015). The MtSOG1A and MtSOG1B homologs found in *M. truncatula* show the presence of all the AtSOG1-characteristic features. By analyzing the peptide sequence, it was possible to observe that as AtSOG1, the C-terminus of MtSOG1A and MtSOG1B have five copies of the SQ motif that is commonly regarded as the preferred site for phosphorylation by ATM and ATR. The *in silico* putative protein-protein interaction studies suggested their involvement in DDR. Indeed, the ATR, ERCC1, GTF2HA, and WEE1 proteins appeared in both AtSOG1 and MtSOG1A/B-derived protein-protein interaction networks. Alongside common characteristics, the two *Medicago* proteins show unique interactions (DNA helicase, FEN1, RAD51, XP-C-like), indicating possible functional differences between these proteins. It is important to understand how SOG1 is distributed among plants. When the AtSOG1 peptide sequence was used to search for SOG1 protein homologs in other plants, putative SOG1 proteins were identified in most of the species covered in Phytozome. Indeed, it is already known that SOG1 homologs are present from moss to angiosperms (Yoshiyama et al., 2014). The clades generated in the phylogenetic tree are

distributed according to the current taxonomy. For the majority of species, it is possible to observe the presence of only a single SOG1 homolog, except for few species covering legumes and trees. No AtSOG1 homologs were identified for *Chlamydomonas reinhardtii*, *Dunaliella salina*, *Volvox carteri*, *Coccomyxa subellipsoidea*, *Micromonas pusilla*, *Micromonas spp.*, *Ostreococcus lucimarinus*. The presence of NAC proteins, although attested in mosses, lycophytes, gymnosperms, and most of the angiosperms, has not been reported for species such as *C. reinhardtii* or other green algae (Zhu et al., 2012).

Following the extensive bioinformatics analyses, the second part of the thesis focused on developing an appropriate experimental system to demonstrate the involvement of miRNAs in the regulation of DDR associated processes. This system had to be original and had to display characteristics of impaired DDR. For this purpose, seed germination and early seedling development were targeted since during seeds desiccation, storage, and imbibition, high ROS levels are being produced as products of internal cellular metabolism and they are known to cause DNA damage (Kranner et al., 2010). Genomic protection and stability are crucial to assure proper seed germination and cell-cycle control and repair of DNA lesions are essential mechanisms contributing to genome maintenance and avoidance of the onset and propagation of mutations. Within the context of the seed germination process, characterized by active cell proliferation, DNA damage must be repaired before the start of cell division to ensure the development of robust plants (Macovei et al., 2017). It is thus necessary that an efficient DNA damage response (DDR) system be activated to deal with these common types of DNA damage. Therefore, it is important to understand the mechanisms of DDR during seed germination and early seedling development. The designed experimental system to induce genotoxic stress, and subsequent DDR activation, was based on the use of two chemical agents: camptothecin (CPT), an inhibitor of Top1 enzyme, and NSC120686 (NSC), an inhibitor of the human TDP1 enzyme. CPT is a cytotoxic drug, a strong inhibitor of nucleic acid synthesis, and a potent inducer of strand breaks in chromosomal DNA. It has been widely studied for its medicinal properties as it constitutes a potent antitumor drug (Wall & Wani, 1996). Together with topoisomerase inhibitors, NSC120686 has been used as a pharmacophoric model to restrain the Tdp1 activity as part of a synergistic treatment for cancer. In plants, the knowledge of its specific functions is still scarce. Macovei et al., (2018) tested the activity of this compound in *M. truncatula* calli in active proliferation to investigate plant Tdp1 genes. These two inhibitors were delivered to *M. truncatula* seeds, alone or in combination, to exploit their function in altering the enzyme-DNA interactions.

The administration of CPT and NSC alone or in combination had no effects on seed germination; however, the CPT and CPT+NSC treatments inhibited seedling development. The seedlings treated with NSC did not show any remarkable differences compared to the control from a phenotypic point of view; this may suggest that the aberrant phenotype observed in CPT+NSC treatment is mainly due to CPT administration. Early studies indicated that CPT treatments result in arrested cells in S phase by inhibiting the progression of the DNA replication forks; this inhibition is due to the presence of the cleavable complexes induced by CPT and

ultimately may lead to cell death. It has been demonstrated that cell death induced by CPT mainly occurs by apoptosis. A significant increase in cell death was also observed in calli treated with NSC (Macovei et al., 2018). Thus, a DNA diffusion assay was performed to evaluate cell mortality in 7-day-old *M. truncatula* seedlings subjected to CPT and NSC treatments. The results showed that the imposed treatments induce an increase in the levels of cell death compared to the non-treated samples, and different cell death profiles are detected according to the treatments. The most severe effects are observed in CPT treatment characterized by high levels of necrosis. Although CPT is widely reported as an inducer of apoptosis, it is not surprising to observe that a fraction of cells undergo necrosis. Agents inducing apoptosis may also cause necrosis, and the distinction between the two forms of cell death in cultures depends on the severity of the insult and/or used concentration (Lennon et al., 1991; McCabe et al., 1997; O'Brien et al., 1998; Mammone et al., 2000). Moderate stress causes apoptotic-like PCD while severe stresses induce necrosis (Reape et al., 2008). This suggests that the concentration of CPT used generated severe stress in the cells. Besides an increase in cell death, an accumulation of DNA damages was observed, thus confirming the induction of genotoxic stress due to the administration of the two compounds. The presence of DNA damage, in form of both SSBs and DSBs, may induce DDR pathways and therefore the activation of processes such as cell cycle arrest and cell death.

Overall, the root of the seedlings was the organ most affected by the imposed treatments. The reduced root length in CPT and CPT+NSC treatments can be explained by examining the types and levels of death. Plants employ PCD when affected by DNA damage, especially in stem cells (Fulcher et al., 2009). Considering that all tissue cells are derived from stem cell niches, it is critical to maintaining the genome integrity of this fraction of cells (Dinneny & Benfey, 2008). Since stem cells are the progenitors of all cells that make up the entire plant body and presumed offspring, mechanisms that prevent their loss due to cell death triggered by DNA damage are present. The solution to this problem might come from the existence of a subpopulation of stem cells in both the shoot and root apical meristems (SAM, RAM) called the quiescent center (QC) in the roots, that divide at a much slower rate than other cells. The QC cells seem to be more resistant to DNA damage than non-QC stem cells (Heyman et al., 2014; Clowes, 1959), a characteristic probably related to their slow-paced cell division. Indeed, when inducing QC cell proliferation, their sensitivity to DNA damage agents increases leading to impaired root growth (Cruz-Ramírez et al., 2013). Therefore, cell cycle slowdown could be the explanation for smaller root sizes in CPT and CPT+NSC treatments in an attempt to decrease exposure to the imposed genotoxic stress. In the event of stem cell loss as a result of DNA damage, *Arabidopsis* QC cells seems to be engaged in a cell division program to supply the lost stem cells, thus enabling rapid recovery of root growth following plant transfer from DNA stressed to non-stressed conditions (Heyman et al., 2013). Similarly, the replacement of impaired root meristem cells by dividing QC cells was observed in X-ray damaged corn roots (Clowes et al., 1959). These observations boost the possibility that, through their cell division, QC cells serve as a sub-pool of stem cells that afford new stem cells with an intact copy of the genome after

genotoxic stress. Therefore, another possible explanation for the aberrant phenotype observed for CPT and CPT+NSC treatments may be that the induced genotoxic stress is severe to the point of not allowing the restoration of a normal phenotype.
In plants, the PCD and DNA repair pathways are under the control of SOG1. The *Arabidopsis* sog1-1 mutant was characterized as a second-site suppressor of the radiosensitive phenotype of seeds defective in the repair endonuclease XPF. Therefore, MtSOG1A and MtSOG1B, identified through the *in silico* analysis, were used as indicators for the efficiency of the designed experimental system. The expression analyses of the two SOG1 genes indicated that they are differentially expressed in CPT/NSC-treated samples. Specifically, the MtSOG1A gene expression was highly induced by the NSC treatments while the MtSOG1B gene expression was highly triggered by the CPT treatment. The increase in their expression suggests that DDR has been activated.
Because the implemented treatments resulted in reduced seedling growth and accumulation of cell death and DNA damage, the expression profiles of several genes involved in DNA repair and cell-cycle regulation, were investigated by qRT-PCR. In the case of CPT administration, a downregulation of TopIα is evident, in agreement with the proposed activity of the inhibitor. An upregulation of TopII gene was registered, indicating its potential backup function when TopIα is inhibited. The Tdp1 (α and β) and Tdp2 genes were downregulated while PARP1 is upregulation. Likely, the upregulated PARP1 is indicative of its involvement in this repair through the activity of pathways alternative to Tdp1. Both MRE11 and NBS1 resulted to be upregulated, indicating the presence and active sensing of DSBs in the cell. Since also MUS81 and ERCC1 genes were upregulated, it seems that these endonucleases also have a major contribution to solving TopI-cc. Among the cell-cycle tested genes, CdkA1 and CycD3 were upregulated, suggesting high gene activity for cell-cycle regulation. Downregulation of CdkB2, CycA1, and CycB2 was observed in *Arabidopsis* tdp1 mutant plants showing severe growth defects (Lee et al., 2010a). The reduced seedling growth observed during CPT treatment may be due to the activity of other cell-cycle regulators that were not investigated in this work. In the presence of NSC, Tdp1α and Tdp1β genes are upregulated, suggesting they are trying to compensate inhibitory effect on the enzyme. The observed downregulation of PARP1 gene, concomitant with the upregulation of Tdp1α, was also evidenced in *M. truncatula* calli exposed to high concentrations of NSC (Macovei et al., 2018). The majority of genes involved in alternative DNA pathways resulted to be downregulated. The upregulation of CycB1, specifically activated by DNA damage in a SOG1-dependent manner (Weimer et al., 2016), indicate for active DNA repair and cell cycle progression. The CPT+NSC treatment showed the inhibition of both Tdp1 and Top1 genes confirming that the treatments hit both TopI and TDP1 activity. Instead, the inhibition of Tdp1 induces the backup function of *Tdp2α* gene, and consequent inhibition of the TopII activity as the TopII gene was downregulated. The presence of CPT+NSC caused an overall downregulation of genes involved in alternative DNA repair pathways, except for *MUS81*. This indicates that the possible DNA-protein crosslinks are being repaired through the activity of MUS81 endonuclease. To summarize, the phenotypic and molecular effect of CPT/NSC

treatments on *M. truncatula* seedling development, the following assumptions are hypothesized:

- during CPT treatment, TopI enzyme is blocked, TopI-DNA covalent complexes accumulate and cause DNA damage. In this situation, Tdp1 and Top1 genes are inhibited while the alternative DNA repair pathways are highly active, and the cell cycle is delayed allowing the repair of the induced DNA damage.
- when NSC is given, the TDP1 enzyme is blocked, still resulting in the accumulation of DNA damage. Although, in this case, the Tdp1 and Top1 genes are active, the alternative DNA repair is inhibited, and the cell cycle is progressing.
- the CPT+NSC combination resulted in the downregulation of most of the investigated genes, affecting both DNA repair and cell cycle progression.

The development of this system has allowed the study of specific miRNAs (mtr-miR156a, mtr-mir172c-5p, mtr-miR2600e, mtr-mir395e, mtr-miR5741) and their putative targets (MtATUBC2, MtACYLTR, MtDNAM, MtE2FE-like, MtRAD54-like) identified as involved in DDR and DDR-related processes from the bioinformatic analyses (Bellato et al., 2019). For these miRNAs, the *in silico* analysis suggested the involvement in different processes such as histone modification (mtr-miR156a), antioxidant defense (mtr-miR2600e), DNA-dependent DNA replication (mtr-miR5741). Among these, miR156 is an evolutionarily conserved miRNA family observed in 51 plant species ranging from mosses to high flowering plants, but characterized by significant evolution and diversification in its sequences, members, and functions (Sunkar & Jagadeeswaran 2008; Cui et al. 2017). Regarding the miRNA172 family, only a few members have been previously identified in plant species including *R. communis*, *V. vinifera*, *A. thaliana*, *P. trichocarpa* (Barvkar et al., 2013). This family has been reported to take part in regulating starch and sucrose metabolism but also flowering, vegetative growth, normal ovule, and seed development (Okamuro et al. 1997; Niu et al., 2013). Together, miR156 and miR172 acting sequentially, regulate developmental timing in *Arabidopsis* (Wu et al., 2009). Furthermore, high-throughput sequencing of *M. truncatula* seedlings found these two miRNAs as involved in salt/alkali stress (Cao et al., 2018). On the other side, miR395 family members were suggested as main regulators of rhizome shoot development. They are also known as general components of the sulfate assimilation regulatory network in *Arabidopsis* and rice (Guddeti et al. 2005; Matthewman et al. 2012). Alongside miRNA conserved families, others are species-specific. This is the case of miR2600 conserved in only few plant species including *Capsicum annum*, and the legumes *C. cajan* and *M. truncatula* (Xu et al., 2015; Nithin et al., 2017). Moving on to miR5741, this miRNA has been reported as targeting genes encoding ethylene-responsive factors and cytokinin dehydrogenase, playing relevant roles in the defense response (McGrath at al., 2005; Siemens et al., 2006).

It is therefore clear that these miRNAs have been studied mainly in plant development and response to various types of stress. However, the qRT-PCR analysis performed in this work of thesis indicates that they are also involved in the response to genotoxic stress, as indicated by their differential expression induced by the CPT/NSC. For example, mtr-miR172c-5p that is upregulated in NSC treated sample is downregulated in CPT treated samples. By observing the expression

profiles of the putatively targeted genes by the selected miRNAs, it is shown that an upregulation of the miRNA is accompanied by a downregulation of the gene predicted to be its target. These observations support the hypothesis that the selected miRNAs target the predicted genes identified by bioinformatic approaches. This information contributes to enrich the information relative to plant miRNAs known for their involvement in the DDR by adding a relevant piece to the puzzle of roles played by these small molecules.

Alongside the poorly investigated role of plant miRNAs in DDR, particular attention has been given to the emerging possibility that plant miRNAs can be transmitted to other species through diet. As a proof of concept, amiRNAs mimicking selected miRNAs abundantly found in the apple fruits were delivered to the human colorectal adenocarcinoma HT-29 cell line and the expression of these microRNAs (mdm-miR482a-3p and miR858) and predicted targets, were evaluated by qRT-PCR. Namely, the RXRα, IL4R, and SMAD3 were predicted as targets of mdm-miR482a while PROM1 and ROCK2 were predicted as targets of mdm-miR858. The results show that the mdm-miR482 putative targets RXRα and IL4R are downregulated upon amiRNA transfection. Although promising, these results obtained from one-shot experiments need further validations. An interesting result that will be further verified is the downregulation of IL4R by the amiRNA mimicking the apple mdm-miR482. This specific receptor is known to have well-defined roles in the immune system. For example, Mirel et al. (2002) evidenced that it is involved in the progression of type 1 diabetes. Furthermore, IL4 receptors show over-expression in many epithelial cancers and might be a promising target for metastatic tumor therapy. Indeed, it is already known that IL4 neutralization determines the attenuation of mammary metastatic tumor growth in the lung by decreasing the pro-tumorigenic properties of both innate and adaptive immune cells of the tumor microenvironment such as CD4(+) T lymphocytes (DeNardo et al., 2009). Understanding the roles of miRNAs in DDR and along with their implications in complex diseases such as cancer (He et al., 2016; Arjumand et al., 2018), pave the way for the development of diagnostic tools or alternative treatments (Huang et al., 2013; Badiola et al., 2015).

In conclusion, the *in silico* analysis allowed the identification of *Medicago truncatula* miRNAs belonging to conserved families and specifically involved in conserved pathways such as DDR. The developed bioinformatics pipeline is useful to explore these connections also in species of agri-food interest such as *Malus domestica*. Focusing on specific DDR-related functions, the hereby presented results significantly contribute to enriching the current knowledge regarding the role of plant miRNAs in DDR both for plant and human cells, considering the trans-kingdom potential. Concerning the wet-lab analyses, since the imposed treatments (CPT, NSC, NSC+CPT) influenced the seedling phenotype, caused accumulation of cell death and DNA damage, upregulation of SOG1 genes, and differential expression of genes involved in DDR-associated pathways, it was possible to conclude that these treatments actively influence DDR. This system was then used to evaluate the expression of specific miRNAs/target genes, thus showing their

specific involvement in DDR-related processes. Furthermore, the *in vitro* transfection of HT-29 cell lines with plant-derived artificial miRNAs suggests a possible functional role of these exogenous miRNAs in human cells. Future studies covering feeding trials in animals and humans will be conducted to ascertain the *in vitro* obtained results.

6. References

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7. List of original manuscripts

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A Bioinformatics Approach to Explore MicroRNAs as Tools to Bridge Pathways Between Plants and Animals. Is DNA Damage Response (DDR) a Potential Target Process?

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MicroRNAs, highly-conserved small RNAs, act as key regulators of many biological functions in both plants and animals by post-transcriptionally regulating gene expression through interactions with their target mRNAs. The microRNA research is a dynamic field, in which new and unconventional aspects are emerging alongside well-established roles in development and stress adaptation. A recent hypothesis states that miRNAs can be transferred from one species to another and potentially target genes across distant species. Here, we propose to look into the *trans*-kingdom potential of miRNAs as a tool to bridge conserved pathways between plant and human cells. To this aim, a novel multi-faceted bioinformatic analysis pipeline was developed, enabling the investigation of common biological processes and genes targeted in plant and human transcriptome by a set of publicly available *Medicago truncatula* miRNAs. Multiple datasets, including miRNA, gene, transcript and protein sequences, expression profiles and genetic interactions, were used. Three different strategies were employed, namely a network-based pipeline, an alignment-based pipeline, and a *M. truncatula* network reconstruction approach, to study functional modules and to evaluate gene/protein similarities among miRNA targets. The results were compared in order to find common features, e.g., microRNAs targeting similar processes. Biological processes like exocytosis and response to viruses were common denominators in the investigated species. Since the involvement of miRNAs in the regulation of DNA damage response (DDR)-associated pathways is barely explored, especially in the plant kingdom, a special attention is given to this aspect. Hereby, miRNAs predicted to target genes involved in DNA repair, recombination and replication, chromatin remodeling, cell cycle and cell death were identified in both plants and humans, paving the way for future interdisciplinary advancements.

Keywords: bioinformatics, DNA damage response, microRNA, networks, trans-kingdom

INTRODUCTION

The classical definition describes microRNAs (miRNAs) as small non-coding, single-stranded molecules that bind to mRNA by sequence complementarity and inhibit gene expression through posttranscriptional regulation (Bartel, 2004; Pasquinelli, 2012). By doing so, miRNAs are involved in many cellular and developmental processes, acting as master-regulators of gene expression. It is well-known that miRNAs are evolutionarily conserved in eukaryotes, although some differences exist between animals and plants, mainly related to their biogenesis and target recognition mechanism (see reviews by Millar and Waterhouse, 2005; Moran et al., 2017). In plants microRNAs are produced in nucleus and exported to cytoplasm, whereas in animals pri-microRNA and pre-microRNA are produced in the nucleus while the microRNA/microRNA* are produced in the cytoplasm. Both plant and animal miRNAs associate with the RISC complex, indispensable for miRNA activity, in the cytoplasm. In animals, pri-miRNAs are first cleaved by Drosha RNase III while in plants this is carried out by Dicer-like (DCL)1. Plant miRNAs have a 2′-O-methylation on the 3′-terminal nucleotide which is not present in animal miRNAs. Considering the target recognition mechanisms, in plants this is based on near-perfect or perfect sequence complementarity (leading mostly to mRNA decay), whereas in animals the sequence complementarity is imperfect, mostly based on the 'seed rule' (base pairing to the 5′ end of miRNAs, especially nucleotides 2–7) (Lewis et al., 2005).

Emerging research proposes a novel and controversial hypothesis indicating that miRNAs can be transferred from one species to another and potentially target genes across distant species. This concept has been developed starting from evidence showing that small RNAs can move from cell to cell (Molnar et al., 2010) and can act in gene silencing (RNA interference) across species (see reviews by Han and Luan, 2015; Weiberg et al., 2015). While the transfer of miRNAs from plants or humans/animals to their pathogens (Valadi et al., 2007; LaMonte et al., 2012; Buck et al., 2014) is less disputed, the situation gets more complicated when addressing the plant miRNA transfer to humans. This is due to several open questions and contrasting results regarding plant miRNA stability, abundance, mode of action, and validation of potential targets in human cells (Dickinson et al., 2013; Tosar et al., 2014; Micó et al., 2016; Cavallini et al., 2018). The first direct indication that ingested plant miRNAs, derived from food, can target genes in a cross-kingdom fashion had been provided by Zhang et al. (2012). The authors showed that a rice miRNA (osa-miR168a) stably exists in the sera and tissues of animals and humans and it specifically targets the liver low-density lipoprotein (LDL) receptor adapter protein 1 (LDLRAP1), decreasing the removal of LDL from plasma. Briefly, this research proposes that plant miRNAs are released from destroyed cells (during mechanical mastication) and transferred to the intestinal epithelial cells, where they could be incorporated into vesicles (exosomes or microvesicles) and enter the circulatory system to be delivered to targeted cells. Plant miRNAs can resist the activity of digestive enzymes and low pH throughout the gastrointestinal tract due to their methylation and high GC content (Zhang et al., 2012; Philip et al., 2015; Zhou et al., 2015). Moreover, immunoprecipitation experiments with anti-AGO2 antibodies have shown that miR168a associates with AGO2 in Caco-2 cells, thus enabling miRNAs' function (Zhang et al., 2012). This was also confirmed in another study where immunoprecipitation data revealed that honeysuckle (*Lonicera japonica*) miR2911 associated with the AGO2 complex in microvesicles (Zhou et al., 2015). In this study, miR2911 has been demonstrated to be resistant to processing and proposed to target genes involved in the resistance to viral influenza. Hence, resistant exogenous plant miRNAs may regulate multiple target genes based on sequence complementarity, similarly to how endogenous miRNAs act (Liu et al., 2017). This concept expands the known types of miRNA functions to key natural bioactive compounds with potential health promoting benefits (depending on the mRNA target). So far, compelling evidence has demonstrated that plant miRNAs are present in human/animal plasma and these miRNAs usually belong to evolutionary conserved families (Vaucheret and Chupeau, 2012; Zhang et al., 2012; Liang et al., 2014; Yang et al., 2015a; Yang et al., 2015b; Cavalieri et al., 2016). Plant miRNAs not only from edible plant species (rice, cabbage, broccoli, watermelon, soybean, strawberry, olive) but also from model (Arabidopsis, poplar) and medicinal plants (Moringa, honeysuckle, turmeric, ginger) had been evaluated for their potential *trans*-kingdom transfer (Zhang et al., 2012; Liang et al., 2014; Zhou et al., 2015; Cavalieri et al., 2016; Chin et al., 2016; Pirrò et al., 2016; Liu et al., 2017; Sharma et al., 2017; Minutolo et al., 2018).

Aside from the biomedical interest, miRNAs *trans*-kingdom interactions can be useful to better understand evolutionary distant conserved pathways. Some examples of preserved pathways between plants and animals include the innate immune signaling pathways (Ausubel, 2005), programmed cell death (PCD)-related pathways (Godbole et al., 2003; Lord and Gunawardena, 2012), some basic functions (e.g. Ca^{2+}ATPase, Ca^{2+}/Na^{+}-K^{+} ion exchanger) of calcium signaling pathway (Nagata et al., 2004), and the DNA damage response (DDR) (Yoshiyama et al., 2013; Nikitaki et al., 2018). Among these, DDR is defined as a complex signal-transduction pathway consisting of DNA damage sensors, signal transducers, mediators, and effectors which in turn activate a series of events (e.g. phosphorylation cascades) that lead to the regulation of downstream processes (e.g. cell cycle checkpoint, DNA repair), common between the plant and animal kingdoms (Yoshiyama et al., 2013). The involvement of miRNAs in the regulation of DDR players is quite recent and insufficiently explored, especially within the plant kingdom. Conversely, studies in human cells have already shown that miRNAs are involved in the regulation of DDR-associated genes and their activity is intricately weaved with traditional elements such as ATM (ataxia-telangiectasia mutated) and p53 (Kato et al., 2009; Landau and Slack, 2011; Wan et al., 2011). In plants, some miRNAs (e.g. osa-miR414, osa-miR164e, and osa-miR408), have been demonstrated to target specific helicases with roles in DNA repair, recombination, replication and translation initiation (Macovei and Tuteja, 2012; Macovei and Tuteja, 2013).

The current work aims to investigate the *in silico trans*-kingdom valence of plant miRNAs as a potential tool to

bridge conserved pathways between plant and human cells, inquiring their implication in DDR. To do so, a multi-faceted bioinformatics approach was developed by combining and evaluating different data- or knowledge-driven resources and tools. The model legume *Medicago truncatula* (barrel medic) has been chosen as target for this analysis because of its potential medicinal properties (high content in saponins) (Tava et al., 2011), sequenced genome and availability of different databases (Goodstein et al., 2012), as well as its conserved synteny among legumes (Gujaria-Verma et al., 2014; Lee et al., 2017) which can offer the possibility of translational applications to other economically relevant species. Moreover, in view of promoting future sustainable agriculture practices and food security, microgreens, defined as seedlings harvested when the first leaves appear, are gaining momentum as novel functional food sources with high nutritional content and health-promoting benefits (Choe et al., 2018). In this context, legume species previously used only as fodder, like *Trifolium* spp., *Medicago* spp. and *Astragalus* spp., are now being proposed as microgreens for human consumption since they had been demonstrated to contain high protein and phytochemical contents as well as low levels of carbohydrates (Butkuté et al., 2018). Hence, starting from a collection of *M. truncatula* miRNAs, we retrieved candidate targets in plant and human transcriptomic datasets and analyzed them with different strategies: *(1)* a gene network-based strategy was used to compare the targeted biological processes in plant and human, using an *Arabidopsis thaliana* homology-based approach for plant network reconstruction; *(2)* an alignment-based strategy was used to identify nucleotide and protein similarities between *M. truncatula* and *Homo sapiens* putative targets; *(3)* another network-based strategy was carried out by using a *de novo* reconstructed *M. truncatula* gene network to further assess the common biological processes targeted in human and barrel medic. All the above-mentioned strategies have been used for the common purpose of identifying shared features (e.g. microRNAs targeting similar processes) between these distantly related organisms.

MATERIALS AND METHODS

The workflow followed in this study is illustrated in **Figure 1** and its parts are discussed below. Three different strategies were employed, namely a network-based pipeline, an alignment-based pipeline, and a *M. truncatula* network reconstruction approach.

Datasets

The list of *M. truncatula* miRNAs was retrieved from miRBase (Kozomara et al., 2019) and included 756 sequences, among which 426 were unique. The human 3′ UTRome sequence dataset was retrieved from the psRNATarget tool web site (Dai et al., 2018) and included 21,233 sequences, among which 18,167 were relative to unique genes. The *M. truncatula* transcript dataset (Mt4.0 v1) was retrieved from the psRNATarget tool web site and included 62,319 transcripts, corresponding to 50,894 unique genes.

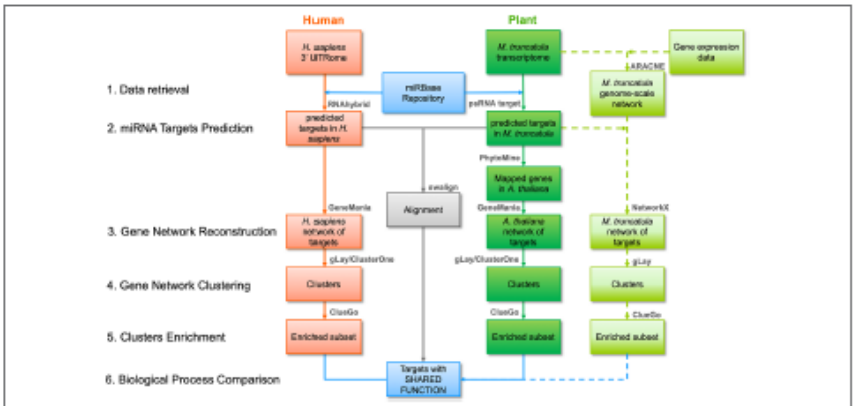

FIGURE 1 | Bioinformatic workflow followed in this work including network- and alignment-based analysis pipelines. The main steps of the network-based pipeline are numbered on the left, at the same level as the pipeline blocks indicating input and output of each step. Red and dark green blocks indicate human and plant inputs/outputs, respectively, and data flow is reported with arrows. The main software tools or functions (detailed in the main text) are summarized above each block. Light green blocks indicate inputs/outputs for the *Medicago truncatula* network-based pipeline, also including genome-scale network construction, and its data flow is reported as dashed arrows. The outputs of the alignment-based pipeline are reported as a single grey block indicating the sequences with significant similarity after alignment. Blue blocks indicate the initial and final data for human and plant in both analysis pipelines.

The gene sequences and the related protein sequences of the predicted targets were retrieved from the NCBI RefSeq database (for human targets) (O'Leary et al., 2016), and from the annotated coding sequence and protein datasets from the *M. truncatula* Genome Database (for plant) (Krishnakumar et al., 2014).

Six microarray datasets from the ArrayExpress (Kolesnikov et al., 2015) repository were used: E-MEXP-1097 (Benedito et al., 2008), E-MEXP-3719 (Verdier et al., 2013), E-MEXP-2883 (Tang, 2014), E-MEXP-3190 (Uppalapati et al., 2012), E-MEXP-3909 (Wang et al., 2016), and E-GEOD-43354 (Limpens et al., 2014). These amounted to a total of 117 raw expression samples (in CEL format) that were used for *M. truncatula* co-expression network reconstruction. The dataset samples measured under perturbed conditions (e.g. salt or drought stress, infections) were excluded. All the considered experiments were conducted on the same microarray platform (Affymetrix GeneChip Medicago Genome array), thereby avoiding genome annotation biases.

miRNA Target Prediction

The psRNATarget (Dai et al., 2018) and RNAhybrid (Kruger and Rehmsmeier, 2006) online tools, specific for miRNA target prediction in plants and mammalians, respectively, were used. The list of *M. truncatula* unique miRNAs was used as input for both tools, together with the *M. truncatula* transcript dataset or the human 3′ UTRome (unless differently indicated). The 3′ UTR region was chosen under the assumption that plant miRNAs can regulate human targets in the same manner as endogenous human miRNAs (Bartel, 2004). This assumption is consistent with a number of recent bioinformatics works, which were in some cases further validated, leading to experimental evidence of cross-kingdom regulation (Shu et al., 2015; Chin et al., 2016; Zhang et al., 2016a; Hou et al., 2018; Zhao et al., 2018a). Despite this commonly performed assumption, it is worth noting that no *golden standard* exists for plant miRNA target prediction in a cross-kingdom context (Lukasik et al., 2018). A small number of other works additionally considered 5′ UTR and/or coding sequences as potential target regions (Liu et al., 2017; Lukasik et al., 2018; Mal et al., 2018). This was motivated by studies in which different transcript regions have been reported as non-3′ UTR targets for both endogenous and cross-kingdom regulations (Li et al., 2018; Wang et al., 2018). With the availability of additional validation studies and models for cross-kingdom regulation, this gap will be filled. Importantly, the proposed workflow can be easily adapted by changing the target sequences files.

The parameters of the two target prediction tools were set to obtain a balanced number of network nodes (about 700 for *A. thaliana* and *H. sapiens*) in the network-based pipeline, and of unique target transcripts (about 1,700 for *M. truncatula* and *H. sapiens*) in the alignment-based pipeline. A highly specific hybridization in seed region, typically occurring in plants, was set in psRNATarget, which was used to find plant target genes for network-based pipeline with the following parameters: number of top targets = 50, Expectation = 2.5, Penalty for G:U pair = 0.5, Penalty for other mismatches = 1, Extra weight in seed region = 1.5, Seed region = 2–13 nucleotides, Mismatches allowed in seed region = 0, HSP size = 19. The list of targets for the alignment-based pipeline was obtained *via* the same parameters as above except the number of top targets which was set to 15. The predicted target list from RNAhybrid was filtered by tuning the sole algorithm parameter that is Minimum Free Energy (MFE), whose threshold was set to −36.5 kcal/mol, while for the alignment-based pipeline it was −34.7 kcal/mol. In both cases, a maximum of 50 targets per miRNA was considered (Zhang et al., 2016a).

Network-Based Pipeline

The lists of predicted targets were used to construct plant and human target networks using GeneMania, (Warde-Farley et al., 2010), and considering all the genetic and co-expression interactions available within the tool. Since GeneMania does not contain *M. truncatula* among the available organisms, the following procedure was used to construct a genetic interaction/co-expression network of *A. thaliana*, by mapping the homologous genes of the *M. truncatula* predicted targets list. The Phytomine tool (Goodstein et al., 2012) of the Phytozome portal (JGI) was used to obtain a mapping from the *M. truncatula* target genes to *A. thaliana* genes, based on homology. Correspondences between the species were considered with a relative threshold similarity above 85%.

Human and plant networks were imported and analyzed using Cytoscape (v.3.7.1) (Shannon et al., 2003) and its applications. Clustering was carried out using the gLay (Su et al., 2010) and ClusterOne (Nepusz et al., 2012) algorithms, considering the networks as undirected and unweighted. ClusterOne was used with the following parameters: minimum size = 50, minimum density = 0.25, unweighted edges, node penalty = 2, haircut threshold = 0, merging method = Multi-pass, Jaccard similarity, overlap threshold = 0.15, seeding method from unused nodes. The gLay algorithm does not have free parameters. For each cluster, enrichment analysis was carried out using ClueGO (Bindea et al., 2009) to find statistically over-represented Gene Ontology (GO) terms in the Biological Process (BP) category, using a right-tail test with the Benjamini–Hochberg correction for multiple testing, and a 75% detail level. GO terms were considered for further analysis if they had p-value < 0.05 and if at least one of the related genes was present in the original target gene list (since GeneMania includes interactor genes not belonging to the input target list). The analysis procedure followed in this network-based pipeline is summarized in **Figure 2**.

Alignment-Based Pipeline

For each miRNA, the nucleotide coding sequence and protein sequence of the predicted transcript targets found in *M. truncatula* and *H. sapiens* were compared *via* sequence alignment. A custom MATLAB R2018a (MathWorks, Natick, MA, USA) script was programmed to automatically carry out this analysis and to evaluate the statistical significance of each comparison. The Smith–Waterman method (Smith and Waterman, 1981) was used to perform local alignment *via* the *swalign* function and get the optimal alignment score (in bits) as output. A random permutation-based statistical analysis was adopted to evaluate the significance of each alignment and to obtain a sequence

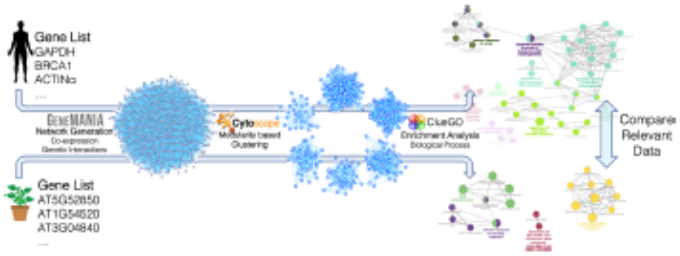

FIGURE 2 | Construction and analysis steps of the miRNA target networks for *Arabidopsis thaliana* and *Homo sapiens*. The lists of miRNA target genes were used to construct genetic interaction/co-expression networks with GeneMania. The resulting networks were analyzed with Cytoscape and its applications. In particular, clustering was carried out with two different modularity-based methods (jLay and ClusterOne) and enrichment analysis was carried out with the ClueGO app to find enriched biological processes in each cluster. The resulting processes found for the two organisms were finally compared, taking into account the related target genes and miRNAs.

length-independent scoring value (*p*-value) (Teng et al., 2014; Tiengo et al., 2015). Specifically, for each sequence comparison, 200 random permutations were constructed for the human nucleotide/protein sequence and an alignment was performed for each randomization. The resulting distribution of bits scores was used to obtain the final *p*-value as the number of alignments giving a bits score higher than the original one, divided by the number of randomizations. Low *p*-values correspond to statistically significant alignments with a considered threshold of 0.05. The analysis procedure followed in this alignment-based pipeline is summarized in **Figure 3**.

Reconstruction of *M. truncatula* Co-Expression Network

Raw expression values were globally normalized using the Robust Multichip Average (RMA) method (Irizarry et al., 2003), and then annotated using the MedtrA17_4.0 *M. truncatula* reference genome assembly. Array probes mapping the same gene were median-averaged and those lacking functional annotation were discarded. Co-expression analysis of the obtained expression panel was performed *via* ARACNE (Margolin et al., 2006) by: *(i)* building the Mutual Information Matrix using the Spearman correlation, and then *(ii)* pruning the obtained interactions among all possible gene triplets with null mutual information. All the analyses were performed in the R environment, using the *limma* (Ritchie et al., 2015) and the *biomaRt* (Durinck et al., 2009) packages for the expression data preparation, and the *minet* package (Meyer et al., 2008) for the co-expression estimation with ARACNE. The obtained adjacency matrix was then used to reconstruct a co-expression network for the miRNA targets of *M. truncatula*, with a custom Python (v.2.7) script, exploiting the *NetworkX* package (Hagberg et al., 2008) to create networks in a Cytoscape-compatible format. The

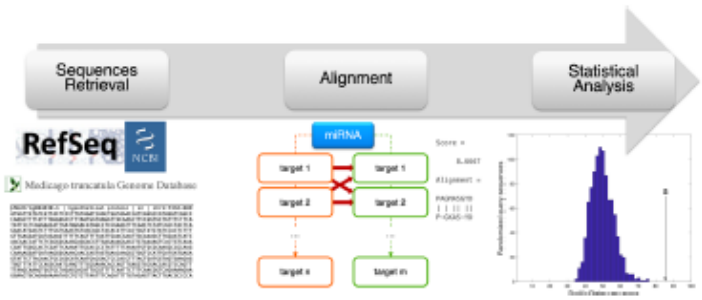

FIGURE 3 | Schematic representation of the alignment-based pipeline. The coding sequence (CDS) and amino acid sequence corresponding to the miRNA target genes were retrieved from online resources (RefSeq and Medicago truncatula Genome Database). For each miRNA, the CDSs and amino acid sequences of human and plant targets were compared via sequence alignment (Smith-Waterman method, by the swalign Matlab function), to compute a similarity score (provided as swalign output) for each human-plant target pair. The statistical significance of the similarity score is finally computed following a randomization method in which, for every alignment, human sequences (CDS or protein) were randomized and the distribution of swalign scores was used to compute the *p*-value.

miRNA targets of *M. truncatula*, obtained as described above (see *miRNA Target Prediction* section), were mapped onto the network to extract their co-expression interactome. Target genes were mapped and all the co-expressed nodes which interact with at least one miRNA target node were included. The resulting sub-network was filtered, eliminating the smallest components, composed of single nodes or less than ten nodes because these are not informative in terms of interactions. The remaining giant component was considered as the final miRNA target gene network. The giant component was analyzed *via* the gLay clustering procedure and, as performed above for the other networks, the obtained clusters were subjected to the ClueGO enrichment step.

RESULTS

Target Prediction

Following the *in silico* target prediction, a list of 3,468 *M. truncatula* transcripts (2,680 unique transcripts and 2,083 unique genes) was obtained. Conversely, 2,297 target transcripts of *M. truncatula*, corresponding to 1,739 unique transcripts and 1,376 unique genes, were considered for the alignment. Analogously, for the network-based analysis, a list of 936 target transcripts (825 unique transcripts and 758 unique genes) was obtained for *H. sapiens*. For the alignment-based pipeline, 2,226 target transcripts, which correspond to 1,754 unique transcripts and 1,549 unique genes, were obtained. The number of targets was tuned to obtain a balanced number of elements between the two species (see below). The target genes could be associated with one or more than one miRNA, as shown in **Supplementary Figure 1**.

Mtr-miRNAs Targeting Shared Functions Between Plants and Humans From the Perspective of the Network-Based Approach

In this pipeline, we focused on the biological processes enriched among the genes targeted by the set of *M. truncatula* miRNAs to uncover shared functions between plant and human. Following the procedure summarized in **Figure 2**, plant and human miRNA target networks were constructed using the GeneMania web tool. While the construction of the human network was straightforward with this tool, the construction of the plant network relied on the mapping from *M. truncatula* targets to homologous genes in *A. thaliana*, thereby enabling to exploit the deep knowledge (datasets and resources) of *A. thaliana*, since *M. truncatula* is not currently supported by GeneMania. The resulting networks were analyzed using two different topology-based graph clustering methods, to decompose the target network, based on highly connected nodes, implying densely-interacting functional modules. The use of two different clustering methods was devised to increase the sensitivity of the pipeline for the detection of functional modules and, subsequently, associated biological processes. The features of the constructed target gene networks are summarized below:

(1) *A. thaliana*—704 nodes (of which 20 were included by GeneMania as interactors), 13,752 edges, 4 gLay clusters, 6 ClusterOne clusters.
(2) *H. sapiens*—753 nodes (of which 21 were included by GeneMania as interactors), 20,795 edges, 5 gLay clusters, 3 ClusterOne clusters.

The target genes and network clusters obtained in this analysis are reported for each species in the **Supplementary Dataset 1** file.

By performing an enrichment analysis for each cluster, we identified the common biological processes (GO terms) targeted by *M. truncatula* miRNAs in both species. The identified shared biological functions include 'vesicle docking involved in exocytosis' (GO:0006904), 'modulation by virus of host morphology or physiology' (GO:0019048), 'cellular response to virus' (GO:0098586), 'positive regulation of posttranscriptional gene silencing' (GO:0060148), and 'branched-chain amino acid metabolic process' (GO:0009081). The miRNAs and predicted target genes associated with the shared GO terms are listed in **Table 1**. Aside from the identical GO terminologies, other common processes were present in both networks (e.g. nucleic acid and amino acid metabolism, response to stress, signaling) (**Supplementary Dataset 1**).

Exocytosis generally implies the active (hence, energy-dependent) transport of newly synthesized lipids and proteins to the plasma membrane along with the secretion of vesicle-enclosed contents to the extracellular matrix. Experimental evidence that exocytosis-related events can be conserved between plants and animals has been recently provided by Zhang et al. (2016b), who demonstrated that specific molecules (namely endosidin 2) are able to inhibit EXO70 proteins, involved in intracellular vesicle trafficking, in both plants and animals. The fact that *M. truncatula* miRNAs are predicted to target functions related to exocytosis in both *A. thaliana* and *H. sapiens*, further indicate the conservation of these pathways between distant taxa. As an example, the KEU (KEULE) and SEC (EXOCYST COMPLEX COMPONENT) genes in Arabidopsis as well as the human SNPH (Syntaphilin) gene are part of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, which is required for vesicle docking and fusion (Lao et al., 2000; Karnik et al., 2015).

The hypothesis that innate immunity is an evolutionarily conserved process, started in the ancient unicellular eukaryote that pre-dated the divergence of the plant and animal kingdoms (Ausubel, 2005), may explain the shared plant and human response to virus. The network-based approach applied in our study allowed to find common players involved in the response to viral attacks in plants (AGO1, AGO2, DCP2, SDE3, DRD1) and humans (BCL2L11, KPNA4, PUM2, FXR1, RIOK3) (**Table 1**). Particularly, the KPNA4 (Karyopherin Subunit Alpha 4) mediates the nuclear import of human cytomegalovirus UL84 (Lischka et al., 2003), PUM2

TABLE 1 | Common biological processes shared between *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	GO ID	*A. thaliana*		*H. sapiens*	
		Gene	miRNA	Gene	miRNA
Vesicle docking involved in exocytosis	GO:0006904	EXO70B1	mtr-miR6244	SNPH	mtr-miR390f-5p
		EXO70D1	mtr-miR2663a		
		EXO70H7	mtr-miR397-5p		
		KEU	mtr-miR6559-3p		
		SEC5A	mtr-miR7698-5p		
		SEC8	mtr-miR2670a		
Modulation by virus of host morphology or physiology	GO:0019048	AGO2	mtr-miR2673a	BCL2L11	mtr-miR6273
		DCP2	mtr-miR6238	KPNA4	mtr-miR169k
			mtr-miR2655b		
Cellular response to virus	GO:0098586	AGO1	mtr-miR168a	BCL2L11	mtr-miR6273
		SDE3	mtr-miR168c-5p	PUM2	mtr-miR160c
			mtr-miR2592a-3p	RIOK3	mtr-miR160a
			mtr-miR2592bm-3p		
Positive regulation of posttranscriptional gene silencing	GO:0060148	DRD1	mtr-miR2650	FXR1	mtr-miR482-3p
				PUM2	mtr-miR160c
Branched-chain amino acid metabolic process	GO:0009081	BCAT3	mtr-miR6212-3p	BCKDK	mtr-miR6273
		CSR1	mtr-miR2660	IVD	mtr-miR2640

(Pumilio RNA Binding Family Member 2) plays a role in cytoplasmic sensing of viral infection (Narita et al., 2014), and RIOK3 (right open reading frame-RIO Kinase 3) is involved in regulation of type I interferon (IFN)-dependent immune response, with a critical role in the innate immune response against DNA and RNA viruses (Feng et al., 2014).

The relationship between miR168a and AGO1 (ARGONAUTE) has been long studied and experimentally validated in plants (Vaucheret et al., 2006), whereas several other targets of the plant miR168a have been identified and/or validated in humans (Zhang et al., 2012; Javed et al., 2017). Aside being involved in miRNA biogenesis and regulation (Mallory and Vaucheret, 2010), AGO proteins have a myriad of other functions including plant antiviral responses and DNA repair (AGO2) (Harvey et al., 2011; Oliver et al., 2014; Carbonell and Carrington, 2015), miRNA-directed target cleavage (AGO5), and RNA-directed DNA methylation (AGO9) (Oliver et al., 2014). Differently, the validated osa-miR168a target in humans is LDLRAP1, with functions in cholesterol metabolism (Zhang et al., 2012), while other predicted targets included RPL34 (Large Ribosomal Subunit Protein EL34), ATXN1 (Ataxin-1), and ALS2 (Alsin Rho Guanine Nucleotide Exchange Factor) with roles in transcription, ribosome biogenesis, and cell trafficking (Javed et al., 2017). Other genes (ST8SIA1, RGS6, IL18RAP, PVR, SYN2, PPFIA1, ZDHHC18, B3GAT1) were predicted in our network-based pipeline to be targeted by mtr-miR168 in humans (**Supplementary Table 1**). This may be due to the fact that, even if miR168a is part of conserved miRNA family, some differences in nucleotide sequences are present among monocot and dicot species and these can alter the structural accessibility and target selection (Lang et al., 2019). When aligning the osa-miR168a with its counterpart in *M. truncatula*, the sequence similarity was of 80.95%, showing important mismatches in the seed region (**Supplementary Figure 2**). This, along with the fact that we took into consideration only the 3′ UTR region, explains the absence of LDLRAP1 among the mtr-miR168a targets in human. We confirmed this by comparing the *M. truncatula* (mtr-) or rice (osa-) miR168a targets found within the human transcript collection (retrieved from NCBI RefSeq) or in the 3′ UTRome, as performed in our pipeline. As expected, we found that no target was detected for both miRNAs in the 3′ UTRome, while targets in the LDLRAP1 coding sequence were found with relevant MFE. In particular, osa-miR168a showed a −35.3 kcal/mol MFE with LDLRAP1, which appeared in the top 5 of the miRNA targets, while mtr-miR168a showed a −33.8 kcal/mol MFE with LDLRAP1 in the 100th position of the lowest-MFE target list (**Supplementary Figure 2**).

miRNAs Targeting Shared Functions in *M. truncatula* and *H. sapiens* Through the Lens of the Alignment-Based Approach

Unlike the network-based pipeline in which over-represented biological processes were searched in the network of all the miRNA target genes, here we focus on sequence similarities among the targets of a given miRNA. In this approach, the analysis included alignments of every single targeted gene (and corresponding protein sequence) between *M. truncatula* and *H. sapiens*, resulting in a total number of 9,626 alignments (**Supplementary Dataset 2**). By applying a threshold *p*-value of 0.05 for nucleotide alignments, 2,735 sequences, corresponding to 115 miRNAs, resulted significant. These miRNAs were predicted to target a total of 315 genes in *M. truncatula* and 801 genes in *H. sapiens*, respectively. Similarly, when this threshold was applied

for the protein alignments, from 697 alignments (including 81 miRNAs) 352 genes were identified in *H. sapiens* and 192 in *M. truncatula*. When considering both the gene and protein sequences, 242 similarities between plant and human transcripts were found, accounting for 93 genes (targeted by 54 miRNAs) in *M. truncatula* and 149 in *H. sapiens* (**Supplementary Dataset 2**).

Focusing on the identification of genes involved in similar functions between the two organisms, the main hits were related to transcription factors (including zinc finger proteins), hormone-responsive elements, and cell division (**Table 2**). The activity of transcription factors (TFs), consisting of the interaction with enhancers to coordinate gene expression, is a common denominator for all living forms. In eukaryotes, another level of regulation is given by miRNAs; these are known to target mostly TFs, at least in plants (Samad et al., 2017). Moreover, coordinated action of TFs and phytohormones guide most plant developmental processes as well as cellular proliferation and dedifferentiation (Long and Benfey, 2006). The predicted targets of miR164 belong to CUP and NAC families of TFs, and these had been previously validated in plants in other works (Fang et al., 2014). However, a piece of interesting information is the fact that this miRNA could target TFs also in human cells. For instance, ZXDC (predicted as a target of mtr-miR164b), belonging to the zinc finger X-linked duplicated (ZXD) family of TFs, is involved in the regulation of histocompatibility (Ramsey and Fontes, 2013). Elsewise, HAND2 (Heart and Neural Crest Derivatives Expressed 2), putatively targeted by mtr-miR2673a, is a member of the helix-loop-helix family of TFs involved in cardiac morphogenesis, vascular development, and regulation of angiogenesis (McFadden et al., 2005). Another interesting fact is that this analysis predicted that conserved miRNA families (miR160, miR166) target genes with roles in hormone regulation in both *M. truncatula* (ABA response element-binding factor, auxin response factor) and *H. sapiens* (DYRK1B, HNF4A). In humans, DYRK1B (Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1B), encoding for a nuclear-localized protein kinase, and HNF4A (Hepatocyte Nuclear Factor 4 Alpha), belonging to the nuclear hormone receptor family, are associated with steroid hormone activity (Sladek et al., 1990; Sitz et al., 2004). Other interesting hits revealed through this approach are presented in **Supplementary Table 2**. An example is represented by mtr-miR2600e, predicted to target an anthocyanin acyltransferase (Medtr2g089765) in *M. truncatula* and the UVSSA (UV Stimulated Scaffold Protein A) gene in *H. sapiens*. Anthocyanins are well-known secondary metabolites with antioxidant function, being able to mitigate photooxidative injury (e.g. UV irradiation) at the cellular and nuclear level by efficiently scavenging reactive oxygen species (Gould, 2004). UVSSA encodes a protein involved in ubiquitination and dephosphorylation of RNA polymerase II subunits, being involved in the transcription-coupled nucleotide excision repair (TC-NER) pathway associated with UV irradiation (Schwertman et al., 2013).

When comparing the network-based and alignment-based approaches, in the case of mtr-miR168a targets, it is possible to evidence the same predicted target in plants (AGO1) along with different predicted targets in humans (**Supplementary Table 2**, **Supplementary Dataset 2**). However, drawing the attention to the 'response to virus' function, it is possible to observe that this was hit with both approaches, as demonstrated by the common predicted target gene PVR (Poliovirus Receptor).

Novel Co-Expression Network Reveals Shared Functions Targeted by mtr-miRNAs in Both *M. truncatula* and Humans

The third approach used in this study pursued the construction of a new *M. truncatula* co-expression network using publicly available gene expression microarray datasets since this organism is not currently supported in readily usable bioinformatic tools for network analysis and construction. An expression panel of 24,777 genes was obtained and used to build a genome-scale co-expression network for *M. truncatula*. The resulting 24,777-node network had 62,857 undirected edges (**Figure 4A**). Among the 2,083 predicted target genes, 1,251 were mapped in this network, resulting in a sub-network of 6,081 nodes and 9,534 edges. The giant component of this sub-network included 5,943 nodes (of which 1,208 were target genes) and 9,405 edges (of which 3,102 were direct interactions among miRNA target nodes), as shown in **Figure 4B**. The clustering procedure found

TABLE 2 | Mtr-miRNAs and their putative target genes related to similar functions in *M. truncatula* and *H. sapiens* as revealed by the alignment-based approach. The genes and their respective accessions are provided for each organism.

mtr-miRNA	*M. truncatula*		*H. sapiens*	
	Accession	Gene	Accession	Gene
mtr-miR166d	Medtr2g086390	ABA response element-binding factor	NM_006484	DYRK1B
mtr-miR160a	Medtr5g061220	auxin response factor	NM_175914	HNF4A
mtr-miR2673a	Medtr2g014260	zinc finger C-x8-C-x5-C-x3-H type protein	NM_001170538	DZIP1L
mtr-miR2673a	Medtr4g082580	WRKY transcription factor 3	NM_021973	HAND2
			NM_032772	ZFN503
mtr-miR164b	Medtr2g078700	CUP-shaped cotyledon protein, putative	NM_001009604	ZNF578
	Medtr4g108760		NM_001040653	ZXDC
mtr-miR164d	Medtr3g435150	NAC transcription factor-like protein	NM_001018052	POLR3H
mtr-miR5287b	Medtr7g088980	cell division cycle protein-like/CDC48 protein	NM_001277742	CYP26B1

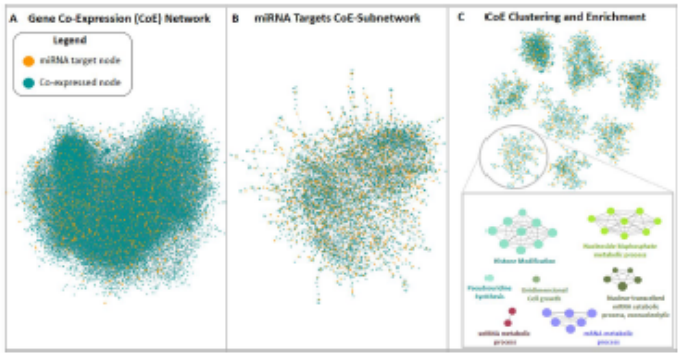

FIGURE 4 | *Medicago truncatula* co-expression network construction and analysis pipeline. **(A)** Genome-scale co-expression network and **(B)** miRNA targets network are shown, where blue nodes represent genes not found among miRNA targets, while orange nodes are miRNA targets. **(C)** Representative set of the clusters resulted from the miRNA targets network analysis. Each cluster was analyzed via enrichment analysis using ClueGO.

45 clusters (**Figure 4C**) which were analysed *via* enrichment analysis. All the resulting GO terms along with the co-expressed genes and associated miRNAs are reported in the **Supplementary Dataset 3** file.

The herein generated network was compared with the first network-based approach made with the tools available for *A. thaliana* to evaluate if the two different network construction procedures lead to the same target biological processes, thereby assessing the robustness of the conclusions for the network-based approach. The GO terms identified within the *M. truncatula* network were mainly related to general processes such as metabolic pathways (e.g., nucleic acids, proteins, and carbohydrates metabolism), plant development (e.g., fruit, seed, embryo development), or hormone signaling (**Supplementary Dataset 3**). On the other hand, the Arabidopsis network was much more varied and specific (**Supplementary Dataset 1**), mostly because *A. thaliana* is *de facto* considered the plant model *par excellence* and hence, much more information, databases, and bioinformatics resources are available in this case. Despite a systematic comparison between the biological processes in the two plants could not be carried out, identical GO terms identified between *A. thaliana* and *M. truncatula* include exocytosis, folic acid metabolism, and thylakoid membrane organization (**Supplementary Table 3**). In this case, it can be underlined also the fact that some miRNAs (e.g., mtr-miR5559-3p, mtr-miR5558-3p, mtr-miR2662, mtr-miR5212-3p) are predicted to target the same genes/functions in both plant species. When comparing the shared biological processes between *M. truncatula* and *H. sapiens*, these are shown to be related to exocytosis, DNA replication, transcription, and modifications, amino acid activation and transport, RNA related processes, histone modification, and protein modifications (**Table 3**). To cite one example, histone modification functions associated with both organisms include the DNA methyltransferase 1-associated protein (Medtr1g086590) in plants and the KANSL1 (KAT8 Regulatory NSL Complex Subunit 1) histone acetyltransferase in humans.

Taken together, the results obtained confirmed that both network-based approaches lead to consistent conclusions, even if *M. truncatula* is characterized by a less detailed Gene Ontology which prevents strong matching between the two plants.

Do mtr-miRNAs Putatively Target Genes Involved in DDR in Plants and Humans?

The three bioinformatic approaches used in the present study allowed to search for common biological processes targeted by *M. truncatula* miRNAs in both plant and human cells. Each approach provided different sets of information that can be either complementary or divergent, based on the assumptions of each used methodology. Besides the results presented so far, we also wanted to focus on a particularly conserved pathway in plants and humans, namely DDR (Yoshiyama et al., 2013; Nikitaki et al., 2018), because information relative to miRNAs targeting this essential process is still scarce, especially when concerning plants. Hence, **Tables 4** and **5** summarize a series of processes related to the DDR pathway and downstream processes in both kingdoms.

DNA repair, recombination, replication, and chromatin dynamics are tightly connected, as modifications of DNA conformation is required in order to allow access of the repair machinery to the damaged sites. This interplay is evidenced also by the fact that several genes are shared among these processes; for instance, the *A. thaliana* DME (Demeter) and DML1 (Demeter-like 1) are associated with both DNA repair (BER-base excision repair, GO:0006284) and chromatin

TABLE 3 | Common biological processes shared between *M. truncatula* and *H. sapiens* as resulted from the network-based approach involving the *M. truncatula* network construction. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*M. truncatula*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
Exocytosis	GO:0006887	Medtr4g102120	mtr-miR5559-3p	GO:0006887	SNPH	mtr-miR399f-5p
		Medtr8g023330	mtr-miR5558-3p	GO:0006904	RIMS3	mtr-miR482-5p
					SYT1	mtr-miR5211
					SYT2	mtr-miR2640
					NOTCH1	mtr-miR6286
					RAB3GAP1	mtr-miR6209
					RPHGAL	mtr-miR2589
					SYT15	mtr-miR166d
DNA replication, transcription, and modifications	GO:0006261	Medtr4g106540	mtr-miR5741a	GO:0090329	INO80	mtr-miR399f-5p
	GO:0090329			GO:2000104	LIG3	mtr-miR6294a
				GO:0008268	HMGA1	mtr-miR6276 mtr-
				GO:0044030	GRHL2	miR2589
				GO:2000678	PER2	mtr-miR169k
				GO:0032786	SIN3A	mtr-miR156b-3p
					BRD4	mtr-miR6286
Amino acid activation and transport	GO:0043038	Medtr7g083030	mtr-miR2657	GO:0009081	BCKDK	mtr-miR6273
	GO:0043039			GO:0009083	IVD	mtr-miR2640
	GO:0006418			GO:0051955	PER2	mtr-miR169k
				GO:0051957	RAB3GAP1	mtr-miR6209
				GO:0009065	NTSR1	mtr-miR408-3p
					TINAGL1	mtr-miR166d
					NANOS2	mtr-miR160c
					PRODH	mtr-miR169d-3p
RNA related processes	GO:0016071	Medtr3g077320	mtr-miR2629f	GO:0050686	CELF1	mtr-miR2670f
	GO:0006397			GO:0006376	CELF2	mtr-miR399f-5p
	GO:0008380			GO:0061014	GIGYF2	mtr-miR166d
	GO:0000375			GO:0061157	TNRC6B	mtr-miR5211
	GO:0000377			GO:0050686	KHSRP	mtr-miR396b
	GO:0000398				MEX3D	mtr-miR2673a
					RNPS1	mtr-miR396b
					SUPT5H	
Histone modification	GO:0016570	Medtr1g086590	mtr-miR395e	GO:0043981	KANSL1	mtr-miR482-5p
	GO:0016573	Medtr4g108080	mtr-miR156a	GO:0043982		
Protein modifications	GO:0043543	Medtr1g086590	mtr-miR395e	GO:0018345	CLIP3	mtr-miR627
	GO:0006473			GO:0006517	ZDHHC18	mtr-miR166b
	GO:0006475			GO:0036507	MARCH6	mtr-miR390
	GO:0018394			GO:0036508	UGGT1	mtr-miR6270a
	GO:0018393			GO:0042532	NF2	mtr-miR6206b

modification-related functions (GO:0006306, GO:0044728), whereas RAD54 (DNA Repair and Recombination Protein), RECA1 (Recombination A 1 protein), and KU80 (helicase Ku80 subunit of KU complex) are coupled with DNA repair (DSB, double-strand break repair, GO:0045003; HR-homologous recombination, GO:0000724) and DNA recombination (GO:0006310) processes (**Table 4**). Similarly, literature available from medical research assigned roles in DNA damage repair and chromatin remodeling to some of the genes predicted as targets of mtr-miRNAs. To cite some examples, PPP4C (Protein Phosphatase 4 Catalytic Subunit), is involved in a myriad of processes spanning from microtubule organization, to apoptosis, DNA repair, DNA damage checkpoint signaling, regulation of histone acetylation (Zhou et al., 2002; Lee et al., 2010), while INO80 (INO80 Complex Subunit) is the catalytic ATPase subunit of the INO80 chromatin remodeling complex, being however related also to DNA DSB repair (Conaway and Conaway, 2009). Functions related to DNA and chromatin/histone modifications were identified also in the *M. truncatula* network-based approach (see **Table 3**). This is the case of Medtr1g086590 (DNA methyltransferase 1-associated protein), Medtr4g108080 (ubiquitin-conjugating enzyme), and Medtr4g106540 (E2F transcription factor-E2FE-like protein) accessions. Within the alignment-based approach, mtr-miR2589 was predicted to target the *M. truncatula* Medtr6g047800 (tRNA methyltransferase complex GCD14 subunit) and the *H. sapiens* SETD1A (SET Domain Containing 1A, Histone Lysine Methyltransferase), functions involved in chromatin organization in both organisms (**Supplementary Table 2**).

Other processes tightly correlated with DDR include cell cycle and cell death (apoptosis/necrosis/programmed

TABLE 4 | Biological processes related to DNA repair, recombination, replication and chromatin remodeling common to *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*A. thaliana*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
DNA repair	GO:0006284	DME	mtr-miR2086-3p	GO:2000779	FOXM1	mtr-miR169d-3p
	GO:0045003	DML1	mtr-miR2661		PPP4C	mtr-miR169k
	GO:0000724	AT1G75290	mtr-miR5240			
		RAD54	mtr-miR172c-5p			
		RECA1	mtr-miR5658-3p			
		ASF1B	mtr-miR1509a-3			
		GMI1	pmtr-miR169l-3p			
		KU80	mtr-miR5272f			
DNA recombination and replication	GO:0006310	ASF1B	mtr-miR1509a-3p	GO:0090329	IND80	mtr-miR399f-5p
		GMI1	mtr-miR169l-3p	GO:2000104	LIG3	mtr-miR5294a
		KU80	mtr-miR5272f	GO:2000678	PER2	mtr-miR169k
		RAD54	mtr-miR172c-5p	GO:0032786	SIN3A	mtr-miR156b-3p
		ROK	mtr-miR5754		BRD4	mtr-miR5266
		RECA1	mtr-miR5658-3p			
		RPA70B	mtr-miR2593a-5p			
Chromatin remodeling	GO:0006306	DME	mtr-miR2086-3p	GO:0043981	KANSL1	mtr-miR482-5p
	GO:0044728	DML1	mtr-miR2661	GO:0043982	HMGA1	mtr-miR6276
	GO:0006305	DRD1	mtr-miR2650	GO:0070828	TNRC18	mtr-miR2589
	GO:0006304	EMB2770	mtr-miR7696c-5p	GO:0031507	GRHL2	mtr-miR2589
	GO:0031056	SDG14	mtr-miR2650	GO:0031936	PHF2	mtr-miR160c
	GO:0031058		mtr-miR2086-3p	GO:0006268	SIN3A	mtr-miR156b-3p
	GO:0031060		mtr-miR7696c-5p	GO:0044030	ZNF304	mtr-miR166a-5p
	GO:0031062			GO:0031935		
	GO:1905269			GO:0031937		
	GO:1902275					
	GO:0080188					

cell death). DNA replication, recombination, and repair are more active during certain phases of the cell cycle and the success of these processes can decide the fate of the cell. The connection between pathways is evidenced by genes that play important functions in both DNA repair/replication, chromatin remodeling, and cell cycle/cell death (**Table 5**). This is the case of the ASF1B (Anti-Silencing Function 1B, histone chaperone) and KU80 functions in plants, involved in the S-phase replication-dependent chromatin assembly (Zhu et al., 2011) and maintenance of genome integrity (West et al., 2002), respectively, and the SIN3A (Histone Deacetylase Complex Subunit Sin3a) and HMGA1 (High Mobility Group Protein A1) genes in humans, with roles associated to chromatin regulation and cell cycle progression (Silverstein and Ekwall, 2004; Pierantoni et al., 2015). While the *A. thaliana* network-based approach has not identified genes (predicted targets of mtr-miRNAs) associated with cell death in plants, this however led to the identification of many putative targets related to apoptosis in human cells. To cite a few, BCL2L11 (BCL2-Like 11 apoptosis facilitator), NOTCH1 (Notch Receptor 1), and TP53BP2 (Tumor Protein P53 Binding Protein 2) are among the most essential apoptotic factors. NOTCH1, part of the Notch signaling pathway, is involved in many processes related to cell fate specification, differentiation, proliferation, and survival, while its activation is related to many types of cancers (e.g. cervical, colon, head and neck, lung, renal, pancreatic, leukemia, and breast cancer) (Xiao et al., 2016). Hence, dietary miRNAs targeting this specific function may have positive implications in sustaining cancer therapies. The alignment-based approach allowed to identify a conserved miRNA (mtr-miR319d-5p) predicted to target genes associated with cell death functions in both *M. truncatula* (DCD-development and cell death domain protein) and *H. sapiens* (MESD, PRR5L) (**Supplementary Table 2**). While MESD (Mesoderm Development LRP Chaperone) is related to the Notch pathway (Hsieh et al., 2003), PRR5L (Proline Rich 5 Like) regulates the activity of the mTORC2 (mechanistic target of rapamycin) complex controlling cell migration (Gan et al., 2012).

Hence, to answer the herein proposed question, the network-based as well as the alignment-based approaches pinpointed mtr-miRNAs predicted to target genes involved in DDR and downstream processes in *A. thaliana*, *M. truncatula*, and *H. sapiens*.

DISCUSSION

In view of the controversies raised by the recent 'dietary xenomiR' hypothesis (Witwer, 2012), bioinformatics studies have the potential to aid the ongoing efforts to reinforce new methodologies and provide the basis for further experimental validation. Model organisms, like *A. thaliana*, are used as guidance systems to explore bioinformatics data-driven questions related

TABLE 5 | Biological processes related to cell cycle and cell death common to *A. thaliana* and *H. sapiens* as resulted from the network-based approach. The ID corresponding to each GO term (GO ID) along with putatively target genes and corresponding miRNAs are provided.

Biological process	*A. thaliana*			*H. sapiens*		
	GO ID	Gene	miRNA	GO ID	Gene	miRNA
Cell cycle	GO:0000075	ASF1B	mtr-miR1509a-3p	GO:1901989	BRD4	mtr-miR5266
	GO:0045930	RAD9	mtr-miR2688b	GO:1901992	EIF4G1	mtr-miR166d
	GO:0007093			GO:1902751	PHB2	mtr-miR5266
				GO:0010971	SIN3A	mtr-miR156b-3p
				GO:0071157	MDM2	mtr-miR169k
					MDM4	mtr-miR5266
Cellular senescence	GO:0090729	KU80	mtr-miR5272f	GO:2000772	ABL1	mtr-miR5276
		TRB1	mtr-miR5568-5p		HMGA1	mtr-miR5276
					VASH1	mtr-miR160c
Apoptosis/cell death	–	–	–	GO:1902108	BMF	mtr-miR2613
				GO:1902110		mtr-miR5266
				GO:1902263	GDNF	mtr-miR2673a
				GO:0060561	BCL2L11	mtr-miR5273
				GO:0001844		mtr-miR5266
				GO:1900117	NOTCH1	mtr-miR5266
				GO:0070231	VDR	mtr-miR5276
				GO:0043525	YWHAG	mtr-miR2673a
				GO:1901028		mtr-miR390f-5p
				GO:1901216	DFFA	mtr-miR390f-5p
				GO:1901030	TP53BP2	mtr-miR390f-5p
				GO:2001238		mtr-miR5266
				GO:0097192		mtr-miR2613
				GO:1900740		mtr-miR160c
				GO:1902686	AKT1	mtr-miR160c
						mtr-miR5266
					DFFA	mtr-miR390f-5p
					KDELR1	mtr-miR166d
					ARHGEF7	mtr-miR2589
					GDNF	mtr-miR2673a
					BAD	mtr-miR5266
						mtr-miR5276
						mtr-miR390f-5p
						mtr-miR2673a
					ABL1	mtr-miR390f-5p
					ITM2C	mtr-miR5266
					PEA15	mtr-miR160c
					TNFRSF12A	mtr-miR160c
					TRAF2	mtr-miR2673a
					CX3CL1	mtr-miR2613
					GDNF	mtr-miR5211
					SPG7	mtr-miR390f-5p

to putative cross-species miRNA targets (Zhang et al., 2016a). The high interest in this field prompted the development of databases able to predict the functional impact of food-borne miRNAs in humans (Chiang et al., 2015; Shu et al., 2015). The DMD (Dietary MicroRNA Database) database covers only very few edible plant species (Chiang et al., 2015), hence there is the need to substantially enlarge the information and include alternative species with a potential impact on food security. Within this framework, the current study aims at identifying plant miRNAs along with their endogenous and cross-kingdom targets to pinpoint conserved pathways between evolutionary distant species. Starting from a list of publicly available *M. truncatula* miRNAs, we made the assumption that any miRNA may have the potential to target genes in both plants and humans. Given that the bioinformatics approaches do not allow the prediction of miRNAs stability and function validation within the organisms, there is the need to further experimentally confirm the proposed hypotheses. The choice of plant species is driven by the fact that *M. truncatula* is at the crossroad between model organisms (in the case of legume research) and economically relevant species, given its potential use as microgreens to support more sustainable agriculture. The presented methodologies can serve both as guidelines to be applied to other plant species as well as to test new hypotheses exploring the potential benefits of food-borne mtr-miRNAs targeting human genes. When considering the conserved families of miRNAs, this study could aid the translational research covering other economically relevant plant species (with 100% sequence similarity) and potential human target genes. As exemplified in **Figure 5**, miR164, miR166, and miR390 have a 100% sequence similarity between *M. truncatula* and other dicot plant species such as tomato (*Solanum lycopersicum*) and apple (*Malus domestica*). Among the selected

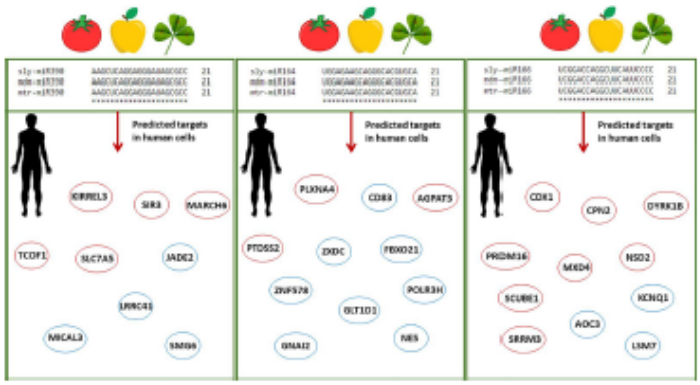

FIGURE 5 | Schematic representation of conserved plant miRNAs potentially targeting human genes. Alignments between three conserved miRNAs (miR390, miR164, miR166) from different plant species, namely *Solanum lycopersicum* (sly), *Malus domestica* (mdm), and *M. truncatula* (mtr), show 100% sequence similarity. The predicted human target genes found in the enriched biological processes of the network-based approach and among the genes with significant sequence similarity in the alignment-based approach are shown in red and blue circles, respectively. Abbreviations: KIRREL3, Kirre Like Nephrin Family Adhesion Molecule 3; SIRT3, Sirtuin 3; MARCH6, Membrane Associated Ring-CH-Type Finger 6; TCOF1, Treacle Ribosome Biogenesis Factor 1; SLC7A5, Solute Carrier Family 7 (Amino Acid Transporter Light Chain, L System), Member 5; JADE2, Jade Family PHD Finger 2; LRRC41, Leucine Rich Repeat Containing 41; MICAL3, Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 3; SMG6, Nonsense Mediated MRNA Decay Factor; PLXNA4, Plexin A4; AGPAT5, 1-Acylglycerol-3-Phosphate O-Acyltransferase 5; PTDSS2, Phosphatidylserine Synthase 2; CD83, Cluster of Differentiation 83; ZXDC, ZXD Family Zinc Finger C; FBXO21, F-Box Protein 21; GLT1D1, Glycosyltransferase 1 Domain Containing 1; GNAI2, G Protein Subunit Alpha I2; ZNF578, Zinc Finger Protein 578; NES, Nestin; POLR3H, RNA Polymerase III Subunit H; CDK1, Caudal Type Homeobox 1; CPN2, Carboxypeptidase N Subunit 2; DIRK1B, Dual specificity tyrosine-phosphorylation-regulated kinase 1B; MXD4, MAX Dimerization Protein 4; NSD2, Nuclear Receptor Binding SET Domain Protein 2; PRDM16, Histone-lysine N-methyltransferase PRDSET Domain 16; SCUBE1, Signal Peptide, CUB Domain And EGF Like Domain Containing 1; SRRM3, Serine/Arginine Repetitive Matrix 3; AOC3, Amine Oxidase Copper Containing 3; KCNQ1, Potassium Voltage-Gated Channel Subfamily Q Member 1; LSM7, U6 snRNA-associated Sm-like protein 7.

examples, miR166 was previously demonstrated to be abundant in different human body fluids and tissues (Lukasik et al., 2018; Zhao et al., 2018b). The putative human targets identified either through the network- or alignment-based approaches could serve as potential candidates to aid medical interventions. To cite one example, inhibition of the AOC3 (Amine Oxidase Copper Containing 3), playing important roles in adipogenesis and putatively targeted by miR166, could result in decreased fat deposition (Carpéné et al., 2007; Shen et al., 2012), hence addressing the big issues related to obesity and the many obesity-associated diseases.

It is important to underline that the experimental design of this study was thought in such a way to potentiate the identification of conserved pathways/players between evolutionary distant species. To do so, three different bioinformatics pipelines were developed (two network-based approaches, considering *A. thaliana* and *M. truncatula* model species, and one alignment-based approach) to confront plant and human targeted biological processes. Form a methodological point of view, the developed approaches enable the exploration of different assumptions supported by robust statistical methods. The network-based approaches rely on extensive knowledge available on the interactions among miRNA target genes in a given species. The knowledge was exploited for the construction of gene co-expression/interaction networks, from which a set of biological processes predicted to be targeted by the plant miRNAs were found. This approach aims to study the regulatory potential of the full list of *M. truncatula* miRNAs. Two network-based approaches were implemented, differing from the point of view of plant network construction. In one approach, the predicted *M. truncatula* target genes were mapped to the genome of *A. thaliana*, which is a supported organism in widely used network construction tools, while *M. truncatula* is not. The other network-based approach relies on the construction of a novel co-expression network for *M. truncatula*, using publicly available expression data, thereby evaluating the robustness of the performed assumptions on the plant network. Finally, the alignment-based approach was radically different, since it only relies on target gene and protein sequences, with no other assumption, and aimed to discover sequence similarities between plant and human targets, individually for each targeting miRNA. In this context, this approach enabled the inference on the potential effect of every single miRNA of the initial list. Importantly, none of the proposed strategies is focused on the prediction of individual target genes: only the ones sharing statistically over-represented processes (in network-based approaches), or having the same targeting miRNA and a statistically significant nucleotide and protein sequence similarity (in alignment-based approaches) were detected and discussed in

this work. The prediction of individual targets relied on existing computational tools used in the early steps of the workflow under assumptions similar to the ones of previous studies (Zhang et al., 2016a), despite no standardized pipeline is well-accepted to accomplish this task due to the lack of genome-wide experimental validations across species. The main limitations of the proposed approaches are that: *(i)* no assumption was made on which miRNAs can be delivered between plants and human, *(ii)* only the 3′ UTR region was assumed to be the target region of plant miRNAs in humans. In addition, we decided to rely on computational methods to predict the binding between miRNA and putative target transcript; alternative approaches could also exploit homology between plant and human miRNAs, which might share the same seed region and then identify human targets based on experimentally validated target genes in human cells (Liu et al., 2017). However, such alternative would have led to the consideration of a smaller number of plant miRNAs, since only the ones with homologous features could have been included in the analysis, thereby losing the possibility to study the whole plant miRNA regulation potential. Nonetheless, we believe that the methodological approaches are sufficiently general to be extended onto the desired miRNA list and candidate target list (e.g. 3′/5′ UTRome, transcriptome, or collection of coding sequences) as input. Moreover, the interpretation of the enriched biological processes identified from network analysis is affected by Gene Ontology terms of different specificity and name (Zhang et al., 2016a), thereby limiting the discovery of all the potentially targeted functions.

From a biological perspective, the employed strategies resulted in both complementary and divergent observations. For instance, 'exocytosis' was a common denominator in all three investigated species (*M. truncatula*, *A. thaliana*, and *H. sapiens*) when using the network-based approaches. On the other hand, the alignment-based approach allowed a more direct identification of miRNAs targeting genes in *M. truncatula* and *H. sapiens* (e.g., same miRNA *vs.* similar/different functions) whereas the generated networks illustrates conserved biological processes (e.g., same function *vs.* same/different miRNAs). The two approaches also indicated connecting elements. For example, mtr-miR168a was predicted to target AGO1 in plants and PVP in humans, functions associated with pathogen (namely, viruses) defence, in both approaches. The miR168a is part of a conserved family of plant miRNAs among different species, but as we seen in **Supplementary Figure 2** and other cited literature (Lang et al., 2019), differences exist between dicot and monocot species. The predicted human targets observed in previously published researches (Zhang et al., 2012; Javed et al., 2017), were not found in the enriched process or sequence similarity with our approaches (see **Supplementary Table 1**). This can have different explanations: *(i)* four sequence mismatches (two located within the seed region) are present between osa-miR168a and mtr-miR168a, including a G at position 14, recently reported to generate a G:U wobble that limits its binding to LDLRAP1 (Lang et al., 2019); *(ii)* only 3′ UTR regions were considered in our study, and since osa-miR168a targets the LDLRAP1 CDS region (Zhang et al., 2012), we did not find this match in the target list; *(iii)* we used the entire length of the miRNA and 100% sequence complementarity instead of only the miRNA seed region (Zhang et al., 2012; Javed et al., 2017). By searching for mtr-miR168a and osa-miR168a targets in the full transcript sequences, a more relevant annealing score to the LDLRAP1 gene was found with osa-miR168a than mtr-miR168a, probably due to the sequence mismatches.

Considering that the purpose of the study was to identify conserved functions between distant species through the lens of mtr-miRNAs, our results report 'vesicle docking involved in exocytosis', 'modulation by virus of host morphology or physiology', 'cellular response to virus', 'positive regulation of post-transcriptional gene silencing', and 'branched-chain amino acid metabolic process' as common biological processes between Arabidopsis and humans (**Table 1**). A different study designed to look into the role of plant miRNAs in inter-species regulatory networks indicated ion transport and stress response as shared functions between Arabidopsis and humans (Zhang et al., 2016a). However, this study took into consideration only 25 miRNAs to construct the relative species-specific networks while we started from a list of 426 *M. truncatula* miRNAs to disclose the full regulatory potential. When considering the alignment-based approach, the most represented predictive targets in *M. truncatula* covered transcription factors and hormone-responsive genes. Interestingly, some of these miRNAs (e.g., mtr-miR160a, mtr-miR164h, mtr-miR166d, mtr-miR2673a) were predicted to target TFs (HAND2, ZXDC) and hormone-related functions (DYRK1B, HNF4A) also in human cells. This is in agreement with the concept that miRNAs may behave in a hormone-like manner since hormones and miRNAs are reciprocally regulated in both plant and animal kingdoms (Li et al., 2018).

Because evidence of miRNAs involvement in the regulation of DDR-related pathways is still limited in plants and considering the conservation of some DDR functions between plants and animals (Yoshiyama et al., 2013; Nikitaki et al., 2018), we decided to focus our attention on these specific pathways. Hence, miRNAs predicted to target genes involved in DNA repair, recombination, and replication, chromatin remodeling, cell cycle, and cell death were hereby identified in plants (see **Tables 4** and **5**). For instance, mtr-miR172c-5p, mtr-miR2638h, mtr-miR5272f, and mtr-miR2086-3p, were predicted to target the Arabidopsis RAD54, RAD9, KU80, and DME genes, respectively. In the *M. truncatula* network-based approach, the 'DNA-dependent DNA replication' (GO:0006261) biological process is represented by Medtr4g106540 (E2F transcription factor-E2FE-like protein) as a predicted target of mtr-miR5741a (see **Supplementary Dataset 3**). Within the alignment-based approach, mtr-miR2589, mtr-miR482-5p, mtr-miR5287b, and mtr-miR319d-5p were predicted to target two methyltransferases (Medtr6g047800, Medtr5g079860), the CDC48 (Medtr7g088980), and DCD genes (Medtr4g084080) (see **Table 2** and **Supplementary Table 2**). All these hits bring an added value for plant science as they associate specific, previously unknown, miRNAs to the regulation of DDR functions. To date, there are only a few reports predicting DDR-associated functions as putative targets of miRNAs; for instance, MRE11 (Meiotic Recombination 11, a DSB repair nuclease) has been predicted as target of miR5261 in *Citrus sinensis* (Liang et al., 2017), or XPB2 (*Xeroderma pigmentosum* type B, an excision

repair helicase) predicted as target of miR122c-3p in *Triticum aestivum* (Sun et al., 2018). In human cell research, miRNAs involvement in the regulation of DDR is much more advanced and it is associated with the development of new therapeutic/diagnostic tools (Hu and Gatti, 2011; He et al., 2016). A number of studies document that p53, the master-regulator of DDR, is targeted by endogenous miRNAs (Hu et al., 2010; Kumar et al., 2011). This is the case of miR-25 and miR-30d, associated with p53 downregulation along with the suppression of downstream interactors p21, BAX, and Puma, hence being involved in apoptotic processes (Kumar et al., 2011). Another example, in relation to DNA repair pathways, indicates that hsa-miR-526b targets the Ku80 mRNA, with subsequent alterations of DSB repair and cell cycle arrest (Zhang et al., 2015). Our bioinformatics approach also revealed mtr-miRNAs predicted to target human genes with roles in DNA repair and related processes (see **Table 4**). To reiterate some examples, PPP4C (putatively targeted by mtr-miR169d-3p) catalyses the dephosphorylation of RPA2 (Replication Protein A2) in response to DNA damage, thus permitting the recruitment of RAD51 (an essential recombinase for the HR repair) to the damaged site (Lee et al., 2010). Likewise, FOXM1 (Forkhead Box M1), predicted as a target of mtr-miR169k, is among the most overexpressed oncoproteins in many types of cancer and therapeutic interventions to suppress its function are of great interest (Halasi et al., 2018). Hence, the identification of a miRNA belonging to conserved plant miRNA families (in this case, miR169) as a putative target of this gene may bring further support to ongoing cancer remedies. In relation to this, also many of the predicted targets associated with apoptosis (see **Table 5**), like the presented example of NOTCH1 (putatively targeted by mtr-miR5266), could have similar implications. The use of plant miRNAs as adjuvants in cancer therapies has been already tested; for example, plant miR159, abundantly found in human serum, has been associated with reduced incidence and progression of breast cancer because it targets the TCF7 (a Wnt signaling transcription factor) gene, causing decreased levels of MYC (Avian Myelocytomatosis Viral Oncogene Homolog) proteins, essential for cell cycle progression (Chin et al., 2016).

In conclusion, the current study provides comprehensive datasets (obtained by combining *ad-hoc* bioinformatics tools) related to *M. truncatula* miRNAs potential to putatively target genes across evolutionary distant species. By focusing on specific DDR-related functions, the hereby presented results significantly contribute to enriching the current knowledge regarding the conservation of DDR in plant and human cells. Considering the implications that some of these putative interactions could have for the biomedical sector, this study also offers additional hypotheses to be further experimentally validated. The developed pipeline can be applied to any species of interest to address species-specific cross-kingdom interactions or to carry out large-scale investigations involving a number of plant/animal species. The application of the proposed methods to other case-studies should take into account the following considerations on data, software, and knowledge availability: *(i)* the miRNAs of a 'donor' organism (e.g., a plant) and the transcriptome of the 'donor' and 'receiving' (e.g., an animal) organism should be available from public datasets or *de-novo* sequencing, annotation and expression studies; *(ii)* the miRNA dataset could be further refined by selecting experimentally known or computationally predicted miRNAs that are protected from degradation during incorporation in the receiver organism. The prediction miRNA targets in both species can be carried out *via* bioinformatic tools online available, although tool specificity for the target species should be taken into account and the parameter(s) of the algorithms should be fine-tuned accordingly, in order to have a balanced number of targets to be analysed in both species. Other target prediction algorithms may be used to overcome the so far weak knowledge on cross-kingdom regulation mechanisms to identify the target transcripts of heterologous miRNAs. For the miRNA target network reconstruction, an homology-based strategy should exploit the homology between the desired organisms and their model organisms in the same kingdom (e.g., *A. thaliana* for plants); on the other hand, *ade-novo* organism-specific co-expression network reconstruction relies on the availability of gene expression data from public datasets (e.g., GEO) or novel microarray/RNAseq experiments. All the networks can be analysed *via* specific software (e.g., Cytoscape) to find clusters of co-expressed genes and to carry out enrichment analyses on the desired gene sets. The experimental validation of the predicted targets can be subsequently performed *via* degradome analysis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MB, AM, and LP conceptualized the study. AM and LP wrote the manuscript. DM performed the network-based approach, MB performed the alignment-based approach, and ES performed the *M. truncatula* network reconstruction and analysis. AM, MB, and CG analysed the generated data. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01535/full#supplementary-material

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REVIEW
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Redox Balance-DDR-miRNA Triangle: Relevance in Genome Stability and Stress Responses in Plants

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Plants are continuously faced with complex environmental conditions which can affect the oxidative metabolism and photosynthetic efficiency, thus leading to the over-production of reactive oxygen species (ROS). Over a certain threshold, ROS can damage DNA. DNA damage, unless repaired, can affect genome stability, thus interfering with cell survival and severely reducing crop productivity. A complex network of pathways involved in DNA damage response (DDR) needs to be activated in order to maintain genome integrity. The expression of specific genes belonging to these pathways can be used as indicators of oxidative DNA damage and effective DNA repair in plants subjected to stress conditions. Managing ROS levels by modulating their production and scavenging systems shifts the role of these compounds from toxic molecules to key messengers involved in plant tolerance acquisition. Oxidative and anti-oxidative signals normally move among the different cell compartments, including the nucleus, cytosol, and organelles. Nuclei are dynamically equipped with different redox systems, such as glutathione (GSH), thiol reductases, and redox regulated transcription factors (TFs). The nuclear redox network participates in the regulation of the DNA metabolism, in terms of transcriptional events, replication, and repair mechanisms. This mainly occurs through redox-dependent regulatory mechanisms comprising redox buffering and post-translational modifications, such as the thiol-disulphide switch, glutathionylation, and S-nitrosylation. The regulatory role of microRNAs (miRNAs) is also emerging for the maintenance of genome stability and the modulation of antioxidative machinery under adverse environmental conditions. In fact, redox systems and DDR pathways can be controlled at a post-transcriptional level by miRNAs. This review reports on the interconnections between the DDR pathways and redox balancing systems. It presents a new dynamic picture by taking into account the shared regulatory mechanism mediated by miRNAs in plant defense responses to stress.

Keywords: redox balance, DDR, miRNA, redox-sensitive TFs, cell cycle checkpoints

INTRODUCTION

The maintenance of the cellular redox balance is a major biological attribute influencing growth, development and survival in plant and animal systems (de Pinto et al., 1999, 2015; Pellny et al., 2009; Chiu and Dawes, 2012). In animal systems, a mild oxidative environment has been observed to activate a signaling pathway leading to cell proliferation (Menon et al., 2003; Menon and Goswami, 2007). Interaction between the epidermal growth factor and their specific receptor stimulates cell proliferation by the generation of a low amount of reactive oxygen species (ROS; Menon and Goswami, 2007). In plants, a strong correlation between the cellular redox state and cell cycle block has been clearly observed in the root quiescent center, a group of spatially defined cells that are blocked in G0 (Jiang and Feldman, 2005; Jiang et al., 2006; Dinneny et al., 2008). An increase in ROS production generally causes a cell cycle arrest before the activation of the cell death program (Chiu and Dawes, 2012; de Pinto et al., 2012). As a common feature of eukaryotic organisms, it has been hypothesized that cell cycle progression is driven by an intrinsic redox cycle consisting in regulated reductive and oxidative phases (Chiu and Dawes, 2012). Glutathione (GSH), the most abundant non-protein thiol in the cell, seems to be a major actor in the redox fluctuations normally occurring during cell proliferation in animal and plant cells (García-Giménez et al., 2013). Alterations in the cell redox potential may also be responsible for the abnormal proliferation of cancer cells which have a "constitutive" decrease in the cellular redox potential, and therapies able to adjust their cellular redox balance have been proposed (Hoffman et al., 2001, 2008). In plants, phythogen toxins blocking cell proliferation induces an alteration in GSH fluxes between nucleous and cytosol (Locato et al., 2015). Thus, sensing the redox state at tissue, cellular and subcellular levels is needed to accurately allow cell cycle progression in the right redox environmental conditions, linking the cell stress response to the cell cycle checkpoint pathway (Pearce and Humphrey, 2001).

The maintenance of the cellular redox balance is also a crucial attribute influencing plant development. Plant embryogenesis has indeed been correlated to a shift in the cell environment toward a more oxidized state (Belmonte and Stasolla, 2007; Stasolla et al., 2008; Becker et al., 2014) and the circadian clock also seems to be regulated at the redox level and vice versa (Lai et al., 2012). Moreover, the cell redox state is intrinsically correlated to the cell metabolic status and consequently it is presumed to be tightly linked to cell energy efficiency. In aerobic organisms, perturbations in the cell redox status are reflected in metabolic efficiency, calculated as the ratio between oxygen consumption and ATP production (Giancaspero et al., 2009). In line with this, in plants, environmental stressing conditions that perturb the cellular redox status have been found to impair the mitochondrial metabolism (Vacca et al., 2004; Valenti et al., 2007). Thus, metabolic efficiency can be monitored by assaying the mitochondrial respiration pathway. In the yeast model, the metabolic cycle, which consists of respiratory (oxidative phase) and fermentative/non-respiratory (reductive phase) phases, seems to be synchronized to cell cycle progression, with mitosis and DNA replication occurring during the reductive phase and G1 during the oxidative phase (Tu et al., 2005). This synchronization may act as a protective mechanism toward genome integrity, thus enabling DNA synthesis to occur in a non-oxidative environment (Chen et al., 2007).

Plant exposure to stressful conditions, both exogenous (solar UV radiation, high soil salinity, drought, chilling injury, air and soil pollutants including heavy metals) and endogenous (metabolic by-products) in nature, can compromise genome integrity. Due to their sessile lifestyle, and the presence, for all the lifespan, of a small population of the same meristematic cells continuously dividing for allowing organism growth, plants have evolved various strategies to cope with environmental constraint conditions (Spampinato, 2017). Among these, the continuous exposure to sunlight represents a dramatic challenge to genome integrity and to genome transmission to the subsequent generation (Roy, 2014). The DDR specifically aims to aid plants to cope with the detrimental effects of genotoxic stress. DDR is a complex signal transduction pathway, which detects DNA damage signals and transduces those signals to execute cellular responses. Both redox systems and DDR pathways are usually tightly regulated through the coordinated activities of cellular oxidants/antioxidants and DNA damage/repair-signaling pathways (**Figure 1**). It is well-known that intracellular ROS acts both as a cellular damaging compound and as a signaling molecule, all depending on its concentration and localization (Foyer and Noctor, 2003; Jeevan Kumar et al., 2015; Mittler, 2017). Links between ROS and DDR pathways have been hypothesized but not yet clearly demonstrated. For instance, studies on animal cells treated with neocarzinostatin (a radiomimetic that causes the formation of double-strand breaks) have shown that ROS induction is partly mediated by increasing levels of histone H2AX, a biomarker for DDR (Kang et al., 2012). Hence, ROS generate DNA damage while being regulated by the DNA damage-signaling pathways.

All the evidence outlined above makes controlling the cell redox balance a major regulator of virtually all plant metabolic re-arrangements occurring in growth, development and defense strategies. Regulation of all the metabolic transitions experienced by DNA (first and foremost, transcription, replication, and repair) within the cells is expected to be tightly connected with redox signaling pathways. Furthermore, the maintenance of cellular redox homeostasis and genomic integrity can be modulated by the activity of microRNAs (**Figure 1**; miRNAs). This review reports on the influence of various redox active systems on DNA damage response pathways and plant transcriptome as well as on post-transcriptional gene expression regulation mediated by miRNA.

DDR AS A KEEPER OF GENOME STABILITY

DNA damage response is an evolutionary conserved, complex network that includes signal transduction pathways composed of sensors, transducers, mediators, and effectors, dedicated to safeguard genome integrity. Several comparative studies have

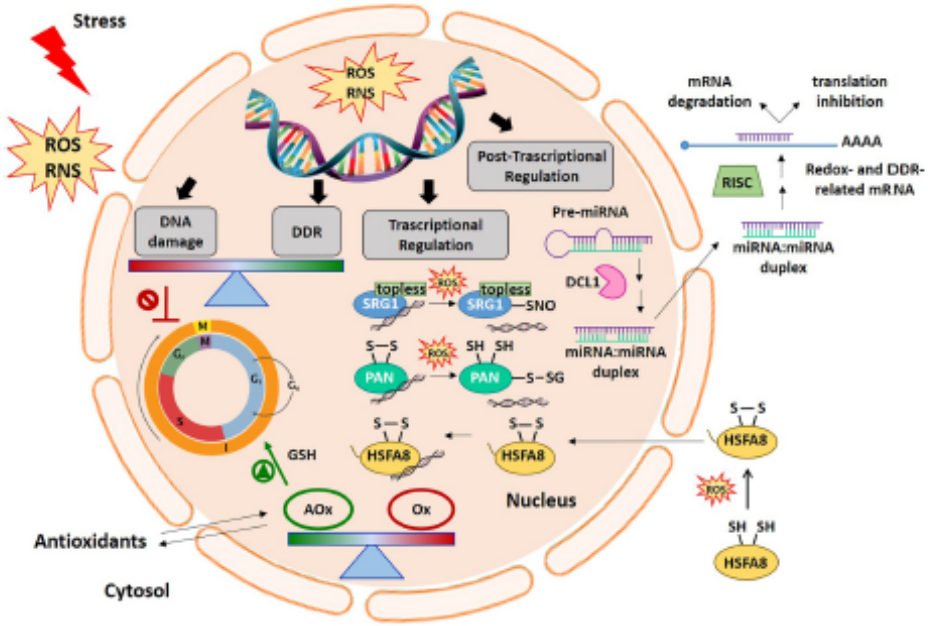

FIGURE 1 | Cellular redox balance-DDR-miRNA triangle. An increase in ROS production generally occurs early under different stress conditions. ROS and redox signals move through different cell compartments. In the nucleus, ROS accumulation can cause DNA damage thus inducing cell cycle arrest. The DDR specifically aims to help plants cope with the negative effects of genotoxic stress. Alterations in antioxidant and oxidant balance in the nucleus are required to promote cell cycle progression in the right redox environment. In this context, ROS and redox signals are involved in the regulation of gene expression at transcriptional and post-transcriptional levels. The picture shows some examples of the redox dependent transcriptional mechanisms involving some redox-sensitive TFs, such as SRG1, PAN, and HSFA8, in gene expression regulation. At the post-transcriptional level, the figure also shows the modulation of redox- and DDR-related target mRNAs by miRNAs. AOx, antioxidants; DCL1, DICER-like1; DDR, DNA damage responses; HSFA8, heat shock factor A8; miRNA, microRNA; Ox, oxidants; PAN, PERIANTHIA; RNS, reactive nitrogen species; ROS, reactive oxygen species; SRG1, SNO-regulated gene 1.

highlighted the conserved features of the core DDR machinery across eukaryotes, including plants and mammals, as well as the presence of unique characteristics in plants (DiRuggiero et al., 1999; Singh et al., 2010; Spampinato, 2017; Nikitaki et al., 2018). Most DDR components are ancestral genes that appeared early in the phylogenetic tree and subsequently expanded and shaped throughout evolution. Based on the detection of a DNA lesion by dedicated sensors, various pathways may be triggered, leading to the activation of cell cycle checkpoints, DNA repair, or programmed cell death (PCD). Endoreduplication, consisting of DNA replication in the absence of cytokinesis, represents a plant-distinctive process, which is also part of DDR (Yoshiyama et al., 2013b).

Most of the knowledge regarding DDR and DNA repair pathways, gained through decades of studies on yeast, bacteria, and mammals, has highlighted its function in plant biology (Spampinato, 2017). Indeed, functional and/or structural homologs of various DDR factors found in animals have been identified in model plants such as Arabidopsis (Spampinato, 2017) and *Medicago truncatula* (Balestrazzi et al., 2011). Some exhaustive examples are: MRE11 (Meiotic Recombination 11), RAD51, NBS1 (Nijmegen breakage syndrome 1) proteins, constituting the MRN complex, and RPA (Replication protein A). The MRN complex is required for double-strand break (DSB) recognition in the DDR pathway involving ATM (Ataxia Telangiectasia Mutated) kinase (Yoshiyama et al., 2013a), while RPA binds to single-stranded DNA (ssDNA) lesions associated with DNA replication in a pathway involving the ATR (ATM and Rad3-related) protein. The ATM and ATR transducers amplify and transduce signals to subsequent effectors through a phosphorylation-mediated cascade of events resulting in the activation of downstream processes (cell cycle arrest with the critical choice between DNA repair and PCD) (Culligan et al., 2004, 2006; Yoshiyama et al., 2013a,b). For instance, ATM and ATR transducers induce phosphorylation of the histone-variant H2AX (Dickey et al., 2009; Yuan et al., 2010)

which, in the γH2AX phosphorylated form, acts as a DNA damage signal and recruits several proteins to the DSB site (Petrini and Stracker, 2003; Yoshiyama et al., 2013a). In yeast and mammals, after ATR activation, serine/threonine-protein kinases CHK1 (checkpoint kinase) and CHK2 were phosphorylated by ATR and ATM, respectively, with a consequent activation of cell-cycle checkpoints (Bartek et al., 2001; Chen and Sanchez, 2004). Arabidopsis appears to have no CHK1 and CHK2 orthologs. Considering that some of the substrates of CHK1 and CHK2 in animals, such as the mediator BRCA1 (breast cancer susceptibility gene 1), and E2F1 (E2F Transcription Factor 1), are also present in plants (Lafarge and Montané, 2003; Inzé and de Veylder, 2006), it has been suggested that other kinases may work as functional homologs of CHK1 and CHK2 (Yoshiyama et al., 2013b). Studies on the Arabidopsis *atm* and *atr* mutants have shown that in addition to the conserved function in DDR, ATM and ATR play a different role in the life of plants (Garcia et al., 2003; Culligan et al., 2004). Yan et al. (2013) reported an intriguing finding linking the plant immune system to DNA damage. They demonstrated that the plant hormone SA induces DNA damage in the absence of a genotoxic agent, and the DDR components, ATR and RAD17 (radiation sensitive) are required for adequate plant immune responses, thus suggesting the role of DDR in the defense against pathogens. In contrast, Rodriguez et al. (2018) reported DNA damage as a consequence of autoimmune response rather than actively produced host-DNA damage aimed at stimulating resistance to pathogens.

The various factors involved in DDR are temporally and spatially regulated and activated through the action of mediators that recruit additional substrates and control their association with damaged DNA (Stewart et al., 2003; Stracker et al., 2009). Several mediators are known in human cells, such as MDC1 (mediator of DNA-damage checkpoint protein 1), 53BP1 (p53-binding protein), BRCA1 (Breast cancer susceptibility 1), related to the ATM pathway, TOPBP1 (topoisomerase 2-binding protein 1), and CLSPN (Claspin), involved in the co-regulation of the ATR pathway. Plants lack counterparts for some DDR mediators (e.g., MDC1 and 53BP1) (Yoshiyama et al., 2013b; Nikitaki et al., 2018). However, there are DDR components exclusively found in plants, such as SMR (Siamese-related) cyclin-dependent protein kinase inhibitors, some chromatin remodelers (CHR complexes), and several DNA and histone methyltransferases such as CMT3 [DNA (cytosine-5)-methyltransferase 3], SDG26 (SET domain group 26), SUVH5 (histone-lysine N-methyltransferase, H3 lysine-9 specific) (Nikitaki et al., 2018). Interestingly, the p53 effector, which is a TF acting as tumor suppressor in animal cells, does not exist in plants. In animals, the master regulator p53 rules the fate of the cell following DNA damage, which triggers cell-cycle arrest and then DNA repair or apoptosis (Helton and Chen, 2007). A similar role in plants has been ascribed to the TF SOG1 (suppressor of gamma response 1), a component of the NAC (NAM-ATAF1/2-CUC2) family (Preuss and Britt, 2003; Yoshiyama et al., 2009). SOG1 regulates more than 100 genes and similarly to p53, induces several pathways including cell cycle arrest, DNA repair, PCD, and endoreduplication (Yoshiyama et al., 2013b; Yoshiyama, 2015). It is thus clear that most of the DDR factors are well preserved in animals and plants, although various key components are unique to plants.

DNA Damage Repair Mechanisms Activated by DDR Pathways

Of the pathways triggered by DDR effectors, DNA repair mechanisms are crucial in maintaining genome integrity. Several pathways are involved in the correction of various types of DNA lesions including: (1) direct repair (DR) or photoreactivation (Jiang et al., 1997), (2) mismatch repair (MMR), (3) base- and nucleotide excision repair (BER, NER) (Shuck et al., 2008; Peña-Diaz and Jiricny, 2012; Jiricny, 2013), (4) double-strand break repair (DSBR), which includes non-homologous end joining (NHEJ), and homologous recombination (HR) mechanisms (Puchta and Hohn, 1996).

The DR is a light-dependent pathway that relies on the activity of flavoenzymes, called photolyases, carrying the two electron-reduced forms of FAD (FADH) as photocatalysts (Sancar, 2003). After binding to the DNA lesion, the enzymes remove the damage following absorption of blue light in the 300–600 nm range (Tuteja et al., 2009). The activity of photolyases is specific to plants, since it seems to be absent in humans and other placental animals (Essen and Klar, 2006). On the other side, MMR is present in all organisms, and corrects replication and genetic recombination errors, which result in poorly matched nucleotides. In eukaryotes, the lesion detected by MutS homolog (MSH) proteins is repaired through enzymatic complexes operating an endonucleolytic cut on the neo-synthetized strand, thus restoring the correct sequence through the action of specific DNA polymerases (Marti et al., 2002; Spampinato, 2017).

The BER mechanism is responsible for the repair of damaged single bases resulting from deamination, alkylation, oxidized bases, abasic (apurinic and/or apyrimidinic, AP) sites, and single-strand breaks (SSBs) (Tuteja et al., 2009). It consists of the excision of the damaged base by a DNA glycosylase followed by the consecutive action of at least three enzymes, an AP endonuclease, a DNA polymerase, and a DNA ligase (Stivers and Jiang, 2003). The 8-oxoguanine DNA glycosylase (OGG1), uracil DNA glycosylase (UNG), and formamidopyrimidine DNA glycosylase (FPG), are some examples of plant DNA glycosylases with roles in stress responses (Gutman and Niyogi, 2009; Macovei et al., 2011). Aside being extensively studied in model plants, the pathway has also been characterized in potato mitochondria where it is mostly involved in the repair of DNA damage related to ROS production (Ferrando et al., 2018). While BER removes small DNA lesions, the NER pathway repairs the main DNA lesions causing extensive distortion in the double helix, such as UV-products and bulky covalent adducts (Kunz et al., 2005). The NER mechanism has mostly been studied in Arabidopsis and rice and investigations on NER genes have also been conducted in other plants such as poplar and sorghum (Singh et al., 2010; Spampinato, 2017). Proteins belonging to the RAD family are involved in DNA lesion recognition in NER. For instance, the RAD23 family of genes has been well characterized in Arabidopsis by developing multiple mutant

plants. The triple and quadruple mutants for *rad23a*, *rad23b*, *rad23c*, and *rad23d* genes have shown clear phenotypic changes resulting in dwarfed plants or reproductive lethally mutants. However, single and double mutants have not shown evident differences, thus suggesting a mostly overlapping function of the four genes.

Repairing the DSBs is mostly carried out by DBSR systems. Studies on DBSR mechanisms have been increasing, not only because of their importance in DNA repair but also as tools to modify plant genomes (Baltes and Voytas, 2015; Sprink et al., 2015). DBSR mechanisms mainly include the HR and NHEJ pathways. HR occurs only when two DNA duplexes contain extensive homology regions, while NHEJ enables DSBs to be repaired in the absence of sequence homology. Given the requirement of a sister chromatid as a template, HR is restricted to the S and G_2 phases of the cell cycle, while NHEJ is active throughout the cell cycle and does not rely on a template (Brandsma and Gent, 2012). The error-free HR pathway uses several enzymes including the ssDNA-binding protein RAD51 recombinase (Chapman et al., 2012; Spampinato, 2017). The balance between the HR and NHEJ pathways is essential for genome stability. Besides the well–characterized Ku–dependent NHEJ pathway (classical non-homologous end-joining, C-NHEJ), an XRCC1(X-ray cross-complementation group)-dependent pathway (alternative non-homologous end-joining, A-NHEJ) has been observed both in humans and in plants (Decottignies, 2013; Bétermier et al., 2014; Williams et al., 2014; Sfeir and Symington, 2015; Spampinato, 2017). C-NHEJ is dominant in the G_1 and G_2 phases of the cell cycle, while A-NHEJ preferentially acts in the S-phase (Karanam et al., 2012; Truong et al., 2013). A-NHEJ takes place in the absence of key C-NHEJ factors, and requires the alignment of microhomologous sequences. The pathway is thus also referred to as microhomology-mediated end-joining (MMEJ). Unlike HR, the lack of a homology sequence in NHEJ leads to an error-prone type of repair, frequently resulting in small insertions, deletions, or substitutions at the break site (Chapman et al., 2012).

DDR in Relation to Redox-Based Mechanisms

Redox-based mechanisms would seem to play a key role in the modulation of DNA damage sensing, signaling, and repair. Although there is extensive knowledge in animal systems (Kim et al., 2013; Mikhed et al., 2015; Somyajit et al., 2017), there are few reports on redox signaling and redox-mediated control of DNA repair in plants (Zhang, 2015). Due to the complexity of such molecular networks and in an attempt to draw a representative picture of the state of the art in the plant kingdom, attention has focused on specific players that have been identified at the crossroads of the redox and DDR pathways.

One case relates to Fe-containing proteins (e.g., Fe–S cluster proteins and hemoproteins) which use Fe as a cofactor and play critical roles in several aspects of genome maintenance, including telomere maintenance and cell cycle control in both animals and plants (Zhang, 2014, 2015). In Arabidopsis, several Fe-containing proteins with key functions in genome stability, including DNA helicases and DNA glycosylases, have been characterized. For example, RAD3 (also known as UVH6), the plant homolog of the human XPD and yeast RAD3 proteins, is an essential helicase required for NER function (Liu et al., 2003). Among the known 26 DNA glycosylases, only DEM (DNA glycosylase DEMETER), DML1 (DEMETER-like 1, AtROS1), DML2, and DML3 proteins contain a Fe-S cluster and participate in DNA methylation (Ortega-Galisteo et al., 2008). The biogenesis of Fe-S proteins requires dedicated cluster assembly pathways (Lill and Mühlenhoff, 2008). The highly conserved cytosolic Fe-S cluster assembly (CIA) machinery is required for the transfer of these clusters to target proteins, including those involved in genome maintenance, and impairment of the CIA pathway possibly compromises genome integrity (Netz et al., 2014). Other pathways are located within subcellular compartments such as ISC (iron–sulfur cluster) in mitochondria and SUF (sulfur mobilization) in plastids (Couturier et al., 2013). Mutations that target genes coding for the CIA subunit, including AE7 (AS1/2 enhancer 7) and ATM3 (ABC transporter of the mitochondrion 3), result in DNA damage accumulation and enhanced HR rates (Luo et al., 2012). Seedlings of the Arabidopsis *ae7* mutant have shown increased sensitivity to the DNA damage agents, methylmethane sulphonate and cisplatin. The *ae7* mutant cells have also been shown to be blocked at the G2/M transition of the cell cycle and revealed increased expression of DDR genes, including PARP (Poly(ADP-ribose) polymerase), BRCA1, GR1 (Gamma response 1), and TOS2 (Ribonucleotide reductase-like catalytic subunit), involved in DSB repair and genome maintenance (Luo et al., 2012). The defective CIA pathway would seem to cause genotoxic damage, which triggers cell cycle arrest and DDR. Similarly, increased sensitivity to genotoxic agents and up-regulation of DDR genes have been observed in the Arabidopsis *atm3* mutant lacking the ATM3 function (Luo et al., 2012).

Chromatin remodeling is also a key aspect since it is necessary for the access of the DDR protein to the damaged DNA site. Evidence of the redox-mediated modulation of chromatin remodelers has been provided in animal systems. Duquette et al. (2018) reported that lysine demethylase 1 (LSD1/KDM1A), a flavin adenine dinucleotide (FAD)-dependent amine oxidase able to demethylate the lysine 4 residue of histone H3, triggers H_2O_2 accumulation as a by-product of its chromatin remodeling activity during the early steps of DDR. This is the first evidence that ROS can be generated *ex novo* in human cells as part of DDR, at a specific damaged site. In addition, the local production of H_2O_2 can control the activity of DNA repair enzymes recruited at the lesion. This suggests that the local redox environment might modulate the two major DBS repair pathways, namely HR and NHEJ (Duquette et al., 2018). It is possible that a similar mechanism also takes place in plant cells. The Arabidopsis genome encodes four LSD1 homologs named LSD1-like (LSDL), of which LSDL1 and LSDL2 control histone H3 methylation only around and within the heterochromatin region containing the floral repressors FLC (FLOWERING LOCUS) and FWA, which is crucial for the timing of the developmental transition to flowering (Jiang et al., 2007). Unlike

for animals, there is currently no evidence of the role of plant LSD1-like proteins in DDR.

In the complex and variegated scenario of intersecting DDR and redox mechanisms, it is also possible that the same protein fulfills a dual role, acting in a redox context as well as maintaining genome stability. In the PARP-like genes, found in eukaryotes, the PARP catalytic domain is associated with other functional domains (Vainonen et al., 2016). The Arabidopsis protein RCD1 (inactive poly [ADP-ribose] polymerase) contains a WWE domain (Trp-Trp-Glu, involved in protein-protein interactions occurring in ubiquitination and ADP ribosylation) (Aravind, 2001) and an RST (RCD-SRO-TAF4) domain also responsible for protein-protein interactions. Proteins that contain this domain combination, specific to plants, are named SIMILAR TO RCD-ONE (SRO) (Jaspers et al., 2010). According to Liu et al. (2014), overexpression of the *TaSRO* gene in Arabidopsis provides increased tolerance to genotoxic stress induced by UV irradiation and H_2O_2 treatments. The authors ascribed genome integrity to the enhanced PARP activity detected in the *TaSRO*-overexpressing cells that positively affected DDR, resulting in higher levels of the ATM ROS sensor. Interestingly, the *TaSRO*-overexpressing cells accumulated more ROS than the control lines, under both non-stressed and stressed conditions, combined with an efficient antioxidant response that ensured redox homeostasis (Liu et al., 2014). Thus, the particular structural features of *TaSRO* enable this protein to play a dual role in the stress response, acting through the modulation of redox parameters and genome maintenance.

Arabidopsis *apx1/cat2* double mutants that constitutively activate DDR at a transcriptional level represent an interesting example of redox-DDR interaction (Vanderauwera et al., 2011). This confers tolerance against various stresses in the double mutants, since the induced DDR is active also in the absence of DNA damage. DDR induction was inhibited under high CO_2 in the double mutants, suggesting that the ROS production derived from photorespiration caused DDR induction at a constitutive level in the double mutants also under standard conditions. In addition, the WEE1 serine/threonine kinase-dependent cell cycle checkpoint was activated in *apx1/cat2* mutants, which suggests that cell cycle arrest is part of the signaling pathway activated by ROS involving DDR induction (Vanderauwera et al., 2011).

ROLE OF REDOX BALANCE IN TRANSCRIPTIONAL CONTROL

Redox Regulated Transcriptome Re-programming

Redox-based mechanisms play a key role in the regulation of gene expression. Several studies based on omics approaches have demonstrated that ROS induce transcriptional modifications by direct or indirect mechanisms. This experimental evidence has been mainly obtained by manipulating cell ROS levels and/or redox balance in pharmacological or genetic contexts. Effective case-by-case studies were obtained by using mutants defective in enzymatic antioxidant systems (such as catalase, ascorbate peroxidases and ascorbate oxidase – AO; Vanderauwera et al., 2005; Gadjev et al., 2006; Pignocchi et al., 2006; Rasool et al., 2017) as well as treatments with ROS-generating systems, with electron transfer inhibitors in chloroplast and mitochondria or oxidative stress triggering agents (Gadjev et al., 2006; Broda and Van Aken, 2018). The transcriptomic changes appear to be finely tuned depending on ROS types and production site within the cell (Locato et al., 2018). In fact, various environmental backgrounds can promote ROS increases, above all in the apoplast, chloroplasts, mitochondria, and peroxisomes. To give some examples: biotic stresses as well as high light (HL), salt, and drought have been related to apoplastic ROS accumulation by the activation of plasma membrane-located NADPH oxidases belonging to the family of respiratory burst oxidase homolog proteins (RBOH; Ma et al., 2012; Kadota et al., 2015; Kurusu et al., 2015; Li et al., 2015; Evans et al., 2016; He et al., 2017; Karpinska et al., 2018); HL also induces chloroplast ROS production (Foyer and Shigeoka, 2011), whereas photorespiration mainly causes ROS production in the peroxisomes (Foyer et al., 2009), and a number of abiotic stresses increase ROS production in mitochondria (Foyer and Noctor, 2003). Controlled fluxes of redox active molecules (oxidants and antioxidants) between organelles and cytosol, regulate redox mechanisms which, in turn, results in the control of gene expression within the nuclei (Locato et al., 2018). This gene expression reprogramming possibly enable plants to by-pass a stressful situation or a metabolic impairment. Sewelam et al. (2014) demonstrated that hydrogen peroxide (H_2O_2) production triggered by the activation of photorespiratory pathway induced a different set of nuclear genes depending on the ROS production site. Their study used Arabidopsis plants overexpressing glycolate oxidase 5 (GO5), producing ROS in the chloroplast under photorespiratory conditions, and a catalase defective line (*cat2-2*), where ROS over-production occurred in the peroxisomes during photorespiration. When ROS were mainly produced in the chloroplasts, the induced genes mostly belonged to the functional categories of the transcription factors (TFs), proteins involved in signaling and metabolic pathways, and in defense or detoxification. Differently, peroxisome-derived ROS mainly induced the expression of genes involved in protein folding and repair (such as chaperones and heat shock proteins – HSPs), along with defense and detoxification processes. Therefore, different ROS responsive genes were identified to be linked to redox impairment occurring in specific intracellular contexts. A meta-analysis (Vandenbroucke et al., 2008) revealed that yeast, plants, and animals share at least four families of H_2O_2-responsive genes: a class of HSPs, GTP-binding proteins, Ca^{2+}-dependent protein kinases, and ubiquitin-conjugating enzymes. Antioxidant genes shows an H_2O_2-dependent up-regulation only in prokaryotes. This probably depends on the fact that in eukaryotes antioxidant genes show a high constitutive expression probably as an evolutionary acquisition. Thus, in eukaryotes, antioxidant systems are mainly controlled at post-translational level (Vandenbroucke et al., 2008). For example, the synthesis of GSH is controlled by post-translational modification based on a

thiol switch mechanism. Oxidative conditions (also determined by stressful conditions) activate the enzyme that catalyzes the limiting biosynthetic step in GSH production, γ-Glutamyl cysteine synthetase (γ-ECS), by disulphide bond formation in the γ-ECS homodimer. This also represents a controlled redox loop involving GSH (reviewed by Yi et al., 2010).

Glutathione is a major redox soluble metabolite controlling the cell redox balance under physiological and perturbed situations in both developmental and defense contexts (Noctor et al., 2012; Hernández et al., 2017; Locato et al., 2017). The effect of GSH on the transcriptome has been investigated in various studies (Cheng et al., 2015 and references therein; Hacham et al., 2014). GSH feeding of Arabidopsis seedlings appears to induce the expression of stress-related genes and down-regulates developmental correlated genes (Hacham et al., 2014). High GSH levels have also often been correlated to increased stress tolerance (Cao et al., 2017; Ferrer et al., 2018; Formentin et al., 2018). Conversely, GSH deficiency in Arabidopsis root meristem less 1 mutant (*rml1*) has been shown to affect root growth and architecture through a massive transcriptome re-programming. This result has been confirmed in other GSH deficient mutants (*cad2-1*; *pad2-1*; *rax1-1*) presenting different mutations in the GSH1 gene, encoding the GSH biosynthetic enzyme γ-ECS, and in condition of GSH depletion by treatment with buthionine sulfoximide (BSO), an inhibitor of GSH biosynthesis. In all these contexts, GSH deficiency affected above all the expression of those genes involved in cell cycle progression, especially those involved in G2-M transition. On the other hand, the expression of several genes related to redox signaling were less modified probably because the GSH redox state did not change in the mentioned experimental conditions. On the other hand, heat shock (HS) responsive genes were down-regulated, suggesting that the lack of GSH affected redox signaling leading to the expression of these genes. This suggests that GSH is generally required in the induction of oxidative-stress related genes. The redox state of nucleus and cytosol in Arabidopsis root cells has also been monitored in *rml1* mutants and in wild type under BSO treatment. In both compartments, the GSH depletion triggered an increase in the redox state, suggesting that the link between root development, growth, and cell redox state is strongly dependent on the GSH level controlling transcriptome re-programming (Schnaubelt et al., 2015). Karpinska et al. (2017) demonstrated that the nuclear redox state is also prone to oxidation when different plant tissues and cell types were treated with inhibitors of mitochondrial and chloroplast electron transfer, which enable oxidative impair within the cells. The authors observed transcriptome re-programming as a consequence of nucleus oxidation, leading to the retrograde regulation of the expression of genes, mainly related to organelle functions. The GSH-dependent control of the nuclear redox state thus appears to be crucial in interconnected signaling networks which are involved in the organelle cross-talk determinant for gene expression regulation. It has also been demonstrated that an increase in GSH, obtained by exogenous treatment or genetically, enhances the translational efficiency of Arabidopsis plants. This enhancement can be inferred from the changes observed in the polysomal fraction profile, which is indicative of the number of active translation events. An increase in the GSH level seems to activate the translation of pre-existing mRNAs of cluster genes related to hormone biosynthesis, proline biosynthesis, stress response, including TFs involved in defense response, root growth, cell cycle, metabolism, and sulfur assimilation. These data are in accordance with the protective role of GSH supplementation against a plethora of different stress conditions. It also suggests an overall control of the translatome and transcriptome of GSH in plants, probably also correlated to the control played by this metabolite in development and cell proliferation (Cheng et al., 2015; Locato et al., 2015).

Another non-enzymatic antioxidant molecule which intracellular concentration affecting the cellular redox state is the ascorbate (ASC). The ASC level and redox state have been correlated to cell proliferation (de Pinto et al., 1999; Pellny et al., 2009; de Simone et al., 2017; Kka et al., 2018), plant development (Foyer and Noctor, 2009; Paradiso et al., 2012; Cimini et al., 2015) and defense (Kiddle et al., 2003; Sabetta et al., 2019). In fact, ASC treatment of quiescent center cells re-activated the cell division process (Liso et al., 1988). However, according to the literature, the possible involvement of ASC in the control of transcriptional events has not been characterized as well as it has been for GSH. A recent system biology study (Stevens et al., 2018) investigated the effect of ASC metabolism perturbation on the transcriptomes, metabolomes, and proteomes of tomato fruits. The study took into account the RNAi lines for AO, L-Galactono-1,4-γ-lactone dehydrogenase (GLD) and monodehydroascorbate reductase (MDHAR), which are all enzymes involved in the control of ASC levels and the redox state. Although in this study the analysis was carried out on a particular non-photosynthetic tissue and reported no differences in metabolite and protein levels, it did reveal the role of the ASC pool in controlling those core genes involved in ribosome biogenesis, structure, translation, and protein folding (Stevens et al., 2018). Another study performed a comparative analysis of the leaf transcriptome of Arabidopsis mutants which showed reduced levels of GSH (*rml1*), ASC (*vitc1*, *vitc2*) and ROS detoxification in peroxisomes (catalase 2 defective mutant; *cat2*) and chloroplasts (thylacoydal ascorbate peroxidase defective mutant; *tapx*) (Queval and Foyer, 2012). It revealed that both low GSH and ASC caused significant transcriptome reprogramming, although deficiencies in the two antioxidants seemed to affect different sets of genes. Interestingly, there was a 30% overlap among the sets of genes regulated by low antioxidant levels and impairment of ROS detoxification systems; whereas only 10% of the genes regulated by H_2O_2 increases observed in *cat2* and *tapx* mutants overlapped (Queval and Foyer, 2012).

The Role of Redox Sensitive TF Regulation in DNA Transcriptional Control

Reactive oxygen species can regulate gene expression by modulating the activity of numerous TFs. Several redox-dependent mechanisms controlling TF activity have been described in plants, although this is still an under-investigated field. Redox regulation may include conformational changes in

TFs and TF-binding proteins (positive or negative regulators), or an alteration in their intracellular compartmentalization as well as redox-dependent TF proteolysis. **Table 1** summarizes the information related to 12 redox-regulated TFs that directly target several genes involved in plant stress responses. A more detailed description of these TFs and their mechanism of action is provided in the sub-chapters below.

Redox Sensitive TF Belonging to the ERF/AP2 TF Family

Different proteins belonging to the ERF/AP2 TF family undergo redox regulation. Of these TFs, the Redox Responsive Transcription Factor1 (RRTF1) seems to be involved in redox homeostasis under adverse conditions. The RRTF1 transcript levels were shown to be strongly and rapidly increased in response to singlet oxygen and other ROS as well as biotic- and abiotic-induced redox signals such as aphid infection, HL, and salt stress exposure (Matsui et al., 2008; Jaspers and Kangasjärvi, 2010; Heller and Tudzynski, 2011; Jiang et al., 2011). The regulation of the activity of this TF is still not well understood. An increase in RRTF1 expression was found after *Alternaria brassicacea* infection and/or H_2O_2 treatment. In this context, WRKY18/40/60 has been shown to be required for this up-regulation (Matsuo et al., 2015). In particular, a dynamic sub-nuclear re-localization of WRKY40 is induced by abscisic acid (ABA) treatment in a phosphorylation-dependent manner. Once in the nucleus, WRKY40 binds the promoter region of RRTF1 thereby controlling its gene expression (Pandey et al., 2010). RRTF1 binds to GCC-box-like motifs located in the promoter of RRTF1-responsive genes, thereby favoring an increased defense response under constraint conditions (Matsuo et al., 2015).

The Related to Apetala-2 (RAP2) TFs are also one of the main groups of redox regulated proteins belonging to the ERF/AP2 family. The Arabidopsis RAP2.4 TF class consists of eight members characterized by highly conserved DNA-binding domains with overlapping and specific functions. These RAP2.4 proteins constitute a regulative network in which RAP2.4a is the transcriptional activator of chloroplast peroxidase activity. Other RAP2.4 proteins may function as important modulators since an imbalance in the RAP2.4 pattern can, either positively or negatively, affect the expression of target genes by altering the RAP2.4a transcription (Rudnik et al., 2017). The RAP2.4a TF undergoes dimerization under slightly oxidizing conditions and regulates the induction of three chloroplast peroxidases, namely 2-Cys peroxiredoxin A (2CPA), thylakoid and stromal ascorbate peroxidase (tAPX and sAPX), as well as other enzymes involved in redox homeostasis, such as CuZn-superoxide dismutase (SOD; Shaikhali et al., 2008). Under severe oxidative stress, RAP2.4a oligomerizes, thus suppressing its DNA-binding affinity and consequently reducing the expression of target genes (Shaikhali et al., 2008). The interaction of RAP2.4a with RADICAL INDUCED CELL DEATH 1 (RCD1) supports the activation of RAP2.4a transcriptional activity (Hiltscher et al., 2014).

Another member of the ERF/AP2 TF family involved in the regulation of gene expression in a redox dependent manner is RAP2.12. This TF is anchored at the plasma membrane within an Acyl-CoA binding protein 1 and 2 (ACBP1/2) under aerobic conditions (Gibbs et al., 2011). Upon hypoxia, the interaction RAP2.12-ACBP1/2 is suppressed and RAP2.12 is translocated to the nucleus by a mechanism involving an N-terminal cysteine (Cys). Once inside the nucleus, RAP2.12 activates the expression of hypoxia-responsive genes, such as pyruvate decarboxylase 1 (PDC1) and alcohol dehydrogenase 1 (ADH1) (Licausi et al., 2011). After re-oxygenation, RAP2.12 is subjected to a redox-dependent proteolysis via the oxygen-dependent branch of the N-end rule pathway (Licausi et al., 2011; Licausi, 2013; Kosmacz et al., 2015). An oxygen-dependent oxidation of the penultimate Cys residues at the N-terminus of RAP2.12 occurs under normoxia conditions. This reaction, catalyzed by plant Cys oxidases, leads to RAP2.12 destabilization (Weits et al., 2014).

Redox Sensitive TF Belonging to the Zinc Finger TF Family

Proteins belonging to the zinc finger TF (ZF-TFs) family can also be redox-regulated. For example, the ZF-TF SNO-regulated gene1 (SRG1), which has been proposed as a nuclear nitric oxide (NO) sensor (Cui et al., 2018). NO is a reactive signaling molecule that modulates the expression of defense-related genes. In response to pathogen attack, a nitrosative burst occurs leading to transient NO accumulation. Following pathogen recognition and NO accumulation, SRG1 is expressed and binds a repeated sequence $ACTN_6ACT$ or $ACTN_4ACT$ within promoters of genes coding for immune repressors. This ZF-TF contains an EAR domain required for the recruitment of the co-repressor TOPLESS, thus favoring the transcriptional suppression of target immune repressors (**Figure 1**). An additional increase in NO levels induces the S-nitrosylation of SRG1, above all at Cys87. The SRG1 S-nitrosylation relieves DNA binding and transcriptional repression, thus enabling the expression of negative regulators of plant immunity (**Figure 1**). The S-nitrosylation of Cys87, and possibly other Cys residues paired to the ZF motifs, may lead to Zn^{2+} release and to conformational changes responsible for the altered activity of this ZF-TF (Cui et al., 2018).

Another redox regulated ZF-TF is the ZINC FINGER OF ARABIDOPSIS THALIANA 12 (ZAT12) which has been suggested to be involved in the abiotic stress signaling network. Under iron (Fe) deficiency conditions, H_2O_2 content showed a marked increase, which leads to the establishment of oxidizing conditions. H_2O_2 may function as a signaling molecule that induces the transcription of the FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT). The increase in the H_2O_2 content occurs in a FIT-dependent manner. Under prolonged Fe deficiency conditions, H_2O_2 reduces FIT transcription and activates the transcription of its direct binding partner ZAT12 (Le et al., 2016). In Arabidopsis, ZAT12 transcription has been shown to be up-regulated as a consequence of superoxide anion (O_2^{-}) treatment (Xu et al., 2017). ZAT12 acts as negative regulator of FIT: in the nucleus, ZAT12 engages FIT through its C-terminal EAR motif in a protein complex thereby altering the balance between active and inactive FIT pools. ZAT12 is also required for the up-regulation of other stress-related genes such, as APX1 and BHLH039 TFs (Davletova et al., 2005; Le et al., 2016). ZAT12 has also been found to undergo proteasome-dependent degradation in the presence of high H_2O_2 levels. The

TABLE 1 | List of redox sensitive TFs and their regulatory mechanism.

TF's family	TF	Redox regulatory mechanism	References
ERF/AP2 TFs	RRTF1	Phosphate-dependent nuclear re-localization of WRKY40 that activate RRTF1 gene expression	Jaspers and Kangasjärvi, 2010; Pandey et al., 2010; Heller and Tudzynski, 2011; Jiang et al., 2011; Matsuo et al., 2015
	RAP2.4a	Conformational state: protein homo-dimerization	Shaikhali et al., 2008; Hiltscher et al., 2014
	RAP2.12	Redox control of the interaction with a binding partner and nuclear re-localization	Gibbs et al., 2011; Licausi et al., 2011; Licausi, 2013; Welts et al., 2014; Kosmacz et al., 2015
ZF-TFs	SRG1	Post-translational modification and redox control of the interaction with a co-repressor	Cui et al., 2018
	ZAT12	Gene expression induction and proteolytic degradation depending on ROS intracellular levels	Davletova et al., 2005; Brumbarova et al., 2016; Le et al., 2016; Xu et al., 2017
bZIP-TFs	PAN	Redox-sensitive DNA-binding controlled by disulphide bridge formation and post-translational modification	Li et al., 2009; Gutsche and Zachgo, 2016
	VIP1	Nuclear re-localization dependent on redox-sensitive interaction with a negative regulator	Takeo and Ito, 2017
	TGA1	Redox-dependent conformational change of the co-activator protein NPR1 that allow its nuclear re-localization and interaction with TGA TFs	Tada et al., 2008; Lindermayr et al., 2010; Kneeshaw et al., 2014; Kovacs et al., 2015
NAC TFs	VND7	Post-translational oxidative modification that affect TF's transactivation activity	Kawabe et al., 2018
HSFs	HSFA8	Redox-dependent conformational change required for nuclear re-localization	Giesguth et al., 2015
	HSFA4A		Pérez-Salamó et al., 2014
	HSFA8B		Yoshida et al., 2010; Huang et al., 2016

EAR motif seems to be crucial for this proteasome-targeting (Brumbarova et al., 2016; Le et al., 2016).

Redox Sensitive TF Belonging to the Basic Leucine Zipper TF Family

The basic leucine zipper TF (bZIP-TFs) is another family including TFs that undergo redox control. A representative example of a redox-sensitive TF belonging to this family is the Arabidopsis TF PERIANTHIA (PAN), which regulates flower organ development and, in particular, the formation of floral organ primordia (Running and Meyerowitz, 1996). PAN was found to bind the AAGAAT motif located in the second intron of the floral homeotic protein AGAMOUS (AG) (Maier et al., 2009). The nuclear interaction of PAN with ROXY1, a plant-specific glutaredoxin (GRX), is crucial for petal development in Arabidopsis (Li et al., 2009). PAN strongly interacts with the AAGAAT motif only under reducing conditions, and redox-sensitive DNA-binding is controlled by the activity of five N-terminal cysteines. Under oxidizing conditions, Cys68 and Cys87, two N-terminal cysteines, can form a disulphide bridge which may alter the conformational structure of this TF, thus changing its ability to bind the DNA (Gutsche and Zachgo, 2016; **Figure 1**). PAN also undergoes redox-dependent post-translational modifications. It has been demonstrated that Cys340, located in a putative transactivation domain, can be S-glutathionylated, thus modifying PAN activity (**Figure 1**). The S-glutathionylation of Cys340 does not affect the PAN DNA binding activity, however, it might indicate an additional redox-dependent strategy capable of altering TF activity (Li et al., 2009; Gutsche and Zachgo, 2016).

The VIRE2-interacting protein 1 (VIP1) is a TF belonging to the bZIP-TF family whose redox-sensitive regulatory mechanism depends on a subcellular relocation due to an altered interaction with a negative regulator. Under control conditions, VIP1

has three phosphorylated serine residues in the HXRXXS motif. In a phosphorylated state, VIP1 can interact with 14-3-3 proteins in the cytosol, and this interaction might inhibit VIP1 nuclear import. Mechanical and hypo-osmotic stress exposure caused de-phosphorylation of VIP1, which resulted in a dissociation of 14-3-3 proteins thereby favoring its nuclear location (Takeo and Ito, 2017).

TGACG-sequence-specific protein-binding (TGA) TFs are bZIP-TFs involved in the redox-regulated activation of defense responses triggering plant immunity under pathogen attack. In Arabidopsis, the salicylic acid (SA)-dependent responses, activated upon pathogen infection, are mediated by the redox-regulated nuclear translocation of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and by an altered interaction between NPR1 and TGA1 and TGA2 TFs (Tada et al., 2008; Kneeshaw et al., 2014). NPR1 is a co-activator protein whose status is tightly controlled by redox changes occurring after pathogen infection or SA treatment (Mou et al., 2003). This protein is kept in the cytosol in a disulphide-bound oligomeric homocomplex. A reduction in the disulphide bond in NPR1 was found to occur in response to SA-induced changes in cellular redox status. The consequent monomerization unmasked a nuclear location signal, which enables the protein to relocate into the nucleus. Thioredoxins h5 and h3 (TRXh5 and TRXh3) reduce the disulphide-binding oligomers thereby favoring NPR1 monomerization and its nuclear translocation (Kneeshaw et al., 2014). In the nucleus, NPR1 seems to interact with TGA TFs and this triggers the expression of defense genes, such as pathogenesis-related protein 1 (PR1) (Tada et al., 2008). NO controls the translocation of NPR1 into the nucleus (Tada et al., 2008) and the DNA binding activity of its interactor protein TGA1 (Lindermayr et al., 2010). The oligomer-to-monomer reaction involves transient site-specific S-nitrosylation. The NO donor S-nitrosoglutathione (GSNO) thus promotes the nuclear accumulation of NPR1, PR1 expression induction and increased GSH concentration upon *Pseudomonas* infection. GSH accumulation has been shown to be crucial not only for cellular redox homeostasis but also for SA accumulation and activation of the NPR1-dependent defense response (Kovacs et al., 2015).

Redox Sensitive TF Belonging to the NAC TF Family

A member of the NAC TF family, named VASCULAR-RELATED NAC DOMAIN 7 (VND7), appears to undergo a reversible oxidative modification (Kawabe et al., 2018). VND7 is involved in xylem vessel cell differentiation (Yamaguchi et al., 2008). Kawabe et al. (2018) found that VND7 is S-nitrosylated at Cys264 and Cys320 located in the C-terminal region near the transactivation domains. The increased S-nitrosylation of VND7 suppresses the transactivation activity of VND7. In this context, a critical role is played by GSNO reductase 1 (GSNOR1) which is thought to be responsible for maintaining cellular S-nitrosothiol homeostasis by regulating the equilibrium between S-nitrosylated proteins and GSNO. The phenotypic traits of the recessive mutant suppressor of the ectopic vessel cell differentiation induced by VND7 (*sevl1*), i.e., an inhibited xylem cell differentiation, have thus been attributed to a loss of function mutation in gsnor1. Consequently, cellular redox state perception by GSNOR1 seems to be important for cell differentiation in Arabidopsis by regulating the post-translational oxidative modification of the TF VND7 (Kawabe et al., 2018).

Redox Sensitive TF Belonging to the HSF, WYRKY, and MYB TF Families

Typical redox sensitive TFs may also be recruited in response to specific adverse environmental situations, for example heat shock factors (HSFs) which activate protective genes in plants subjected to high temperatures or other stress conditions. HSFs recognize the heat stress elements (HSEs) located in promoters of heat-induced targets. Plants have numerous classes of HSFs that are encoded by 21 genes in Arabidopsis (Scharf et al., 2012). HSFs remain inactive in the cytosol by interaction with HSPs. This interaction masks the nuclear location signal and the oligomerisation domain. Under stress conditions, HSPs act as molecular chaperones and HSFs oligomerize and are translocated into the nucleus where they modulate the expression of target genes (Hahn et al., 2011; Mittler et al., 2012; Scharf et al., 2012). A redox-dependent translocation of HSFA8 from the cytosol to the nucleus has been described in Arabidopsis plants subjected to H_2O_2 treatment (Giesguth et al., 2015). Two Cys residues act as redox sensors in AtHSFA8: Cys24, which is located in the DNA binding domain, and Cys269, which is located in the C-terminal part of the protein. Disulphide bond formation between Cys24 and Cys269 may cause a drastic conformational change and induce AtHSFA8 translocation into the nucleus probably by its release from multi-heteromeric complexes (**Figure 1**). In single mutants (AtHSFA8-C24S and AtHSFA8-C269S) and in the double mutant (AtHSFA8 C24/269S), HSFA8 nuclear translocation is thus suppressed under oxidative stress (Giesguth et al., 2015). Similarly, Arabidopsis HSFA4A, described as an H_2O_2 sensor, has been reported to form homodimers (or homotrimers). This mechanism is thought to be required for the transactivation activity of this TF (Pérez-Salamó et al., 2014). HSFA4A expression is enhanced by numerous adverse conditions known to induce ROS accumulation such as salt, paraquat, heat/cold treatment, drought, hypoxia and several pathogens (Sun et al., 2001; Libault et al., 2007; Peng et al., 2012). HSFA4A, in turn, seems to modulate transcriptional activation of a set of target genes involved in mounting defense responses to abiotic and biotic stress conditions, such as APX, HSP17.6A, ZAT6, ZAT12, CTP1, WRKY30, and CRK13. In Arabidopsis-related species, the formation of redox-sensitive disulphide bonds of Cys residues may be a requirement for HSFA4A homodimerization. In addition, Ser309, located between two activator domains, has been identified as the preferential phosphorylation site catalyzed by MPK3 and MPK6 (Pérez-Salamó et al., 2014).

HSFA6B is another redox-regulated HSF which might play a role in the ABA-dependent pathway under salt and dehydration (Yoshida et al., 2010; Huang et al., 2016). HSFA6B is a protein located in both the cytosol and nucleus under normal growth conditions. After ABA or leptomycin treatment, there is an increase in its nuclear location. In the nucleus, HSFA6B may interact with other HSF proteins such as HSFA1A, HSFA1B,

and HSFA2, thereby forming hetero-oligomeric complexes and significantly activating the transcriptional activity of defense genes such as HSP18.1-CI, DREB2A, and APX2 (Huang et al., 2016). HSFA6B seems to have a functional redundancy with the HSFA6A protein during salt and drought stresses. HSFA6A is present at the nucleus and cytosol simultaneously under physiological conditions. However, after salt and drought treatment, HSFA6A has been mainly detected in the nucleus. HSFA6A functions as a transcriptional activator of target genes involved in the enhancement of stress tolerance by its C-terminal moiety. This TF is, in turn, transcriptionally activated by various TFs such as ABF/AREB proteins, MYB96, MYB2, MYC2, and WRKY TFs under salt and drought stress in Arabidopsis (Abe et al., 2003; Seo et al., 2009; Niu et al., 2012; Hwang et al., 2014). In addition, the VOZ1 protein may interact with the DNA-binding domain of HSFA6A under normal growth conditions; however, under high salinity conditions, VOZ1 expression slightly decreases together with its protein content. Thus, freed from interaction with VOZ1, HSFA6A protein can function as a positive regulator of the gene expression involved in tolerance acquisition (Hwang et al., 2014).

A core set of ROS-responsive transcripts has been identified in the systemic acquired acclimation response of Arabidopsis following HL application. Four different TFs, namely GATA8, WRKY48, WRKY53, and MYB30, were found to control HL-dependent transcriptome re-programming. The expression of these TFs peaked 2 min after HL exposure both in local and systemic leaves. They were found to be associated with ROS/Ca^{2+} waves generated under these stress conditions (Zandalinas et al., 2019). MYB30 also regulates oxidative and heat stress responses by modulating cytosolic Ca^{2+} levels in response to H_2O_2 variations through annexin expression modulation. During ROS/Ca^{2+} wave propagation, MYB30 binds the promoters of ANN1 and ANN4, and represses their expression thereby regulating cytosolic Ca^{2+} levels (Liao et al., 2017). WRKY48 and WRKY57 are involved in pathogen- and drought- induced defense responses (Xing et al., 2008; Van Eck et al., 2014; Sun and Yu, 2015) and GATA8 acts as a positive regulator of Arabidopsis seed germination (Liu et al., 2005).

The examples discussed above suggest that under biotic and abiotic stress conditions, ROS cause drastic changes in nuclear gene expression by altering the activity of specific TFs that regulate the synthesis of proteins related to plant stress adaptation.

THE IMPLICATION OF MIRNAS IN REDOX- AND DDR-ASSOCIATED PATHWAYS

Gene expression can be modulated also at post-transcriptional level. At this regard, miRNAs, a type of short non-coding RNAs, have been indicated as promising candidates in the precise regulation of genes by targeting messenger RNAs (mRNAs) for cleavage or directing translational inhibition. miRNAs are generally produced from a primary miRNA transcript, the pri-miRNA, through the activity of nuclear RNase DICER-LIKE 1 (DCL1), while mature miRNAs are incorporated into a protein complex named RISC (RNA-induced silencing complex) (**Figure 1**; Reinhart et al., 2002; Bartel, 2004).

In human cells, recent studies have investigated the interactions between DDR components, redox signaling pathways, and miRNAs (for reviews see Hu and Gatti, 2011; Wan et al., 2014; Bu et al., 2017). An interplay between miRNAs, DDR and redox signaling pathways is possible. Indeed, both DDR and redox signaling can modulate miRNA expression, while miRNAs can directly or indirectly modulate the expression of proteins that are part of DDR and redox signaling. Understanding the roles of miRNAs in DDR and redox signaling along with their implications in complex diseases such as cancer (He et al., 2016; Arjumand et al., 2018), or throughout the aging process (Bu et al., 2016, 2017), are viewed as diagnostic tools or alternative therapeutic treatments (Huang et al., 2013; Badiola et al., 2015).

The situation is quite different in plants, where only very few studies have started to address this complex picture. Yet, miRNAs have been extensively studied in terms of stress responses and exhaustive reviews regarding this aspect are available (Noman et al., 2017; see recent reviews by Djami-Tchatchou et al., 2017; Wang et al., 2017; Islam et al., 2018). To understand the roles of miRNAs within the redox balance-DDR-miRNA triangle, recent literature was consulted to examine the direct or indirect implication of miRNAs in ROS production/scavenging and DDR pathways, based on their predicted/validated target genes.

miRNAs and ROS

As above described, ROS are by-products of cellular metabolic processes that can act as secondary messengers in specific signaling pathways. In humans, miRNAs targeting central regulators of the ROS signaling pathway have been identified, such as the Nuclear Factor Erythroid-Derived 2-Like 2 (Nrf2), or Tumor Necrosis Factor-Alpha (TNFa), and ROS scavengers, such as SOD or CAT (Wang et al., 2014). Similarly, studies in plants have revealed the presence of miRNAs targeting genes involved in ROS production and scavenging (**Table 2**). The influence of miRNAs in these processes can be classified as (1) direct, when directly targeting genes coding for proteins with oxidant or antioxidant properties, and (2) indirect, when the targeted genes affect redox signaling pathways downstream. miR529 is an example of an indirect influence. This miRNA targets some of the genes belonging to the SQUAMOSA promoter-binding protein-like proteins (SPLs), a plant specific transcription factor involved in regulating plant growth and development (Rhoades et al., 2002). Recently developed rice lines overexpressing *MIR529a* have been shown to have increased resistance to oxidative stress imposed by applying exogenous H_2O_2, because of enhanced levels of SOD and peroxidase (POD, POX) enzymes (Yue et al., 2017). The authors demonstrated that the over-accumulation of miR529a resulted in an enhanced seed germination rate, root tip cell viability, chlorophyll retention, and reduced leaf rolling rate during exposure to H_2O_2. Regarding the miR529a targets, out of the five predicted genes (*OsSPL2*, *OsSPL14*, *OsSPL16*, *OsSPL17*, *OsSPL18*) only two, *OsSPL2* and *OsSPL14*, were

TABLE 2 | List of miRNAs targeting genes with roles in ROS production and scavenging.

miRNA	Species	Targeted genes	Related stress	References
miR395	*Arabidopsis thaliana* *Brassica napus* *Oryza sativa* *Nicotiana tabacum*	*ATPS, SULTR2;1*	Nutrient deficiency Heavy metal	Matthewman et al., 2012; Zhang L. W. et al., 2013; Jagadeeswaran et al., 2014; Panda and Sunkar, 2015; Yuan et al., 2016
miR396b	*Poncirus trifoliata*	*ACO*	Cold	Zhang et al., 2016
miR397	*Arabidopsis thaliana* *Oryza sativa* *Lotus japonicus*	*LAC*	Nutrient deficiency H_2O_2	Li et al., 2011; De Luis et al., 2012; Zhang Y. C. et al., 2013; Wang et al., 2014
miR398	*Arabidopsis thaliana* *Vitis vinifera* *Triticum aestivum* *Phaseolus vulgaris* *Medicago truncatula*	*CDS1, CDS2, Nod19, COX5b*	Heavy metal Drought Salinity	Trindade et al., 2010; Naya et al., 2014; Kayihan et al., 2016; Lang et al., 2017; Li J. et al., 2017; Li L. et al., 2017
miR408	*Arabidopsis thaliana* *Oryza sativa* *Nicotiana tabacum* *Medicago truncatula*	*PCY, PLC, LAC, UCC, UCL8*	Biotic stress Drought Salinity γ-irradiation	Trindade et al., 2010; Zhang et al., 2017; Pan et al., 2018; Song et al., 2018
miR414	*Panicum virgatum*	*CAT isozyme B, FAO, NADH_UbQ/plastoQ_OxRdtase, HSP, COX*	–	Xie et al., 2010
miR474	*Citrus sinensis* *Zea mays*	*PDH, NAD-dependent malic enzyme*	Boron deficiency Submergence	Zhang et al., 2008; Lu et al., 2014
miR477	*Panicum virgatum* *Triticum aestivum*	*Fd-GOGAT*	Drought	Xie et al., 2010; Akdogan et al., 2016
miR528	*Oryza sativa* *Agrostis stolonifera*	*PCY-like, LAC, MCOs, GALTs, AO*	Drought Salinity Heavy metals	Liu et al., 2015; Yuan et al., 2015; Wu et al., 2017
miR531	*Panicum virgatum* *Triticum aestivum*	*HSP 17.9, POD62, POX, CYP P450, ACO1*	Environmental pollutants	Xie et al., 2010; Li J. et al., 2017
miR9773	*Triticum aestivum*	*CYP P450*	Environmental pollutants	Li J. et al., 2017
miR1121	*Triticum aestivum*	*CAT-1, POD6, MT3-like*	Environmental pollutants	Li J. et al., 2017
miR9653b	*Triticum aestivum*	*LOX-like protein*	Environmental pollutants	Li J. et al., 2017
miR1132	*Panicum virgatum*	*CYP87A15*	–	Xie et al., 2010
miR1436	*Panicum virgatum* *Oryza sativa*	*POD2*	Heat	Xie et al., 2010; Mangrauthia et al., 2017
miR1535	*Panicum virgatum*	*CYP72A63*	–	Xie et al., 2010
miR2102	*Panicum virgatum* *Oryza sativa*	*CYP P450, SOD, COX VI, POD, ACO1*	Arsenic	Xie et al., 2010; Sharma et al., 2015
PC-5p-213179-14	*Zea mays*	*POD*	Low seed vigor	Gong et al., 2015
PN-2013	*Triticum aestivum*	*MDHAR*	Biotic stress	Fang et al., 2014
novel_miR_120	*Brachypodium distachyon*	*NDH1α* subunit 12	H_2O_2	Lv et al., 2016
novel_miR_4	*Brachypodium distachyon*	*CYP P450 734A1*	H_2O_2	Lv et al., 2016
novel_miR_234	*Brachypodium distachyon*	*FTR*	H_2O_2	Lv et al., 2016
novel_miR_197	*Brachypodium distachyon*	*CYP P450 90D2*	H_2O_2	Lv et al., 2016

downregulated in seedlings overexpressing *MIR529a*, therefore suggesting that these two were the direct targets of miR529a. This also induced the upregulation of other stress-related genes such as *OsCPR5* (Constitutive expression of pathogenesis-related genes 5), proline synthase (Os10g0519700), amino acid kinase (LOC_Os05g38150), peroxidase precursor (LOC_Os04g59150), and *OsVPE3* (Vacuolar processing enzyme-3). Based on these findings, the authors proposed a potential complex network of miR529a-SPLs-downstream genes in the ROS signaling pathway in response to oxidative stress (Yue et al., 2017).

Table 2 summarizes the information related to 23 miRNAs that directly target several genes with roles in ROS production/scavenging in various plant species. Of these, the most studied in relation to oxidative stresses are miR395 and miR398. The predicted and validated targets of miR395 are the ATP sulfurylase (ATPS) and low-affinity sulfate transporters SULTR2;1 (Matthewman et al., 2012; Jagadeeswaran et al., 2014). ATPS catalyzes the activation of sulfate by transferring sulfate to the adenine monophosphate moiety of ATP to form adenosine 5′-phosphosulfate and pyrophosphate (Patron et al., 2008). SULTR2;1 is responsible for the internal transport of sulfate from roots to shoots (Takahashi et al., 2000). The modulation of miR395 thus seems ideal to address the sulfate assimilation pathway and

develop crops with increased efficiency of sulfate uptake (Yuan et al., 2016). A key question is how this is related to redox signaling. When sulfate reaches chloroplasts and mitochondria, it is reduced first to sulphite and then to sulfide, which is essential for the synthesis of cysteine and methionine, two fundamental amino acids for supporting redox reactions in plants. The reduced form of cysteine functions as an electron donor, while its oxidized form acts as an electron acceptor. This different redox state allows to hypothesize a role of redox signaling in inducing nutrient-related or stress-responsive miRNAs. Above all, it refers to the intracellular thiol redox status, which regulates a variety of cellular and molecular events such as the activity of proteins, signal transduction, transcription and several other cellular functions (Panda and Sunkar, 2015). Another well-studied example is miR398, which targets the metal-induced superoxide dismutases, CDS1 and CDS2, in a number of different species (see **Table 2**; Trindade et al., 2010; Naya et al., 2014; Kayhan et al., 2016; Leng et al., 2017; Li J. et al., 2017; Li L. et al., 2017). Because of its role in regulating this important ROS scavenger enzyme, miR398 has been found to be involved in plant responses to a multitude of stresses, including drought (Trindade et al., 2010), salinity (Feng et al., 2015), metal-induced toxicity (Xu et al., 2013), and other pollutants such as sulfur dioxide (SO_2) (Li L. et al., 2017).

Other miRNAs (e.g., miR414, miR531, miR1121, miR1436, miR2102) that target other ROS scavenging enzymes such as CAT, SOD, POD, and POX have been identified and their involvement in the plant stress response has been proven (Xie et al., 2010; Sharma et al., 2015; Li J. et al., 2017; **Table 2**). A particular example is miR414, which targets a myriad of genes with different functions in plant stress metabolism and antioxidant responses. As shown by Xie et al. (2010) in switchgrass, miR414 was predicted to target 44 different mRNAs, several of which dealing with oxygen/ROS including CAT isozyme B, polyamine oxidase (PAO), cytochrome *c* oxidase (COX), and NADH-ubiquinone oxidoreductase B16.6 subunit (NADH_UbQ/plastoQ_OxRdtase).

miRNAs and DDR

The ability of DDR to sense DNA damage, transduce signals and promote repair, depends on the coordinated action of a series of factors. Of these, the MRN complex represents the first "line of defense" as it acts as a sensor of damage signaling by recruiting DDR-related proteins, including ATM and other mediators, to the DSB sites (Goldstein and Kastan, 2015).

In human cell research, miRNAs are being investigated for their modulator role in the regulation of DDR (for review see Hu and Gatti, 2011; He et al., 2017). For example, miR-18a and miR-412 have been proved to negatively regulate ATM expression and reduce the capacity of DNA damage repair in tumorigenic cells challenged with irradiation or chemotherapy (Song et al., 2011; Mansour et al., 2013). Other studies have demonstrated that miRNAs are involved in the post-transcriptional regulation of p53 (Hu et al., 2010; Kumar et al., 2011), the master-regulator of DDR that drives the fate of the human cell directing it to DNA repair, cell cycle arrest, apoptosis, or senescence. For instance, miR-25 and miR-30d have been shown to interact with p53, and, as a consequence, its downregulation leads to the suppression of some of its target genes (*p21*, *BAX*, *Puma*) resulting in reduced apoptosis (Kumar et al., 2011). Downstream effectors, such as the DNA repair pathways, are also influenced by miRNAs at least in animals, as shown in several studies investigating human cancer cell lines. Moreover, examples of miRNA involvement in NHEJ (e.g., miR-101) or HR repair mechanism (e.g., miR-107, miR-103, miR-222) have been reported in animal and plant cells (Yan et al., 2010; Huang et al., 2013; Neijenhuis et al., 2013). In the case of the hsa-miR-526b, which targets the Ku80 mRNA, in addition to DSB repair, the plant cell cycle progression is also affected in the G_0/G_1 phase (Zhang, 2015).

In plants few studies have addressed the potential role of miRNAs in the regulation of DDR-associated genes. Most of this evidence comes from high-throughput transcriptomic studies dedicated to investigating specific stress responses/adaptations. **Table 3** summarizes a collection of miRNAs predicted to target several genes with different roles in the DDR pathway.

In a study on changes in miRNA expression during magnesium (Mg)-induced starvation in oranges roots, the authors collected different miRNAs affecting several functions, ranging from the antioxidant response, adaptation to low-phosphorus and activation of transport-related genes, to DNA repair (Liang et al., 2017). The study identified the MUTL-homolog 1 (MLH1) and MRE11 as targets of miR5176 and miR5261, respectively. The *MLH1* gene is part of the MMR pathway, one of the DNA repair defense systems responsible for maintaining genome integrity during cell division. Previous studies in yeast have identified four MutL homologs that form functionally distinct heterodimers, of which Mlh1/Pms1 and Mlh1/Mlh2 are involved in the correction of different types of DNA mismatches (Wang et al., 1999). In plants, the MLH1 has been less investigated compared with other MutL/MutS homologs. However, interaction between MLH1 and MLH3 has been shown to be required for the formation of double Holliday junctions and normal levels of meiotic crossovers in Arabidopsis plants (Jackson et al., 2006). Thus, identifying a miRNA capable of suppressing the activity of MLH1 would also help to better clarify the functions of this gene. The particular case of miR5176 showed that its induction under Mg-deprived conditions resulted in the activation rather than the inhibition of MLH1 associated with enhanced MMR activity in response to Mg-deficiency (Liang et al., 2017). This could be due to other post-transcriptional modifications or the activation of alternative regulatory mechanisms. In addition, the *MRE11* gene that encodes DNA repair and meiosis proteins belonging to the MNR complex, was identified as being targeted by miR5261, and induced in Mg-deprived roots. In this case, the down-regulation of miR5261 resulted in enhanced levels of MRE11

TABLE 3 | List of miRNAs targeting genes with roles in DNA damage response.

miRNA	Species	Targeted genes	Related stress	References
miR1127a	*Triticum aestivum*	SMARCA3L3	–	Sun et al., 2018
miR2275	*Triticum aestivum* *Prunus persica*	CAF1	Drought	Esmaeili et al., 2017; Sun et al., 2018
miR122c-3p	*Triticum aestivum*	XPB2	–	Sun et al., 2018
miR5179	*Citrus sinensis*	MUTL-homolog 1	Mg-deficiency	Liang et al., 2017
miR5261	*Citrus sinensis*	MRE11	Mg-deficiency	Liang et al., 2017
miR528b	*Hordeum bulbosum*	RFA1C	Salinity	Liu and Sun, 2017
miR403	*Helianthus annuus*	AGO1, AGO2	Salinity	Ebrahimi Khaksefidi et al., 2015; Kumar et al., 2018
miR2102	*Panicum virgatum* *Oryza sativa*	TFIID subunit 10	Arsenic	Xie et al., 2010; Sharma et al., 2015
miR477	*Panicum virgatum* *Triticum aestivum*	RAD23	Drought	Xie et al., 2010; Akdogan et al., 2016
novel-mir_222	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_120	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_98	*Brachypodium distachyon*	TFIID subunit 12	H_2O_2	Lv et al., 2016
novel-mir_69	*Brachypodium distachyon*	RAD50	H_2O_2	Lv et al., 2016
novel-mir_147	*Brachypodium distachyon*	SMUBP-2	H_2O_2	Lv et al., 2016
novel-mir_4	*Brachypodium distachyon*	SAGA29	H_2O_2	Lv et al., 2016
miR414	*Oryza sativa*	OsABP helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013
miR408	*Oryza sativa*	OsDSHCT helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013
miR164e	*Oryza sativa*	OsDEH helicase	Salinity γ-irradiation	Macovei and Tuteja, 2012; Macovei and Tuteja, 2013

and, as a consequence, better detection of DNA damage and repair of DSBs.

In another study aimed at determining miRNAs responsive to H_2O_2 during seedling development in *Brachypodium distachyon*, a novel miRNA called novel_mir_69 was identified as targeting the *RAD50* mRNA (Lv et al., 2016). Using next generation high-throughput sequencing, a total of 144 known and 221 new miRNAs were identified as being responsive to H_2O_2-induced stress in *B. distachyon*. In addition to *RAD50*, other genes with a role in DNA damage repair were shown to be targeted by several other newly identified miRNAs in this study. For instance, the DNA-binding protein encoded by *SMUBP-2* was predicted to be targeted by novel_mir_147, the novel_mir_4 targeting the SAGA-associated factor 29 homolog, while the transcription initiation factor IID (TFIID) was predicted to be targeted by novel_mir_120. The SMUBP-2 is a transcription regulator which also has a 5′ to 3′ helicase activity. Its RH3 helicase domain and AN1-like zinc finger domain have been shown to bind single-stranded DNA (Lim et al., 2012). The SAGA-associated factor 29 homolog is a chromatin reader component of the transcription regulatory histone acetylation (HAT) complex (Kaldis et al., 2011). On the other hand, TFIID is a key component of the transcription pre-initiation complex (PIC), responsible for recognizing and binding to specific promoter DNA sequences (e.g., TATA elements). Studies on yeast have demonstrated that both TFIID and SAGA can be sequentially recruited at the DNA damage site in a differential manner, based on the type of stress induced (Ghosh and Pugh, 2011). For instance, when the methylmethane sulphonate mutagenic agent was used, the induced genes underwent transcription complex assembly sequentially, first through SAGA and then through a slower TFIID recruitment. However, when heat shock was applied, the induced genes used both the SAGA and TFIID pathways rapidly and in parallel. Similarly, studies in plants have demonstrated that TFIID associates with essential proteins involved in DNA repair and chromatin remodeling, such as MRE11 and TAF1 (TATA-binding protein Associated Factor 1, histone acetyltransferase), in an attempt to maintain genome integrity under genotoxic stress conditions (Waterworth et al., 2015).

The fact that miRNAs were predicted to directly or indirectly interact with chromatin remodeling associated genes further adds to the complicated layers of regulation of this complex phenomenon. In a bioinformatics study on switchgrass, TFIID mRNA was predicted to be targeted by miRNAs (miR2102) (Xie et al., 2010). In the same study, another DNA repair gene, namely *RAD23*, was predicted to be targeted by miR477. The *RAD23* gene, encoding for the UV excision repair protein RAD23 homolog A, is involved in the NER pathway. By interacting with several other components of the DNA repair machinery, it also plays an important role in BER DNA damage recognition (Sturm and Lienhard, 1998). In Arabidopsis, RAD23 have also been demonstrated to have an essential role in the cell cycle, morphology, and fertility of plants through their involvement in ubiquitination pathways (Farmer et al., 2010). Another component of the NER pathway

predicted to be targeted by miRNAs is *XPB2* (homolog of *Xeroderma pigmentosum* complementation group B2). In a transcriptome analysis performed during anther development in male sterile wheat lines, *XPB2*, a DNA repair helicase, was shown to be targeted by tae-miR1122c-3p (Sun et al., 2018). The induced expression of *XPB2*, acting as a DNA damage detector, has been suggested to be necessary for DNA damage repair during pollen formation. It is worth noting that this study used a particular wheat line (337S), which is sensitive to both long-day-length/high-temperature and short-day-length/low-temperature, to investigate the miRNA involvement in the regulation of male sterility by looking at the pre-meiotic and meiotic cell formation (Sun et al., 2018). Besides *XPB2*, other DNA repair and chromatin remodeling associated genes have been identified as targets of miRNAs. For instance, tae-miR2275 targeted the *CAF1* (CCR4-associated factor 1), involved in early meiosis, whereas tae-miR1127a targeted the *SMARCA3L3* (a new member of SWI/SNF factor SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A, member 3-like 3), believed to be involved in the progression of meiosis in male reproductive cells. In yeast, the CCR4-Not (Carbon Catabolite Repressed 4-Negative on TATA-less) complex has been shown to be involved in replication stress and DNA damage repair, as well as maintaining heterochromatin integrity (Mulder et al., 2005; Cotobal et al., 2015). The SWI/SNF chromatin-remodeling complex is, instead, an essential component of chromatin remodeling, and its involvement in DNA damage response is dependent on the CCR4–Not complex (Mulder et al., 2005). By showing interactions between tae-miR2275-*CAF1* and tae-miR1127a-*SMARCA3L3*, this study demonstrated that the diversified roles of SMARCA3L3 and CAF1 in DNA repair and chromatin remodeling helped to maintain chromatin and genome integrity during meiosis (Sun et al., 2018).

Other miRNAs that putatively control different helicase genes have been identified by *in silico* analysis in rice (Umate and Tuteja, 2010). Of these, osa-MIR414, osa-MIR408 and osa-MIR164e have been experimentally validated as targeting the *OsABP* (ATP-Binding Protein), *OsDSHCT* (DOB1/SK12/helY-like DEAD-box Helicase), and *OsDBH* (DEAD-Box Helicase) genes (Macovei and Tuteja, 2012). The expression of miRNAs and their targeted genes correlated negatively in response to salinity stress and gamma-irradiation treatments, which caused DNA, damage (Macovei and Tuteja, 2012, 2013). Given that helicases are enzymes that catalyze the separation of double-stranded nucleic acids in an energy-dependent manner, they are involved in a wide range of processes such as recombination, replication and translation initiation, double-strand break repair, maintenance of telomere length, nucleotide excision repair, and cell division and proliferation (Tuteja, 2003). Hence, by targeting a wide range of helicases, as shown by the literature cited here, miRNAs are responsible for regulating all this array of processes associated with helicase activities.

An interesting aspect of miRNAs is their capacity to regulate their own biogenesis. This happens by targeting ARGONAUTE genes (AGO1 and AGO2), as in the case of miR403 and miR172 (Ebrahimi Khaksefidi et al., 2015). Aside from their involvement in small RNA pathways and epigenetic silencing phenomena (Schraivogel and Meister, 2014), AGOs have also been shown to be associated directly or indirectly with DNA repair (Oliver et al., 2014). The particular case of miR403 and miR172 shows that in addition to targeting AGO, they also interact with DML1 and DML3 (involved in DNA methylation), thus suggesting the multiple role of these miRNAs in small RNA pathways and DNA methylation (Ebrahimi Khaksefidi et al., 2015).

CONCLUSION

This review has explored the interconnections between the molecular mechanisms controlling the cell redox balance and gene expression regulation, occurring at transcriptional and post-transcriptional levels, as well as the maintenance of genome integrity (**Figure 1**). In particular, evidence here reported, underline the influence of the redox signaling in the modulation of molecular pathways activated in response to developmental and environmental stimuli. Interestingly, specific players involved in redox sensing and homeostasis, influence plant metabolism at different levels. During evolution, plants, as all other living organisms, have developed capability for using specific molecular players in a cross-cutting manner both in developmental processes, in defense responses activated by environmental stimuli and in DNA replication and repair. GSH and correlated thiol systems represent a case in point of key actors controlling the redox buffering capability of plant nuclei and they are crucial also for DNA replication and repair, and consequently cell cycle progression, as well as for the regulation of gene expression in different contexts (Diaz-Vivancos et al., 2010; Martins et al., 2018; Ratajczak et al., 2019). Moreover, numerous TFs, regulating the expression of genes involved in plant development, DDR or in the activation of stress-related responses, are described to be redox-regulated. The activity of these TFs is mainly influence d by alterations in the cell redox balance, which lead to conformational changes and their possible subcellular re-location. Recently, evidence of the involvement of a continuously increasing number of miRNAs in several processes is opening new scenarios on the complexity of redox signaling and homeostasis. Although some miRNAs targeting genes with different roles related to defense systems, development and DDR pathways have been predicted or validated in different plant species, this field requires further investigation. Interestingly, some miRNAs have been predicted to target genes belonging to the above-indicated pathways. Examples include miR408 and miR414, which target the helicases involved in DNA repair as well as several genes implicated in the redox system (see **Tables 2, 3**). Similarly, miR528 is predicted to target RFA1C (replication A 70 KDa DNA-binding subunit C), involved in DNA replication and efficient DNA repair and recombination (Longhese et al., 1994), as well as antioxidant-related genes (e.g., phytochromes, oxidases). Switchgrass miR477 has also been shown to target the Rad23 DNA repair associated factor as well as Ferredoxin-Dependent Glutamine-Oxoglutarate Amidotransferase (Fd-GOGAT), acting as electron donor in

glutamate metabolism (Xie et al., 2010). The evidence here reported highlight an interconnectivity between the redox and DDR pathways created by a network of miRNAs. Further studies aimed at clarifying these complex regulatory networks are strongly encouraged.

AUTHOR CONTRIBUTIONS

AB, AM, and VL drafted the manuscript. SC, CG, AM, and VL wrote the manuscript. SC and AM created the figures and tables. AB and LD revised and critically improved the manuscript.

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MINI REVIEW
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Plant miRNA Cross-Kingdom Transfer Targeting Parasitic and Mutualistic Organisms as a Tool to Advance Modern Agriculture

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MicroRNAs (miRNAs), defined as small non-coding RNA molecules, are fine regulators of gene expression. In plants, miRNAs are well-known for regulating processes spanning from cell development to biotic and abiotic stress responses. Recently, miRNAs have been investigated for their potential transfer to distantly related organisms where they may exert regulatory functions in a cross-kingdom fashion. Cross-kingdom miRNA transfer has been observed in host-pathogen relations as well as symbiotic or mutualistic relations. All these can have important implications as plant miRNAs can be exploited to inhibit pathogen development or aid mutualistic relations. Similarly, miRNAs from eukaryotic organisms can be transferred to plants, thus suppressing host immunity. This two-way lane could have a significant impact on understanding inter-species relations and, more importantly, could leverage miRNA-based technologies for agricultural practices. Additionally, artificial miRNAs (amiRNAs) produced by engineered plants can be transferred to plant-feeding organisms in order to specifically regulate their cross-kingdom target genes. This minireview provides a brief overview of cross-kingdom plant miRNA transfer, focusing on parasitic and mutualistic relations that can have an impact on agricultural practices and discusses some opportunities related to miRNA-based technologies. Although promising, miRNA cross-kingdom transfer remains a debated argument. Several mechanistic aspects, such as the availability, transfer, and uptake of miRNAs, as well as their potential to alter gene expression in a cross-kingdom manner, remain to be addressed.

Keywords: agriculture, cross-kingdom, microRNAs, mutualism, pathogen, plant

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INTRODUCTION

Plants have evolved sophisticated mechanisms to adapt to environmental changes and to interact with different organisms. Many of these strategies are based on the activation and repression of large sets of genes, and miRNAs are important regulator molecules in this scenario. They may be induced or repressed to subsequently regulate the expression of target genes through post-transcriptional silencing or translational inhibition of their mRNA targets. MicroRNAs are defined as small,

non-coding, single-stranded RNAs acting as regulators of multiple biological and physiological processes. In plants, these small molecules derive from stem-loop precursors that are processed through a Dicer-like (DCL) enzyme and loaded, in association to Argonaute (AGO) proteins, into the RNA-induced silencing complex (RISC) that serve to direct them to their target site where cleavage of mRNAs or inhibition of translation happens (Jones-Rhoades et al., 2006).

Nowadays, miRNAs are starting to be envisioned for their ability to move not only within an organism, but also across kingdoms and influence gene expression in evolutionary distant organisms (LaMonte et al., 2012; Cheng et al., 2013; Shahid et al., 2018; Zhang et al., 2019a). The presence of a methyl group on the ribose of the last nucleotide together with the association with RNA binding proteins and packing into exosomes may contribute to the stability and transfer of plant miRNAs across kingdoms (Valadi et al., 2007; Zhao et al., 2012). The miRNA cross-kingdom transfer may be favored by the conserved features of the RNA silencing machinery among eukaryotes, though taxon-specific variations exist. Such differences are mainly related to the ability of organisms to incorporate RNA molecules, systematically transmit the RNA signals to other tissues and to the magnitude and duration of the RNA silencing response (Winston et al., 2007; Shannon et al., 2008; Wang et al., 2015; Wang et al., 2016). Most examples of miRNA cross-kingdom transfer come from plant-pathogen/parasite interactions (Zhang et al., 2016; Wang et al., 2017a; Zhang et al., 2019a). The cross-kingdom transfer of endogenous plant miRNAs to pathogens or parasites may inhibit their invasive powers while the miRNA transfer from parasitic eukaryotes to plants may suppress the immunity of the host plants. In the case of symbiotic/mutualistic relations, the miRNA transfer from plants may influence essential processes such as growth and development (Zhu et al., 2017).

Understanding the complex network of interactions between plants and eukaryotic organisms and the translation of these information from the bench to the field can pave the way for the development of new technologies. In view of this, miRNA-based strategies exploiting the potential of plant miRNAs to move across kingdoms and silence specific genes in distantly related organisms, are gaining ground. The use of artificial miRNAs (amiRNAs) can be regarded as valuable tools that can complement the already existing technologies to face the global climate changes and associated agricultural challenges (Chen et al., 2013; Mitter et al., 2016).

The current minireview focuses on the latest information related to cross-kingdom miRNA addressing plant-parasite/mutualistic relations. Specific examples of cross-kingdom transferring plant miRNAs and potential gene targeting are provided and their potential implication in improving agricultural practices are discussed. Since this is still a highly debated topic, where both positive and negative results are available with regard to plant miRNA stability, abundance, and especially cross-kingdom targeting ability, several open questions are being proposed relative to methodological and mechanistic issues.

PLANT-PARASITE MIRNAS CROSS-KINGDOM TRANSFER: ALTERNATIVE TOOLS TO FIGHT PLANT PESTS AND DISEASES

Among plant diseases, agricultural crop infection by fungal pathogens annually cause multimillion dollars losses. While the most used methods to combat fungal-borne diseases are fungicides and chemical sprays, these have negative impacts on human health and surrounding environment (Almeida et al., 2019). The cross-kingdom miRNAs delivery between plants and fungi may represent alternative, environmental-friendly approaches to fight fungal diseases and confer crop protection (Wang et al., 2016). To date, miRNAs have been observed to move in a cross-kingdom manner from plants to fungi and vice versa (**Table 1**). An example of plant miRNA transfer to pathogenic fungi is constituted by miR159 and miR166 from cotton (*Gossypium hirsutum*), shown to confer resistance to *Verticillium dahlia* (Zhang et al., 2016). These miRNAs, found in fungal hyphae isolated from infected cotton tissues, were predicted to hit the virulence-related proteins HiC-15 (isotrichodermin C-15 hydroxylase) and Clp-1 (Ca^{2+}-dependent cysteine protease). The targets were validated by transiently expressing miRNA-resistant HiC-15 and Clp-1 in tobacco and *V. dahliae*. Subsequent analysis of *V. dahliae* mutants confirmed that the targeted fungal genes had an important role to play during fungal virulence and that they were specifically targeted by the miRNAs exported from the infected cotton plants to achieve silencing, hence conferring resistance to the fungal pathogen (Zhang et al., 2016). An example of fungal miRNA delivery to host plants is the case of a novel miRNA-like RNA from *Puccinia striiformis* f. sp. *tritici* (*Pst*), the agent causing the wheat stripe rust disease, able to act as a pathogen effector and suppress wheat innate immunity (Wang et al., 2017a). Pst-milR1, identified by high-throughput analysis of *Pst* sRNA library, was predicted to target the β-1,3-glucanase *SM638* (pathogenesis-related 2) gene in wheat. Co-transformation analyses and RACE (rapid amplification of the

TABLE 1 | Examples of cross-kingdom miRNA transfer related to plant parasitic and mutualistic relations.

Donor	Receiver	Relation	miRNA	Target	Function	Reference
G. hirsutum	*V. dahlia*	Parasitic	miR159 miR166	*HiC-15* *Clp-1*	Fungal virulence	Zhang et al., 2016
P. striiformis	*T. aestivum*	Parasitic	pst-miR1	*SM638*	Innate immunity	Wang et al., 2017a
A. thaliana	*P. xylostella*	Parasitic	miR159c novel-7703-5p	*BLH6/P1* *RPO2*	Pupae development	Zhang et al., 2019a
B. campestris	*A. mellifera*	Mutualistic	miR162a	*TOR*	Caste development	Zhu et al., 2017

cDNA ends) validation in tobacco leaves confirmed that *SM638* was targeted by Pst-miR1.

When considering insect pests, the cross-kingdom transfer of miRNAs has been investigated for its communication role between plants and plant-feeding insects, such as *Plutella xylostella* (diamondback or cabbage moth) (Zhang et al., 2019a). RNA sequencing analysis has evidenced the presence of 39 plant miRNAs in the moth hemolymph. The plant-derived miR159a, miR166a-3p, and the novel-7703-5p were predicted to influence cellular and metabolic processes in *P. xylostella* through binding and suppressing *BJHSP1*, *BJHSP2* (basic juvenile hormone-suppressible protein 1 and 2), and *PPO2* (polyphenol oxidase subunit 2) genes. QRT-PCR analyses carried out following treatment with the specific miRNA agomir sequences, demonstrated the downregulation of the predicted targets whereas a luciferase assay proved the binding to their respective targets. Further insect development studies revealed that treatments with agomir-7703-5p resulted in the development of abnormal pupae and decreased adult emergence rates (Zhang et al., 2019a).

Other examples focused on showing the presence of plant-derived miRNAs in insect pests. For instance, Zhang et al. (2012) investigated this aspect in several *Lepidoptera* and *Coleoptera* species subjected to controlled feeding experiments. This study focused on determining the presence of conserved miR168 sequences in insects by means of northern blot and deep sequencing; while northern blot analyses were negative, the deep sequencing data revealed the presence of miR168 in moderate quantities. Hence, the authors discuss the possibility of sample contamination evidencing the existence of some artefacts during sequencing data analysis (Zhang et al., 2012). Deep sequencing was used to reveal plant miRNAs in cereal aphids (*Schizaphis graminum*, *Sipha flava*) causing serious losses in sorghum (*Sorghum bicolor*) and switchgrass (*Panicum virgatum*) crops (Wang et al., 2017b). Thirteen sorghum miRNAs and three barley miRNAs were detected and predicted to target aphid genes playing important roles in detoxification, starch and sucrose metabolism.

MiRNA cross-kingdom transfer probably occurs also during the interplay between plants and parasitic nematodes (phytonematodes) (Jaubert-Possamai et al., 2019). Plant-parasitic nematodes are responsible for considerable crop losses worldwide. Understanding how plants respond to these organisms is necessary to bridge the gap between agricultural production and the growing food demand. Most of the scientific literature on gene silencing mechanisms comes from nematodes, specifically from *Caenorabditis elegans*. However, these studies mostly focus on the ability to uptake double strand RNAs (dsRNAs) from the environment (Huang et al., 2006; Tian et al., 2019) rather than on the cross-kingdom transfer of plant miRNAs. Many studies have investigated the involvement of plant miRNAs and their corresponded gene targets in response to phytonematodes infection (Hewezi et al., 2008; Li et al., 2012; Lei et al., 2019; Pan et al., 2019). Transcriptomic analyses evidenced extensive reprogramming of gene expression at the nematode feeding sites, modulated by plant miRNAs; also, some conserved miRNAs were shown to have analogous roles in feeding site formation in different plant species (Jaubert-Possamai et al., 2019).

The cited examples depict a promising research area. Understanding the complex interactions between host plants and parasitic organisms would pave the way for the development of new technologies for a more sustainable control of plant pests and diseases.

MIRNAS CROSS-KINGDOM TRANSFER IN PLANT MUTUALISTIC INTERACTIONS

Several studies on mutualistic relations have regarded many miRNAs target processes related to hormone-responsive pathways and innate immune function (Formey et al., 2014; Wu et al., 2016). The majority of these processes correspond to turning off defense pathways that would otherwise block fungal or bacterial proliferation within plant tissues (Plett and Martin, 2018). In a recent study, Silvestri et al. (2019) have looked into the symbiosis between the arbuscular mycorrhiza (AM) *Rhizophagus irregularis* and the model legume *Medicago truncatula*, showing the presence of fungal microRNA-like sequences potentially able to target plant transcripts. The *in silico* analysis, verified through a degradome analysis, predicted more than 200 plant genes as putative targets of specific fungal sRNAs and miRNAs, many of which had specific roles in AM symbiosis. For instance, three miRNA-like sequences (*Rir*-miRNA-like 341, 342, and 828) shown to be up-regulated in the intraradical phase were suggested to be responsible for the regulation of AMF genes required to manipulate fungal or host plant gene expression. The predicted target genes encode for a DHHC-type zinc finger protein (AES89412), integral membrane family protein (AES91391), and carboxy-terminal region remorin (AES81367).

In recent years, evidence that plant miRNAs target genes in a trans-kingdom fashion in pollinator insects is steadily accumulating. Currently available studies report pre-eminently on dietary intake of plant miRNAs by honey bees (*Apis mellifera*) (Ashby et al., 2016; Gismondi et al., 2017; Zhu et al., 2017). The plant-pollinator relationship is partly mutualistic considering the nutrients intake in exchange for the pollination service that enables plant reproduction. The presence of plant miRNAs in honey was reported by Gismondi and collegues (2017) who detected and quantified several miRNAs belonging to conserved families (miR482b, miR156a, miR396c, miR171a, miR858, miR162a, miR159c, miR395a, miR2118a) in different types of honey. The authors found that the most enriched in plant-miRNAs was the honey obtained from sweet chestnut (*Castanea sativa*) flowers. In bees, the dietary intake of pollen-derived miR162a was proven to regulate caste development at larval stage (Zhu et al., 2017). It was shown that miR162a targets *TOR* (target of rapamycin) mRNA downregulating its expression at the post-transcriptional level. Interestingly, this mechanism was found to be conserved in *Drosophila melanogaster* (common fruit fly), a non-social type of insect (Zhu et al., 2017).

Nonetheless, contrasting results are also reported. Although Masood et al. (2016) observed accumulation of plant miRNAs after pollen ingestion in adult bees, they did not find any evidence of biologically relevant roles of these plant miRNAs in bees. Likewise, expression analysis of pollen-derived miRNAs ingested by bees, revealed the absence of substantial uptake and systemic delivery of miR156a, highly expressed in bee-bread and honey (Snow et al., 2013). In a different system, silkworm (*Bombyx mori*) and mulberry (*Morus* spp.) was used as model to study the proposed miRNA-mediated crosstalk between plants and insects (Jia et al., 2015). Sanger sequencing and digital PCR demonstrated the presence of mulberry-derived miRNAs in silkworm tissues while the administration of synthetic miR166b did not influence silkworm physiological progress.

CROSS-KINGDOM TRANSFER OF AMIRNAS FOR AGRICULTURAL PURPOSES

The knowledge acquired on endogenous miRNAs as regulators of gene functions within and among organisms led researchers to develop increasingly sophisticated agricultural technologies based on miRNAs. Among these, the amiRNA (artificial miRNA) strategy was developed to produce specific miRNAs that can effectively silence designated genes (Zhang et al., 2018). One of the main characteristics of amiRNAs is the conserved secondary foldback structure that has to be similar to that of a typical pre-miRNA. In this case, the original structure of the miRNA-5p:miRNA-3p sequence will be replaced by an engineered miRNA targeting a designated mRNA, and the most preferred structures are those existing in conserved miRNA families. In this way, amiRNAs can be engineered to target any mRNA with higher specificity compared to other strategies like dsRNA overexpression or siRNA accumulation. Since pre-amiRNA processing results in a single amiRNA targeting a designated sequence, this eliminates the off-target effects and the production of secondary siRNAs is quite limited (Manavella et al., 2012). A highly relevant attribute for agricultural purposes is the fact that amiRNAs are stable and inheritable. Moreover, the amiRNA-mediated silencing is believed to pose less problems regarding bio-safety and environmental security with respect to other strategies (Liu and Chen, 2010; Toppino et al., 2011), due to the small size of the inserts and reduced probabilities for horizontal transfer. Aside the study of gene functionality (Schwab et al., 2006; Warthmann et al., 2008), amiRNA technology has been applied to knock out genes from insect pests, nematodes, viruses, and other phytopathogens (Niu et al., 2006; Fahim et al., 2012; Guo et al., 2014; Kis et al., 2016; Wagaba et al., 2016).

Several pre-miRNAs have been used as backbones to synthesize artificial miRNAs in engineered plants with the aim to control agricultural pests (**Table 2**). This strategy is based on the possibility of miRNAs to be transferred through diet across kingdoms and the ability of these small molecules to exercise their biological activity in recipient organisms. Indeed, the miRNAs in the transgenic plant may be taken up by plant feeding organisms and then suppress selected genes such as those related to metabolism, development but also to pathogenesis/parasitism by exploiting the endogenous silencing machinery of the plant feeding organism. Essential genes either involved in pathogen metabolism, or causing resistance to plant toxins, or encoding effectors involved in pathogenicity, have been considered as potential targets. For instance, enhanced resistance to the aphid *Myzus persicae* was reported in transgenic plants expressing amiRNAs targeting the *MpAChE2* (aphid acetylcholinesterase 2) gene (Guo et al., 2014). The *AChE* gene encodes for hydrolase enzyme that hydrolyses the neurotransmitter acetylcholine and plays vital roles in insect growth and development (Kumar et al., 2009). In a recent investigation, amiRNA-based technology targeting AChE was also applied by Saini and co-workers (2018) to defeat *Helicoverpa armigera*. They demonstrated that the silencing of *HaAce1* gene by host-delivered amiRNAs disrupted growth and development in the polyphagous insect. Another example relates to the use of amiR-24 targeting the 3′-UTR of the *chitinase* gene. Transgenic tobacco plants producing amiR-24 were fed *H. armigera* caterpillars, resulting in delayed molting and enhanced lethality (Agrawal et al., 2015). In a different study, amiR15 was used to design transgenic rice plants resistant to the striped stem borer, *Chilo suppressalis* (Jiang et al., 2016). The amiR15, design staring from the insect specific miRNA, *Csu*-miR-15, targets the *CsSpo* (Cytochrome P450 307a1) and *CsEcR* (Ecdysone receptor) genes involved in the ecdysone signaling pathway. Feeding trials carried out using the transgenic miR-15 rice resulted into increased mortality and developmental defects in the targeted insect pest. The effect of amiRNAs was studied also on the *Avr3a* gene, the target transcript of *Phytophtora infestans*. AmiRNAs targeting different regions of the *Avr3a* gene imparted moderate type of late blight resistance into two transformed Indian potato cultivars (Thakur et al., 2015).

AmiRNA delivery may be considered as a species-specific pesticide and as a potential and powerful alternative to the chemical strategies used so far. This miRNA-based technology may be considered as an alternative method for intragenic crop

TABLE 2 | Examples of cross-kingdom transfer of artificial microRNAs (amiRNAs) from transgenic plants to their respective pathogens/parasites.

Modified plant	Pathogen/parasite	Target	Function	Reference
N. tabacum	*M. persicae*	*MpAChE2*	Synaptic transmission	Guo et al., 2014
A. thaliana	*H. armigera*	*HaAce1*	Synaptic transmission	Saini et al., 2018
N. tabacum	*H. armigera*	*Chitinase*	Chitin synthesis	Agrawal et al., 2015
O. sativa	*C. suppressalis*	*CsSpo CsEcR*	Embryonic development	Jiang et al., 2016
S. tuberosum	*P. infestans*	*Avr3a*	Fungal virulence	Thakur et al., 2015

engineering causing less public concern. For beneficial insects, such as honey bees, amiRNA-based technology may be used to counteract virus infections by feeding them in large field treatment with amiRNAs able to reduce the expression of viral genes. Apart from transgenic plants permanently expressing amiRNAs, amiRNAs sprayed onto leaves in conjunction with miRNAs enriched soil can minimize pest damages (Cagliari et al., 2019).

CONCLUSIONS AND FUTURE PERSPECTIVES

Plant pathogens place a global burden on major crops being responsible for reduced crop yields with great repercussions on food production and food security (Savary et al., 2019). On the other hand, promoting the investigation of mutualistic relations has the potential to better assist sustainable agricultural practices (Duhamel and Vandenkoornhuyse, 2013).

As shown in the presented examples, understanding the miRNA cross-kingdom transfer and mode of action could contribute to decrease the pathogenicity of fungi and pests, hence promoting better plant productivity. In the case of insects (pests or pollinators), administration of plant miRNAs (through genetic engineering, nanoparticles, or spraying) may actively contribute to population control, reducing the prevalence of pests while enhancing the preponderance of pollinators. In this context, researches could be envisioned to grasp on how plant miRNA trans-kingdom regulation could be used to avoid the extinction of bees, as exemplified in the studies demonstrating their involvement in cast development (Zhu et al., 2017). But, to progress this debated field, many questions still need to be addressed and many additional steps must be taken to elucidate plant miRNAs uptake and potential cross-kingdom gene targeting. In this highly-technological era, the rapid progress of bioinformatics studies and tools to predict cross-kingdom miRNA targets (Mal et al., 2018; Bellato et al., 2019) sets the stage to advance new hypothesis to be subsequently experimentally tested. Nonetheless, many of the existing questions demand solid proofs from wet lab analyses. From the point of view of experimental design, questions related to the most appropriate techniques (deep sequencing, digital PCR, qRT-PCR) and references (samples and/or genes) to be used for cross-kingdom miRNA studies still need to be addressed and uniformized accordingly (Chan and Snow, 2016; Zhang et al., 2019b). Once these issues are settled, we can then proceed to investigate other challenges; for instance, why some plant miRNAs seem to be more stable and abundant than others? Are the levels of host plant miRNAs found in pathogen species high enough to exert a physiological impact? How do plant miRNAs reach their targets in the receiving organism? Considering the miRNAs mode of action (targeting mRNAs based on sequence complementarity), their impact on the receiving organism can variate depending on the targeted genes; hence, studies covering both favorable and unfavorable effects need to be encouraged to promote best-informed scientific solutions.

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AM conceptualized the minireview. CG, PL, and AM wrote the manuscript.

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Oxidative Stress and Antioxidant Defence in Fabaceae Plants Under Abiotic Stresses

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Abstract Legumes, grown worldwide under various climate conditions, are strongly appreciated for their nutritional value and impact in terms of environmental benefits and enhanced sustainability. Several environmental and anthropogenic factors can affect legume crop productivity, among which are the deleterious abiotic stresses, namely drought, salinity, temperature, heavy metals. Different abiotic stresses impair legume growth and performance by triggering a common scenario within the cell that is extensive oxidative damage. Thus, a better understanding of the molecular mechanisms underlying the oxidative stress response in legumes will lead to innovative agronomic and scientific developments, promoting the future competitiveness of the system. The chapter will present and discuss the state of the art concerning the hallmarks of oxidative damage and plant antioxidant response as well as the impact of oxidative injury on genome integrity. The focus will be on the DNA damage response and the way plants use this complex molecular network to cope with stress. Besides dissecting the cellular mechanisms, an in-depth evaluation of the several environmental and anthropogenic factors that are stress determinants is provided. In this context, the role of emerging players as miRNAs will be discussed. This chapter provides new insights on legume profiles of antioxidant stress response resulting from 'omics', covering issues of model legumes *versus* legume crops.

1 Hallmarks of Oxidative Damage

The evolution of photosynthesis has led to substantial changes in the composition of the Earth's atmosphere since carbon dioxide was progressively replaced by oxygen. The new living organisms were able to adapt to oxygen and its reduction intermediates, the cytotoxic reactive oxygen species (ROS), through the parallel evolution of antioxidant defence mechanisms that carefully regulate ROS levels within the cell (Apel and Hirt 2004; Gutteridge and Halliwell 2018; Xie et al. 2019). Oxidative

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stress is a complex array of chemical and physiological events associated with all the different types of biotic (pathogen infection) and abiotic stresses (e.g. drought, salinity, extreme temperature, metal toxicity) that higher plants face during their life cycle (Xie et al. 2019). This condition results from the ROS overproduction and accumulation and compromises crop yields (Moller et al. 2007; Farooq et al. 2009; Challinor et al. 2014; Zorb et al. 2019).

Oxidative stress impacts the agronomic performance of grain legume crops that represent a fundamental source of dietary protein and essential mineral nutrients, particularly in the developing countries (Zhu et al. 2005; Bohra and Singh 2015; Bohra et al. 2015; Considine et al. 2017). In the context of global climate changes, adverse environmental conditions combined with improper agricultural land management are expected to challenge the global productivity of grain legume crops, thus limiting their benefits to soil fertility as well as their essential role in the cereal-legume-based cropping systems (Foyer et al. 2016; Considine et al. 2017).

Free radical species as O_2^- (superoxide radical), $OH^{\cdot}$ (hydroxyl radical), $HO_2^{\cdot}$(perhydroxyl radical) and non-radical molecules (H_2O_2, hydrogen peroxide; $^1O_2$, singlet oxygen) drive oxidative stress (Apel and Hirt 2004). The main sites of ROS production within the plant cell, in the presence of light, are chloroplasts and peroxisomes, whereas mitochondria represent the predominant source in the dark (Xia et al. 2015; Corpas et al. 2015). Other sites of ROS production have been identified, e.g. the plasma membrane where this event is mediated by the NADPH-dependent oxidase (Sharma et al. 2012), the endoplasmic reticulum (with the involvement of cytochrome P450) (Mittler 2002) and the apoplast (Hu et al. 2006). Excessive ROS levels cause oxidative injury to membrane lipids, proteins and nucleic acids, impairing the cell structure and metabolism, and ultimately lead to programmed cell death (Mittler and Blumwald 2015). Thus, the balance between ROS accumulation and scavenging is a critical parameter for plant survival and growth under environmental stress conditions. The hallmarks of oxidative stress damage can be precisely identified through the biochemical and molecular profiles of specific cellular components.

1.1 Lipid Peroxidation

At the level of cellular membranes, lipid peroxidation occurs when highly reactive ROS, such as hydroxyl radicals and singlet oxygen, abstract a hydrogen atom from polyunsaturated fatty acids to form lipid hydroperoxides. The latter are unstable and decompose to generate reactive aldehydes and ketones. Malondialdehyde (MDA) is a breakdown product of polyunsaturated fatty acid, and the MDA levels are regarded as a quantitative hallmark of lipid peroxidation induced by oxidative damage (Moller et al. 2007). Membrane lipids are sensitive targets, and the extent of ROS attack can be measured under adverse environmental conditions.

Lipid peroxidation is a reliable indicator of cell membrane damage, reported in several studies focused on the response to drought and heat stresses in alfalfa

(*Medicago sativa* L.) (Naya et al. 2007; Wang et al. 2009), white clover (*Trifolium repens* L.) (Lee et al. 2007), cowpea (*Vigna unguiculata* L.) (Nair et al. 2008) and *Lotus japonicus* L. (Sainz et al. 2010). Lipid peroxidation has been reported as one of the parameters used to assess the degree of oxidative damage triggered by heavy metals, as in the case of exposure to lead in pea (*Pisum sativum* L.) plants (Dias et al. 2019). MDA was also used as oxidative stress marker to monitor the response of chickpea (*Cicer arietinum* L.) roots to cadmium (Kar 2018). On the other hand, stress-tolerant genotypes can be selected, based on their ability to maintain low levels of lipid peroxidation, as reported for the drought-tolerant alfalfa varieties (Zhang et al. 2019). Similarly, lipid peroxidation has been used as hallmark to test the impact of salt stress on the *Medicago truncatula* L.-*Sinorhizobium meliloti* symbiosis and to evaluate the protective role of pre-treatments with the polyamines spermine and spermidine (Lopez-Gomez et al. 2017).

Another interesting use of this oxidative stress marker has been described in the context of seed germination. Doria et al. (2019) recently provided MDA profiles during the early step of water up-take by *M. truncatula* seeds in order to assess the impact of imbibitional damage on the lipid membranes. Lipid peroxidation helps the evaluation of an effective seed priming, the pre-sown treatment used by seed technologists to improve seed vigour (Bailly et al. 2000; Paparella et al. 2015). Polyamines are valuable priming agents, which are able to accelerate and increase germination of white clover seeds (Li et al. 2014). The treatment with polyamines triggered the pre-germinative metabolism, enhancing the antioxidant defence. Exogenous spermidine was able to improve the seed tolerance to water deficit during germination, and this was evidenced by the lower lipid peroxidation levels detected, compared to the untreated samples (Li et al. 2014).

1.2 Protein Oxidation

The irreversible protein oxidation is a major event that regulates the protein biological function and fate (Oracz et al. 2007). When free Fe^{2+} or Cu^{+} react with hydrogen peroxide, the resulting metal-catalysed oxidation generates highly reactive hydroxyl radicals (Fenton reaction; Halliwell 2006) that, in turn, oxidise amino acids. Protein carbonylation is induced through the interaction with reactive aldehydes generated by cell membrane lipid peroxides (Moller et al. 2011; Fedorova et al. 2014). Glycation represents a different type of oxidative post-translational modification, caused by the reaction of lysine and arginine residues with reducing sugars or their oxidation products. Legume root nodules contain high levels of Fe-proteins (nitrogenase, cytochromes and leghemoglobin) that can easily undergo oxidation (Becana et al. 2000). The occurrence of these oxidative post-translational modifications has been investigated in bean (*Phaseolus vulgaris* L.) nodule proteins (Matamoros et al. 2018). Metal-catalysed oxidation of amino acids led to malate dehydrogenase inactivation and leghemoglobin aggregation. The glycated proteins identified included

the key nodule enzymes sucrose synthase, glutamine synthetase and glutamate synthase (Matamoros et al. 2018). Patterns of carbonylated proteins in maturing seeds of *Medicago truncatula* reported by Satour et al. (2018) revealed a correlation between carbonylation levels and seed deterioration.

2 The Plant Antioxidant Response

Plants have evolved highly complex defence mechanisms to limit the distructive effects of ROS. Such mechanisms contribute to the cellular redox homoeostasis through enzymatic and non-enzymatic antioxidants. The coordinated action of these players improves the cell ability to detoxify ROS, thus providing protection against oxidative damage (Mittler 2002; Gill and Tuteja 2010; Sharma et al. 2012).

The antioxidant enzymes mostly investigated include SOD (superoxide dismutase) which is able to catalyse the dismutation of O^{2-} radicals into O_2 and H_2O_2. Depending on the metal cofactor required to activate the catalytic site, they are classified as MnSODs, FeSODs and CuZnSODs. These classes can be distinguished based on different molecular features (e.g. their sensitivity to inhibitors) and subcellular locations. SOD isoforms are found in legume nodules where they play an essential role in removing the O^{2-} radicals released from the oxidation of legemoglobin, as shown for alfalfa MnSOD (Becana and Salin 1989) and soybean CuZnSOD (Puppo et al. 1982). The FeSOD isozyme has been identified first in cowpea nodules (Becana et al. 1989) and then in alfalfa, common bean and mung bean nodules (Becana et al. 2000). The tetrameric hemoproteins catalases are found as multiple isozymes, mainly located in peroxisomes and glyoxysomes (Scandalios et al. 1997). Catalase is located in the peroxisomes of determinate nodules, where it removes H_2O_2 generated by uricase and other oxidases (Kaneko and Newcomb 1987).

All the components of the ascorbate-gluthatione (AsA-GSH) cycle (Noctor and Foyer 1998; Asada 1999) are found in the cytosol of nodule cells (Dalton et al. 1986, 1992, 1993a, b; Dalton 1995). The AsA-GSH cycle includes four enzymes, namely ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) that act as a highly effective H_2O_2 detoxification route (Sofo et al. 2010). Non-enzymatic antioxidants consist of major cellular redox buffers, such as AsA, GSH, flavonoids, tocopherols, tocotrienols, carotenoids and alkaloids. AsA participates in several processes of plant growth and development (Noctor and Foyer 1998) and is found in legume nodules where it removes ROS (Matamoros et al. 1999). The thiol tripeptide GSH is a ROS scavenger, participating in the AsA-GSH cycle for H_2O_2 removal in chloroplasts and nodule cytosol (Dalton 1995), as well as in sulphur transport and storage, stress tolerance and heavy metal detoxification (Noctor and Foyer 1998). The leaves, roots and seeds of some legumes contain homoglutathione (hGSH) instead of or in addition to GSH (Klapheck et al. 1988). The relative abundance of GSH and hGSH in different legume tissues and species is determined by the corresponding thiol tripeptide synthetases. GSH and hGSH play protective roles during N_2 fixation

(Matamoros et al. 1999). Nodules possess other compounds with antioxidant properties, e.g. soybean nodules contain α-tocopherol (Evans et al. 1999). Polyamines are organic polycations involved in plant growth, but they are also powerful ROS scavengers (Fujihara et al. 1994). Uric acid, another potent antioxidant compound, is an intermediate of purine degradation and ureide synthesis found in peroxisomes (del Rio et al. 1998). Flavonoids and other phenolics are abundant in nodules, where they can inhibit lipid peroxidation by intercepting the peroxyl radicals formed in nodule membranes (Moran et al. 1997).

The antioxidant defence is essential for legume seed viability and successful germination under stress conditions, as reported in *M. truncatula* (Macovei et al. 2011a). The free radical scavenging activity of *M. truncatula* seeds was significantly higher at four and eight hours following rehydration, in presence and absence of PEG (polyethylene glycol)-mediated osmotic stress (Macovei et al. 2011a). *APX* and *SOD* genes, encoding cytosolic ascorbate peroxidise and superoxide dismutase, were up-regulated in *M. truncatula* seeds, as part of the antioxidant response (Balestrazzi et al. 2011a, b; Macovei et al. 2010, 2011b). The role played by antioxidant mechanisms in the response to specific abiotic stresses, namely drought stress, soil salinity, heat stress and heavy metal toxicity, will be described with more details in Paragraph 9.4.

3 Oxidative Injury and Genome Integrity: The DNA Damage Response

The integrity of genetic information is compromised under severe stress conditions that trigger oxidative DNA damage and impair genome stability. This results into reduced plant growth and crop productivity. To overcome genotoxic injury, plants have evolved an efficient repair machinery that responds to damage perception and signaling networks, the so-called DNA damage response (DDR) (Yoshiyama et al. 2013; Nikitaki et al. 2018). DDR has been mainly investigated in the model plant *Arabidopsis*; however, in recent years, *M. truncatula* has become a versatile system for the study of DNA damage sensing and repair, particularly, in relation to seed germination and seed quality issues (Pagano et al. 2017, 2019 and 2020), and hopefully, this knowledge will be translated to the most relevant legume crops (Parreira et al. 2018).

Macovei et al. (2011a) reported on the involvement of the *M. truncatulaOGG1* (8-oxoguanine DNA glycosylase/lyase) and *FPG* (formamidopyrimidine-DNAglycosylase) genes in the seed repair response, as part of the base-excision repair (BER) pathway (David et al. 2007). In *M. truncatula*, the *MtOGG1* and *MtFPG* genes were up-regulated during seed imbibition, with a peak at the timepoint of rehydration in which the highest levels of oxidative DNA damage, measured in terms of 8-oxoguanine accumulation, were recorded (Balestrazzi et al. 2011b). The contribution of nucleotide excision repair (NER) pathway to genome maintenance during

M. truncatula seed imbibition has been highlighted as well (Macovei et al. 2010, 2011b).

To date, *M. truncatula* is the plant system mostly used for the study of tyrosyl-DNA phosphodiesterases (TDPs) (Macovei et al. 2018a, b; Confalonieri et al. 2013; Faè et al. 2014; Sabatini et al. 2015, 2017; Araujo et al. 2016). The Tdp1 and Tdp2 DNA repair enzymes are involved in the processing of a wide range of 5′-and 3′-end blocking DNA lesions, among which the cytotoxicstabilised topoisomerase/DNA covalent complexes (Pommier et al. 2014). Differently from animals where Tdp1 is encoded by a single copy gene, plants possess a small *Tdp1* gene family, first identified in *M. truncatula* by Macovei et al. (2010). Both the *Tdp1α* and *Tdp1β* genes were up-regulated during seed imbibition, and *in planta*, in response to osmotic and heavy metal stress (Macovei et al. 2010). The involvement of plant *Tdp1* genes in the complex DDR response of *M. truncatula* cells has been evidenced using ionising radiation (Donà et al. 2014), antisense strategy and RNA-Seq-based analysis (Donà et al. 2013, 2017; Sabatini et al. 2015). Transgenic lines over expressing the *MtTdp2α* gene revealed enhanced tolerance to genotoxic stress caused by PEG6000 and copper (Confalonieri et al. 2013; Faè et al. 2014).

The knowledge concerning DNA damage response during seed development is still scarce. A transcriptomic profiling of the expression of genes related to DNA damage response/chromatin remodeling mechanisms was performed in *P. vulgaris* seeds at four distinct developmental stages (Parreira et al. 2018). In late embryogenesis, the observed up-regulation of genes related to DNA damage sensing and repair suggested for a tight control of DNA integrity. At the end of filling and onset of seed dehydration, the up-regulation of genes involved in sensing of DNA double-strand breaks indicated that genome integrity is challenged (Parreira et al. 2018).

4 Environmental and Anthropogenic Factors as Stress Determinants

4.1 Drought Stress

Water deficit, the main limiting factor for the successful establishment of crop cultivation, is playing a dramatic role in the current scenario of climate change (Boyer et al. 2013). Drought stress affects plant growth, reducing crop production, especially during grain filling and the reproductive phase. The extent of decrease in yield depends on the intensity and duration of drought stress, crop developmental stage and genotypic variability. Agricultural forage production requires adequate water supply, and this is problematic in semi-arid climates where insufficient water supply severely limits the production of forage legumes (Hopkins and Del Prado 2007; Nadeem et al. 2019a).

Drought resistance is achieved through reduced water loss combined with a more efficient water up-take, whereas drought tolerance relies on the action of osmoprotection and antioxidant mechanisms (Farooq et al. 2009; Fang and Xiong 2015). One of the biochemical changes occurring when plants experience these harmful conditions is ROS accumulation that is balanced through the antioxidant mechanisms. Enhanced antioxidant protection and declined lipid peroxidation are tightly associated with superior drought tolerance in legumes. Alfalfa avoids drought by reaching the deep moistured soil (Huang et al. 2018); however, several complex cultivar-specific morphological, physiological and molecular traits, including a robust antioxidant response, generally contribute to alfalfa drought resistance (Wang et al. 2009, 2011; Maghsoodi and Razmjoo 2015; Quan et al. 2016). Under oxidative stress, antioxidants may increase more during the recovery phase than in the stress phase, as observed in bean (Yasar et al. 2013), pea (Mittler and Zilinskas 1994; Osman 2015), soybean (Guler and Pehlivan 2016), chickpea (Patel et al. 2011) and cowpea (Carvalho et al. 2019). In the presence of drought stress, SOD, APX, GR, GST, GPX and POD activities are increased in resistant cultivars of common bean and horse gram (*Macrotyloma uniflorum* L.) (Saglam et al. 2011; Bhardwaj and Yadav 2012). The response of legumes at the onset of drought can vary; however, the final yield will significantly be reduced (Nadeem et al. 2019a).

4.2 Soil Salinity

Soil salinity exerts a deleterious impact on crops, including legumes, in terms of oxidative stress, genotoxicity, ionic imbalance, nutrition deficiency and osmotic stress (Murillo-Amador et al. 2007; He et al. 2015; Nadeem et al. 2019b). Composition of legume grains and grain yield is also altered (Manchanda and Garg 2008). This major constraint to crop production affects about 20% of the total irrigated land area in the world (FAO). Soil salinity is mainly caused by excess sodium chloride used for irrigation (Flowers and Flowers 2005).Salinity stress disrupts metabolic pathways by impairing key enzyme activities, while triggering ROS accumulation. Salt-tolerant legumes rely on effective antioxidant defence systems to scavenge the toxic-free radical species (Hernandez et al. 1999; Kukreja et al. 2005; Farooq et al. 2015). Under salinity stress, SOD, MDHAR, DHAR, GR and APX activities are significantly increased in salt-tolerant pea cultivars (Hernandez et al. 2000) and, similarly, a salt-tolerant common bean cultivar showed enhanced APX and CAT activities (Yasar et al. 2008).

4.3 Heat Stress

Temperature extremes (heat stress and cold stress) can severely damage crop plants at all stages of development, resulting in loss of productivity. Legumes, such as chickpea, lentil (*Lens culinaris* L.), mung bean (*Vigna radiata* L.), soybean and peas, are characterised by different degrees of sensitivity to high- and low-temperature stresses, and this compromises their performance at different developmental stages (germination, seedling emergence, vegetative phase, flowering and pod/seed filling phase) (HanumanthaRao et al. 2016; Sharma et al. 2016). In the case of mung bean, it has been reported that each degree rise in temperatures above optimum reduces the seed yield by 35–40% (Sharma et al. 2016). Increasing atmospheric CO_2 concentration along with temperature also limits plant growth, particularly in C_3 plant species, like mung bean (Nair et al. 2008). Heat stress decreases the activity of SOD, CAT and APX and increases ROS levels and membrane damage in the aerial parts of soybean, chickpea and mung bean plants (Djanaguiraman and Prasad 2010; Kumar et al. 2013; Nahar et al. 2015). Application of exogenous GSH enhanced heat stress tolerance in mung bean seedling by modulating antioxidant systems (Nahar et al. 2015), whereas higher levels of antioxidant metabolites have been detected in heat-tolerant chickpea and soybean genotypes under heat stress (Kumar et al. 2013; Chebrolu et al. 2016).

4.4 Heavy Metal Stress

Heavy metal environmental pollution has become a serious concern for living organisms and ecosystems. Heavy metals accumulated in soil are difficult to degrade and remove, and their further accumulation in plant tissues can cause higher plant-tissue toxicity (Nagajyoti et al. 2010). Heavy metal toxicity significantly affects plant growth as it suppresses antioxidant enzyme activity (Duan et al. 2018). The use of forages grown on metal-contaminated soil can increase the risk of heavy metals entering the food chain and affecting human health. The response to heavy metals has been investigated in legumes, highlighting the role of antioxidant players. *P. sativum* plants exposed to lead (Pb) in soil showed increased GR, APX and CAT activities in leaves and roots that were triggered by the phytohormone jasmonic acid (Dias et al. 2019). Similarly, in chickpea plants exposed to increasing vanadium (V) concentrations, the antioxidant enzyme activities (SOD, CAT, POD) were increased in a dose-dependent manner (Imtiaz et al. 2018).

5 Novel Players in the Response to Oxidative Stress: MicroRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules (21–22 nt) which are able to regulate gene expression at post-transcriptional level. They can silence the expression of a specific gene by matching to its messenger RNA (mRNA), triggering degradation or, in some cases, repressing the translation step (Yu et al. 2017; Tyagi et al. 2019). miRNAs have been retrieved in several legume species including *M. truncatula*, soybean, chickpea, common bean and *L. japonicas* (Subramanian et al. 2016; Szittya et al. 2008; Arenas-Huertero et al. 2009; Hu et al. 2013; Zheng et al. 2016; Pan et al. 2016; Wu et al. 2017). In a study performed on drought-sensitive and drought-tolerant soybean seedlings, Kulcheski et al. (2011) identified 256 miRNAs among the novel MIR07 and MIR11. The relative expression of MIR07 was increased under water deficit in both the drought-sensitive and drought-tolerant genotypes. In contrast, MIR11 expression showed a genotype-dependent response, being stable in the tolerant plants. Other soybean miRNAs involved in drought tolerance were also identified by Zheng et al. (2016). High-throughput sequencing was used by Jatan et al. (2019) to detect and characterise small RNAs in drought-tolerant chickpea roots in presence or absence of the rhizobacterium *Pseudomonas putida*, under water deficit. This study suggested that bacterial inoculation might play a crucial role in the modulation of miRNAs and their target genes in response to drought stress (Jatan et al. 2019). The genotype-dependent response of miRNAs to abiotic stresses was further assessed by Barrera-Figueroa et al. (2011) who investigated and compared the impact of water deficit on drought-tolerant and drought-sensitive cowpea cultivars. MicroRNAs also mediate the response to heat stress, as reported in common bean (Naya et al. 2014) and alfalfa (Matthews et al. 2019).

Members of the miR156 family control the *Squamosa promoter-binding protein-like* (*SPL*) genes in the context of plant growth under abiotic stresses. Alfalfa plants overexpressing miR156 and RNAi-mediated knockdown of the *SPL13* gene showed increased tolerance to heat stress associated with increased non-enzymatic antioxidant content (Matthews et al. 2019). A significant improvement in drought tolerance was also observed in alfalfa plants overexpressing miR156, concomitant with enhanced accumulation of proline, abscisic acid and antioxidants (Arshad et al. 2017).

The state of the art related to the miRNA word in legumes is rapidly expanding and databases resulting from the use of high-throughput technologies which are continuously enriched with huge amounts of information. The legume miRNAomes need to be integrated with tissue-specific transcriptomes and proteomes captured in response to abiotic stresses and/or ameliorating treatments (e.g. priming).

6 Insights on Legume Profiles of Antioxidant Stress Response Provided by 'Omics'

In the last decades, the approaches used to study biological systems and explain their behaviours have changed. The reductionist vision based on functional biology and aiming at model simplification is being progressively complemented and substituted by more holistic views based on system biology and aiming at huge dataset integration. Key functional and regulatory genes involved in abiotic stress resistance have been identified using next-generation technologies and related bioinformatic tools that enable the rapid and cost-effective analysis of whole genomes and transcriptomes in major crops.

In a recent review, Abdelrahman et al. (2018) provided an exhaustive overview of the knowledge gained on the molecular mechanisms underlying abiotic stress resistance in legume crops. Microarray-based gene expression analyses were initially used for transcriptome studies in legumes, as in the case of soybean (Le et al. 2012; Ha et al. 2015; Tripathi et al. 2015; Ramesh et al. 2019), *L. japonicus* (Asamizu et al. 2005), alfalfa (Gao et al. 2016), *M. truncatula* (Cheung et al. 2006), chickpea (Deokar et al. 2011) and candidate genes for drought, salinity, cold and heavy metal stress resistance were identified. Garg et al. (2016) performed a comparative transcriptome analysis of drought- and salinity-tolerant/sensitive chickpea genotypes in response to drought or salinity at different developmental stages. The study highlighted the extensive transcriptional reprogramming occurring in the different chickpea genotypes, showing enhanced drought sensitivity during the early reproductive stage, followed by pronounced salt stress sensitivity at the late reproductive stage (Garg et al. 2016).

High-throughput Illumina Hiseq 2500 sequencing allowed to identify drought-responsive miRNAs from alfalfa roots and leaves, providing 281 novel, predicted miRNAs (Li et al. 2017). Based on this study, drought-induced miRNA-related pathways were established, and the most representative families (miR166, miR159, miR482 and miR2118) with regulatory roles in legumes under drought were assessed (Li et al. 2017). Genome-wide analysis revealed auxin-responsive miRNAs differentially expressed in soybean roots in response to salt stress (Sun et al. 2016) as well as miRNAs involved in post-transcriptional regulation of gene expression in chickpea roots under salt and drought stresses (Khandal et al. 2017).

7 Model Legumes Versus Legume Crops

The choice of a model organism is essential to establish a representative experimental system, and it is functional for the definition of specific timepoints, physiological stages and treatments in which the phenomena of interest are to be studied. Two legume species in the galegoid clade (cool season legumes), *M. truncatula* and *L. japonicus*, which belong to the tribes Trifolieae and Loteae, respectively,

were selected as model systems to investigate legume genomics and biology (Cook 1999; Stougaard 2001). Differently from the major crop legumes, *M. truncatula* and *L. japonicus* own a small genome, ideal for carrying forward and reverse genetic analyses and well suited for studying biological issues relevant to crop legumes, among the abiotic and biotic stress tolerances.

M. truncatula was originally chosen for the study of rhizobia-legume symbiosis and the molecular genetics of nitrogen fixation in legume root systems (Barker et al. 1990). Subsequently, the interest towards *M. truncatula* as a model organism has progressively increased because of other appealing features. Its phylogenetic relationship and genetic similarity with other legumes, along with his small sequenced genome (~500 Mbp) and its diploidy (2n = 16) makes it a suitable model system for molecular genetics. Furthermore, its autogamy, its short generation time (~3 months) and its in vitro regenerative capacities add practical advantages to its cultivation, both in field/greenhouse and in vitro (Frugoli and Harris 2001).

L. japonicus is a perennial temperate pasture species, closely related to birdsfoot trefoil (*Lotus corniculatus* L.) with features useful for genomics (e.g. a short life cycle of 2–3 months, self-fertility, diploidy (2n = 12) and a small genome of 472.1 Mb). Although both *L. japonicus* and *M. truncatula* belong to the 'temperate' or 'galegoid' legume group, *L. japonicus* forms determinate nodules as observed in 'tropical' or 'phaseoloid' legumes such as soybean and common bean. *L. japonicus* is widely used to study plant–microbe interactions, due to its ability to establish a range of different types of relationship with symbiotic and pathogenic microorganisms, and it has been demonstrated to be amenable to genetic analyses (Handberg et al. 1992; Sato and Tabata 2006; Mun et al. 2016).

Efforts and investments made to decipher *M. truncatula* and *L. japonicus* genomics have brought to significant advances in basic and applied research, facilitating knowledge transfer from the best-characterised models to related food and feed legumes (Zhu et al. 2005). Comparative genome mapping has revealed macrosynteny or the conserved gene order between species as well as microsynteny (conserved gene content and order at sequence level over a short, physically defined DNA contig) (Zhu et al. 2005). The conserved genome structure between *M. truncatula* and crop legumes has allowed for map-based cloning of genes required for nodulation in crop legumes (Zhu et al. 2005). Similarly, these approaches are currently used to speed up the identification of stress-tolerant phenotypes and design of new breeding strategies to ameliorate legume crop adaptation to adverse environments. In this context, a promising, integrated strategy aimed at the identification of stress-tolerant legume germplasm has been recently described by Menendez et al. (2019). The link between plant physiology and big data resulting from 'omics' was examined in order to highlight the gene-to-metabolite networks involved in the abiotic stress interactions dealing with the ROS scavengers polyamines. These approaches are expected to accelerate the identification of stress-tolerant phenotypes and the design of new biotechnological strategies to increase their yield and adaptation to marginal environments, making better use of available plant genetic resources.

8 New Avenues for Improving Abiotic Stress Tolerance in Legumes

Genome resources (physical maps, functional genomics tools) facilitate the isolation of key genes contributing to abiotic stress tolerance in several legume species, providing the opportunity for crop improvement. Thus, biotechnologists and breeders will be able to target more rapidly and precisely the desired agronomic traits. The molecular profiling of germplasm collections reflecting the global biodiversity will promote the discovery of novel players in abiotic stress tolerance that will be included breeding programs. In this context, the focus is directed towards the 'orphan' or underutilised legumes, suited to withstand harsh environments (e.g. arid regions) (Cullis et al. 2018). Orphan legumes include groundnut (*Arachis hypogaea*), grass pea (*Lathyrus sativus*), bambara groundnut (*Vigna subterranea*), cowpea (*Vigna unguiculata*) and marama bean (*Tylosema esculentum*). They are staple food crops in many developing countries, with little economic importance, not extensively improved by breeders (Foyer et al. 2016); however, these plants have evolved successful survival strategies using a combination of different traits and responses. To date, orphan legumes represent a unique source of information for breeders who are looking for traits able to allow for survival in extreme environments.

Genome editing stands as a powerful tool for legume crop improvement, whose potential is still underexploited. To date, a few studies are available that describe the use of CRISPR/Cas9 for editing drought-tolerance-related genes in legumes (Cai et al. 2015, 2018). The CRISPR-based approach requires a deep characterisation of the target gene(s), their function and regulatory mechanisms, and this might delay the use in legume crops that still own limited information on stress-related genes.

Due to their peculiar physiology, legumes are expected to play pivotal roles in the mitigation of the effects of climate changes as well as in the contribution to sustainable farming. Their broad genetic diversity will be the source of alleles with relevance in novel adaptive traits. Researchers from different disciplines in plant science must integrate their expertise to address the open questions and develop new tools. Traditional and modern breeding must join their efforts and integrate their tools in order to accelerate genetic improvement.

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11

Oxidative Stress and Antioxidant Defense in Germinating Seeds

A Q&A Session

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11.1 Introduction

Seeds are fundamental from both an ecological and agronomical point of view, as they represent not only the main propagating vectors but also a highly effective strategy to survive harsh environmental conditions and allow successful transmission of genetic information to the next generation (Waterworth et al. 2015). Starting from the primordial state of development on the parent plant, seeds undergo various types of endogenous and/or exogenous stresses that may undermine cellular structures/functions. As a consequence, Reactive oxygen species (ROS), defined as chemically reactive chemical species containing oxygen, are continually produced during all phases of seed development, from seed dehydration to storing and germination, posing different outcomes on seed longevity and quality (Chen et al. 2012). Accumulations of ROS and NOS (nitrogen oxygen species) in seeds have been well documented in several species and at different developmental stages. Moreover, the literature listing the roles of hydroxyl radicals ($OH^{\bullet}$) (Schopfer et al. 2001; Richards et al. 2015; Wang et al. 2018), hydrogen peroxide (H_2O_2) (Gidrol et al. 1994; Caliskan and Cuming 1998; Hite et al. 1999; Schopfer et al. 2001; Bailly et al. 2002; Morohashi 2002; Lariguet et al. 2013; Biju et al. 2017; Ellouzi et al. 2017; Ni et al. 2018), superoxide radicals ($O_2^{\bullet -}$) (Gidrol et al. 1994; Schopfer et al. 2001; Chen et al. 2009; Kong et al. 2015), nitric oxide (NO) (Caro and Puntarulo 1999; Sarath et al. 2007; Wang et al. 2015; Gadelha et al. 2017; Yadu et al. 2017; Mao et al. 2018), and many other reactive molecules in the plant stress response, is in continuous expansion.

The effects and the roles of reactive radicals on the several aspects of seed physiology constitute a complex and variegated picture whose comprehension remains fundamental given the importance of seeds for plant reproduction and human use. These address ROS activity in various seed developmental stages as well as the implications of

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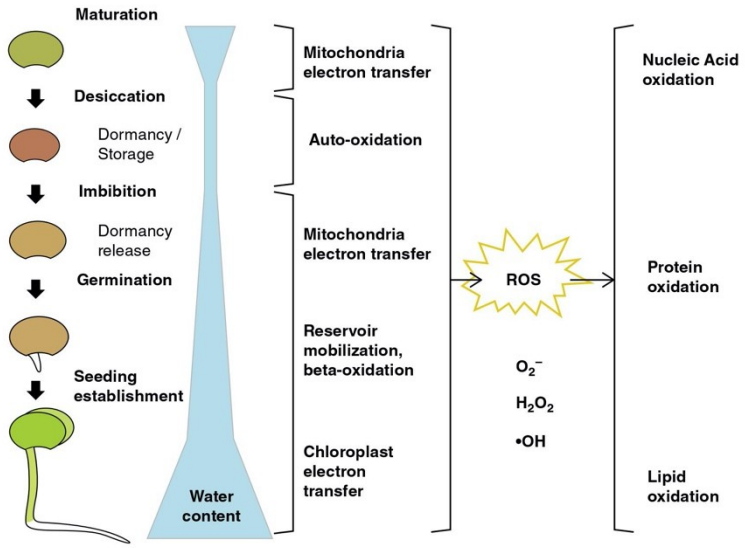

Figure 11.1 Timing and mechanisms of ROS production in seeds and their main effects on cell macromolecules.

different molecular players (Figure 11.1). New models, describing ROS not merely as damaging agents but also as key factors (e.g. signaling molecules) that regulate plant immunity and development, are emerging (Filippou et al. 2016; López-Cruz et al. 2017; Pucciariello and Perata 2017). Because of this duality, the presence and diffusion of ROS throughout the cell compartments have to be spatially and temporally regulated in order to avoid damage and, in the same time, enable them to fulfill their biological functions (Mittler et al. 2011; Wrzaczek et al. 2013). Indeed, integrative approaches carried out in the *Arabidopsis* model system had contributed to the identification of a large set of genes involved in the regulation of ROS (e.g. production vs scavenging), with more than 150 genes participating in this "ROS gene network" (Mittler et al. 2004, 2008).

In the present chapter, we propose a Q and A session focusing on fundamental questions relating ROS to seed germination. The structure of the chapter follows the *"3 W/3H"* approach and hence, concentrates on the following six questions: (i) Where are the ROS production sites in seeds? (ii) Where does ROS act at a molecular level? (iii) How do seeds protect themselves from ROS overdose? (iv) How does ROS influence seed dormancy? (v) How does the crosstalk between ROS and phytohormones influences seed germination? (vi) What are the roles of ROS in seed priming and seed longevity? As the starting question "What are ROS?" is already addressed in the introduction, the next sub-chapters will be discussed the six proposed questions.

11.2 Where Are the ROS Production Sites in Seeds?

Although many mechanisms of ROS production are common to different tissues, a number of them are particularly prominent in seed-specific physiological states, e.g. water uptake during imbibition, radicle protrusion preceding seedling establishment (Bailly 2004). At the cellular level, several components are considered as preferred sites for ROS production, and these tally for mitochondria, peroxisomes, cell membrane and apoplast.

The reactivation of metabolism during seed imbibition causes an enhanced H_2O_2 accumulation, resulting from electron leakage within the mitochondrial electron transport chain. It has been calculated that in mitochondria, approximately 2–3% of oxygen (O_2) molecules can be converted to H_2O_2 (Chance et al. 1973; Puntarulo et al. 1988; Kranner et al. 2010). Indeed, a transient burst in $O_2^{\bullet-}$ and H_2O_2 has been documented during seed imbibition and a second $O_2^{\bullet-}$ burst during radicle elongation stage (Kranner et al. 2010). Aside mitochondria, peroxisomes and glyoxysomes represent other major sources of ROS in seeds mainly due to the type of enzymatic reactions carried out inside these cellular components. For instance, lipid peroxidation results in the formation of H_2O_2 whereas $O_2^{\bullet-}$ radicals can be produced from the oxidation of xanthine (Corpas et al. 2001; Del Rio et al. 2002). The activity of glyoxysomes, specialized peroxisomes found in oily seeds, reaches a peak in the early stages of seedling development with the mobilization of reserve lipids and their conversion to sugars (Huang et al. 1983). The enzymatic activity of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidases (NOX), responsible for electron transfer from cytoplasmic NADPH to O_2, is an important source of H_2O_2 in the plasma membrane and it is regarded as being involved in the oxidative changes observed in plant-pathogen interactions (Grant and Loake 2000). The cell wall and apoplast constitute additional sites for the accumulation of H_2O_2 under biotic stress response, mainly because of the enzymatic reactions catalyzed by peroxidases and amine oxidases (Bolwell and Wojtaszek 1997; Bolwell et al. 2002). Furthermore, ROS production can result from the non-enzymatic autoxidation of lipids, in particular during the dry storage of seeds, when metabolism and, consequently, enzymatic activities are notably reduced (Priestley 1986; Wilson and McDonald 1986). In addition to the multiple production sites, ROS are able to diffuse in different cell compartments through transmembrane aquaporins and peroxiporins (Henzler and Steudle 2000) and, being highly reactive molecules, can be converted into other types of ROS that interact with many biological molecules. Such variables have to be taken into account in order to study the physiological effects of each reactive species.

11.3 Where Does ROS Act at a Molecular Level?

The occurrence of oxidative damage has been reported for a large set of biological macromolecules, such as lipids, DNA, RNA and proteins (Figure 11.1) (Harman and Mattick 1976; Osborne 1994; Bailly 2004; Rajjou and Debeaujon 2008; Rajjou et al. 2008) and has been positively correlated to loss of seed longevity (Harrison and McLeish 1954; Justice and Bass 1978; Groot et al. 2012).

11.3.1 ROS vs. Lipids

Lipids are particularly subject to oxidation both in metabolically active seeds and during dry storage. Yet, in dormant seeds, the enzymatic reactions normally occurring within all metabolic pathways are decidedly more reduced due to the dehydrated environment present in mature seeds or during dry storage (Priestley 1986; Wilson and McDonald 1986). Nonetheless, even in these conditions, alternative ROS sources can still lead to relevant oxidative damage, mainly through the auto-oxidation processes such as Amadori and Maillard reactions (Sun and Leopold 1995; Murthy and Sun 2000), protein carbonylation (Arc et al. 2011) and lipid peroxidation (Priestley et al. 1986; Wilson and McDonald 1986; Bailly 2004). Such ways of ROS production and its subsequent damage to macromolecules are among the main processes involved in seed aging and loss of seed vigor observed during dry storage, driving to economically relevant consequences (Buitink and Leprince 2008).

Following seed imbibition and dormancy release, all metabolically active cell compartments can become potential ROS sources. Among these, glyoxysomes reach a peak of activity immediately after germination, when the metabolic pathways involved in the conversion of reserve lipids to sugars are particularly necessary for seedling establishment (Huang et al. 1983). In this context, the oxidation of components from any type of lipid membranes can result in the production of H_2O_2 and $O_2^{\bullet -}$, respectively (Corpas et al. 2001; Del Rio et al. 2002). The negative effects of extensive oxidation of membrane lipids has been demonstrated in sunflower seeds, in which the loss of catalase (CAT) activity and the subsequent inability to detoxify H_2O_2 resulted in a significant loss of seed vigor and viability (Bailly et al. 1996; Bailly et al. 1998; Bailly et al. 2002).

11.3.2 ROS vs. Proteins

Proteins are sensitive to oxidative damage mainly because of $OH^{\bullet}$ that can react with many amino acids and functional groups. On the other hand, some enzymes, such as the ones involved in the Calvin-Benson cycle, can be inactivated by the reaction of their thiol groups with H_2O_2 (Charles and Halliwell 1980). Overall, different protein types can be affected by oxidative damage, including ion channels, receptor proteins and membrane transporters, leading to irreversible impairment of cell metabolic activities (Halliwell and Gutteridge 1999).

In seeds, the imbibition phase (characterized by rapid water uptake) has been associated with significant changes in the redox state of proteins, mainly in the embryo and endosperm; the state of these proteins is being transformed from an oxidized form (S-S) to a reduced one (–SH) (Buchanan and Balmer 2005). In particular, seed storage proteins (SSP), including the abundant globulins, represent one of the main targets of oxidation in seeds. In this context, they can also act as ROS scavengers, preventing oxidative damage to other classes of proteins (Job et al. 2005; Arc et al. 2011).

A specific type of protein modification introduced by oxidative reactions is protein carbonylation. Many amino acidic residues, namely arginine, lysine, proline and threonine, can undergo oxidation events interfering with the protein function; e.g. carbonylated proteins often show increased susceptibility to proteolysis (Rivett and Levine 1990; Berlett and Stadtman 1997; Dukan et al. 2000; Dunlop et al. 2002).

Carbonylation of *Arabidopsis* 12S globulin has been observed in both aged and unaged seeds. Mutations in the genes coding for 12S globulin have been linked to a severe impairment of seed longevity, suggesting a role of this SSP in seed aging processes (Nguyen et al. 2015). Furthermore, the carbonylation seems to be selective, acting on the α-subunit in unaged seeds, and on both α and β subunits in aged seeds (Job et al. 2005; Rajjou et al. 2008; Kalemba and Pukacka 2014). Proteomic approaches also revealed that protein carbonylation is a targeted phenomenon, with specific subsets of proteins being preferentially carbonylated. As an example, 12S cruciferins, the most prominent class of SSPs in *Arabidopsis thaliana* seeds, have been found to undergo carbonylation during the early germination phases and this was thought to be positively correlated with the efficiency of protein cleavage and mobilization; also, in this case, some cruciferin isoforms were more susceptible to carbonylation than others (Arc et al. 2011; Rajjou et al. 2012). Apart from storage proteins, many enzymes have been shown to be specifically targeted, e.g. glycolytic enzymes, aldose reductase, methionine synthase, mitochondrial ATP synthase, the large chain of the chloroplastic ribulose bisphosphate carboxylase, the large chain and many translation factors and molecular chaperones (Sano et al. 2016). Hence, carbonylation has been proposed as a marker for the overall oxidative state of cell proteome (Ballesteros et al. 2001; Das et al. 2001; Mostertz and Hecker 2003; Johansson et al. 2004) and sensitive assays for its detection and measurement have been developed (Levine et al. 1990, 1994).

Seeds have evolved a number of strategies to counteract oxidative damage to proteins. Molecular chaperones, including heat-shock proteins, allow the correct folding and provide protection against oxidative stress (Nguyen et al. 2015). The positive effects of such defense mechanisms have been demonstrated by overexpressing the sunflower heat-shock transcriptional factor A9 (*HaHSFA9*) in tobacco. The resulting transgenic tobacco plants developed seeds with a substantially improved seed longevity (Prieto-Dapena et al. 2006). The oxidized methionine residues of many proteins can be restored by methionine sulfoxide reductases (Weissbach et al. 2005), whose positive effects on seed longevity had been shown in two *Medicago truncatula* genotypes (Chatelain et al. 2013). Thioredoxins constitute another class of enzymes involved in the regulation of disulfide proteins. Their influence on the reduction of disulfide bounds aims at increasing protein solubility and susceptibility to proteolysis, thus facilitating the mobilization of storage proteins (Buchanan and Balmer 2005), as demonstrated in *M. truncatula* (Alkhalfioui et al. 2007).

11.3.3 ROS vs. Nucleic Acids

Particularly, ROS accumulation is the primary cause of DNA strand breaks due to modifications of bases and deoxyribose units (Bray and West 2005). The occurrence of considerable DNA strand breaks has been reported as a major impairment of seed longevity and vigor (Cheah and Osborne 1978). The most common oxidative base modification is guanine hydroxylation to produce 7,8-dihydro-8-oxoguanine (8-oxoG), a potentially mutagenic base (Bray and West 2005; Biedermann et al. 2011). On the other side, also deoxyribose, purines, and pyrimidines can be subjected to oxidative modifications (Breen and Murphy 1995).

Base Excision Repair (BER), the pathway involved in the repair of 8-oxoG (Bray and West 2005; Biedermann et al. 2011; Chen et al. 2012), is one of the processes taking part

in the DNA Damage Response (DDR) in all plant tissues, including seeds. Such responses are important to maintain an enhanced germinability and longevity (Balestrazzi et al. 2011a, b; Waterworth et al. 2015). Indeed, the upregulation of genes involved in BER pathway (e.g. formamidopyrimidine-DNA glycosylase -*FPG*-, 8-oxoguanine DNA glycosylase/lyase-*OGG1*, tyrosyl-DNA phosphodiesterases -*Tdp1α* and *Tdp1β*) has been reported during *M. truncatula* seed imbibition (Macovei et al. 2010; Macovei et al. 2011a). Similarly, genes with known roles in nucleotide excision repair (NER), like the transcription elongation factor IIS (*TFIIS*), were upregulated in *M. truncatula* and *A. thaliana* seeds subjected to stress conditions (Macovei et al. 2010; Murgia et al. 2015). In addition, upregulation of genes involved in the antioxidant response and ROS scavenging has been reported both during imbibition under physiological conditions and osmotic stress; among these are *APX* (ascorbate peroxidase) and *SOD* (superoxide dismutase) genes, required for H_2O_2 and $O_2^{\bullet -}$ scavenging, (Balestrazzi et al. 2011a; Macovei et al. 2011a, b) and *MT2* (type 2 metallothionein), involved in the seed response to stress (Donà et al. 2013). In maize germinating seeds, an epigenetic control has been highlighted in aleurone cells, where the *SOD* gene promoter was hyperacetylated in response to gibberellins, while abscisic acid (ABA) promoted hypoacetylation and caused an impaired germination (Hou et al. 2015).

The single-stranded structure of RNA is even more susceptible to oxidative damage than the DNA double helix. Its cytoplasmic location and the lack of specific repair pathways makes RNA much more prone to degradation and the translational block resulting from its massive damage has been suggested as an important cause of longevity loss in imbibed seeds (Rajjou et al. 2008; Bazin et al. 2011).

11.4 How Do Seeds Protect Themselves from ROS Overdose?

Nowadays the ability of seeds to germinate fast and uniformly, along with the seedling capacity to establish a successful harvest, represents a priority to achieve high crop yields (Paparella et al. 2015; Macovei et al. 2017; Pagano et al. 2017). The endogenous production of ROS related to the cellular metabolism may compromise the optimal crop production, affecting these traits by overaccumulation. In addition, given the sessile lifestyle, plants are exposed to several abiotic and biotic stresses that may further increase ROS production (Gill and Tuteja 2010; Balestrazzi et al. 2011b). Hence, in order to protect themselves, plants, as well as seeds, have evolved several defense mechanisms. Here, we summarize some passive and active mechanisms, alongside the molecular implication of DDR systems in seeds.

11.4.1 Passive Mechanisms

After maturation, orthodox seeds can survive long periods of storage in dehydrated and dormant conditions. During dormancy, and especially at low temperatures, cytoplasm passes from a metabolically active fluid state to a viscous glassy state in which the mobility of metabolites, macromolecules, and cellular structures is severely restricted by the reduced water content. This reduction of metabolic activity limits the production of ROS typical of mitochondria, chloroplasts, and other cellular compartments, thus

preventing extensive damage to cellular components before germination occurs (Buitink and Leprince 2008). Nevertheless, in dry conditions, non-enzymatic sources of oxidative damage are still active, namely lipid peroxidation (Wilson and McDonald 1986), Amadori and Maillard reactions (Murthy and Sun 2000), thus requiring detoxification systems to prevent extensive cellular damage.

As during the seeds dehydrated state the activity of antioxidant enzymes is strongly impaired, many orthodox seeds have evolved non-enzymatic systems to scavenge the ROS produced during storage. This is also because many classes of molecules are accumulated during seed maturation. Polyphenols, like flavonoids, are among the most common and relevant non-enzymatic antioxidants that accumulate in the seed coat, endosperm, and embryo as a consequence of specific developmental signals. Polyphenols have been shown to significantly contribute to maintaining seed longevity in *Arabidopsis* seeds (Debeaujon et al. 2000). Other polyphenols, like proanthocyanidins, can accumulate in the seed coat and are supposed to participate in seed hardening. Indeed, an increased accumulation of proanthocyanidins was observed in the seed coat of *Arabidopsis* seeds maturated on mother plants subjected to low temperature (16 °C instead of the standard 22 °C) (Sano et al. 2016). During seed desiccation, the reaction catalyzed by TT10 (Transparent Testa 10), a laccase-like enzyme, oxides the soluble proanthocyanidins and produce quinonic compounds that cross-link to the cell wall forming a stronger barrier against water, mechanical damage, microorganisms and hostile environments (Pourcel et al. 2007). This cross-linking and subsequent seed browning was documented in cotton, pea and *Sida spinosa* seeds (Pourcel et al. 2005).

Besides polyphenols, other molecules were implicated in the seed ROS scavenging. Such is the case of tocopherols, also known as Vitamin E, which protect membrane lipids from non-enzymatic oxidation during dry storage. As an example, different *Arabidopsis* genotypes with impaired tocopherol biosynthesis were shown to retain a reduced longevity (Sattler et al. 2004; Giurizatto et al. 2012). Still in *Arabidopsis*, seed longevity had also been related to the accumulation of lipocalins. These represent a class of small hydrophobic proteins that prevents lipid oxidation and enhance adaptation to stress. A number of genes encoding for lipocalins were identified. For example, *AtTIL* (temperature-induced lipocalins), induced by high temperature, and *AtCHT* (chloroplastic lipocalins), present in the chloroplast, are involved in such mechanisms of resistance to oxidative stress (Boca et al. 2014). Reduced glutathione is another abundant regulator of the intracellular redox environment in seeds; its abundance and redox state have been proposed as a marker for seed aging (Kranner et al. 2006; Nagel et al. 2015). In addition, SSP particularly abundant in the seeds of many species, represent one of the primary targets of oxidation. For this reason, besides their role as reserve molecules, an additional role as ROS scavengers has been proposed for these type of proteins (Job et al. 2005; Arc et al. 2011).

11.4.2 Active Mechanisms

The activity of the enzymatic machinery implicated in ROS detoxification is strongly limited during seed dormancy because of the dehydrated glassy state of the cytoplasm in most orthodox seeds (Rajjou et al. 2012). In these conditions many sources of oxidative stress deriving from the cellular metabolism are inactive, despite alternative sources like lipid auto-oxidation, which can lead to extensive structural damages and

impairment of seed vigor. In addition, during the quiescent state, the activity of DDR pathways is also limited by the anhydrous conditions.

Following seed imbibition and consequent metabolism reactivation, the metabolic pathways leading to ROS production, as well as the enzymatic machinery involved in their detoxification, resume their activities. In plants, a set of different enzymes and signaling molecules are generally involved in ROS detoxification, e.g. superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidase (GPX), glutathione reductases (GSR), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDAR), thioredoxins (TRX), peroxiredoxins (PRX), and glutaredoxins (GRX) (Bailly 2004; Kumar et al. 2015). All these enzymes are also produced and active at different levels during seed germination. Protection from stress is also expected during seed maturation and desiccation in order to grant an enhanced longevity. In this case, polyphenol oxidases (PPOs), catechol oxidases (CO), laccases (LAC), and peroxidases (PODs), enzymes involved in the oxidation of flavonoids, were shown to accumulate in the seed coat, leading to browning and impermeabilization to water (Pourcel et al. 2007). The presence of such enzymes has been highlighted in the embryo testa of *Arabidopsis* and *Glycine max* seeds (Moïse et al. 2005; Pourcel et al. 2005).

11.4.3 DDR and ROS in Seeds

As ROS overaccumulation is among the main causes of DNA decay in many organisms, one case that needs special attention is represented by the levels of damage that ROS can induce to the DNA double helix as well as the activation of specific repair pathways (Roldán-Arjona and Ariza 2009; Balestrazzi et al. 2011a, b). In this regard, it was shown that the perception of DNA lesions, essential to also ensure cell viability in seeds, can drive the suppression of the cyclin-dependent kinase (CDK) activity and result in a delay or arrest of the cell cycle (Kitsios and Doonan 2011).

Because the maintenance of genome integrity is essential to preserve the genetic information and its faithful transmission to subsequent generations (Waterworth et al. 2016), this aspect is fundamental in the case of seeds. In this regard, plants evolved sophisticated mechanisms, grouped under the umbrella of DDR, which enable them to cope with the detrimental effects of genotoxic stress. Because seeds are susceptible to elevated levels of genotoxic stress, seed vigor and viability are strongly conditioned by the efficiency of the repair machinery. In fact, the intense activation of DNA repair pathways during the pre-germinative metabolism triggered by imbibition is critical for de novo DNA synthesis in embryo cells (Bray and West 2005; Waterworth et al. 2015; Macovei et al. 2017). At this stage, upregulation of several DNA repair genes involved in BER and NER pathways (Macovei et al. 2010; Balestrazzi et al. 2011a, b; Macovei et al. 2011a, b; Pagano et al. 2017) as well as in the main pathways for the repair of double strand breaks (DSBs), namely homologous recombination (HR) and non-homologous end joining (NHEJ) (Waterworth et al. 2015, 2016) was highlighted. Waterworth et al. (2016) identified ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM RAD3-RELATED (ATR) as important sensor kinases playing crucial roles in seed germination. Both ATM and ATR are main players of the DDR pathway, being responsible for DNA damage sensing and hence, the orchestration of the entire process (Maréchal and Zou 2013; Yoshiyama et al. 2013; Nikitaki et al. 2018). Both the

case of ATM and ATR are demonstrative examples of the involvement of DDR genes in seed longevity and thus, seed quality. In particular, ATM has been identified as the main factor that controls the progression from the seeds' dry state to the germination phase, through its intrinsic role as a surveyor of genomic integrity. Specifically, ATM operates by controlling the DNA replication during seed imbibition. Its activation is induced by the presence of DSBs, a highly deleterious form of damage that can cause chromosomal aberrations (Waterworth et al. 2016). The transcriptional control of the cell cycle inhibitor SIAMESE-RELATED 5 (SMR5) mediates ATM signaling (Yi et al. 2014). It was also shown that SMR5 is responsible for the delay in the germination of aged seeds (Waterworth et al. 2016). The strong DSB-specific transcriptional response is also detectable in unaged seed. While ATM is activated as a result of DSB, ATR operates when single-stranded regions of DNA arise during replication (Nakabayashi et al. 2005).

Thus, DDR influences the rate of germination showing a link between levels of genomic damage and seed vigor and viability, two determinant aspects of plant survival and productivity. In this framework, ROS are intrinsically influencing the physiological process of seed development and quality but also, they exacerbate the genotoxic stress under adverse environmental conditions.

11.5 How Does ROS Influence Seed Dormancy?

Based on their ability to survive desiccation after ripening, seeds are broadly classified into two main categories: orthodox and recalcitrant. Recalcitrant seeds are unable to survive dry conditions after their maturation and subsequently cannot stand long storage periods without deteriorating. Unlike recalcitrant seeds, orthodox seeds can better survive desiccation events, and this allows them to maintain their viability throughout maturation and long periods of dry storage, in a state of dormancy (Roberts 1973; Ooms et al. 1993; Walters, 1998 ; Rajjou and Debeaujon 2008; Angelovici et al. 2010).

Dormancy is defined as an adaptive trait to increase seed lifespan and survival in dry environments and to enhance plant reproductive capacity, since it allows seeds to persist in the soil after dispersal, delaying almost indefinitely their germination until the environmental conditions are suitable for seedling establishment (Sano et al. 2016). Dormant seeds are able to survive harsh conditions such as low temperature, long periods of dry storage and many other kinds of biotic and abiotic stress. A key aspect to achieving survival in dry conditions is seed desiccation; this implies the transformation of the cytoplasm from a fluid and metabolically active state to a glassy viscous condition in which mobility of cellular components and enzymatic reactions are severely limited. As many of the pathways leading to ROS production in metabolically active tissues are inactive during this phase, the possibility of oxidative damage is restricted thus, resulting in an enhanced desiccation tolerance and longevity in orthodox seeds (Buitink et al. 2000; Buitink and Leprince 2008). A combination of late embryogenesis abundant (LEA) proteins, sucrose, and raffinose family oligosaccharides (RFOs) accumulates in desiccating seeds substituting water and stabilizing glassy cytoplasm (Koster and Leopold 1988). These had been also proposed as $OH^{\bullet}$ scavengers (Nishizawa et al. 2008).

In dormant seeds, ROS can be produced non-enzymatically through Amadori and Maillard reactions (Murthy and Sun 2000), lipid peroxidation (Wilson and McDonald 1986), and protein carbonylation (Arc et al. 2011). These autoxidation processes lead to the progressive loss in viability and vigor also observed during long-term storage in orthodox seeds. Following seed imbibition, cytoplasm rehydrates and regain its fluid state. This leads to metabolism reactivation and subsequently ROS accumulation (Vranová et al. 2002; Apel and Hirt 2004). On the other hand, with the resumption of all enzymatic activities the proteins also involved in the active ROS scavenging and macromolecule repair resume their activity. Coherently with these considerations, two bursts in H_2O_2 levels have been observed during early seed development before desiccation and following imbibition (Bailly et al. 2008).

The growing body of evidence related to the implication of ROS in the regulation of dormancy has led to the individuation of many agents and chemicals able to break dormancy. For instance, methyl viologen (MV) interrupts dormancy by inducing ROS production as well as patterns of protein carbonylation similar to those observed after natural seed ripening (Oracz et al. 2007, 2009; Whitaker et al. 2010). In *Bidens pilosa*, the administration of Fenton reagents induced the production of $OH^{\bullet}$ and has reduced germination timing (Whitaker et al. 2010). Conversely, diphenylene iodonium (DPI), acting as a NOX inhibitor, when administered to radish seeds had caused a reduction in the levels of $O_2^{\bullet -}$ (Schopfer et al. 2001); additionally, inhibition of germination was observed in many species, including *Arabidopsis*, barley, and cress (Müller et al. 2009a, b; Ishibashi et al. 2010).

11.6 How Does the Crosstalk Between ROS and Phytohormones Influences Seed Germination?

Physiology and timing of dormancy induction and release largely vary in different species and relies on many balanced parameters such as hormonal control, water uptake, environmental conditions and physical resistance of the seed coat to the rupture during radicle protrusion. Most species, including the model plant *A. thaliana*, exhibit a physiologically induced state of dormancy controlled by the balance of endogenous hormonal signals and released following after-ripening dry storage or rehydration under specific humidity, light, and temperatures. Generally, the establishment of a dormant state is enhanced by low temperature and low humidity levels and its subsequent interruption requires specific conditions or treatments. For example, in *A. thaliana* seeds dormancy release requires imbibition under cold (4 °C) and dark conditions in a procedure referred to as stratification (Baskin and Baskin 2004; Long et al. 2015).

Besides the deleterious effects of oxidative damage, there is evidence that ROS may cover important roles in certain physiological processes, participating in crosstalk networks along with hormonal signals (Figure 11.2). More specifically, at certain levels, ROS accumulation acts as a positive signal for dormancy regulation and release by interacting with ABA, the main dormancy-promoting hormone, and gibberellins (Bailly et al. 2008). The binary role of ROS is particularly evident for H_2O_2, which can act both positively and negatively on dormancy release (Kibinza et al. 2006; Oracz et al. 2007).

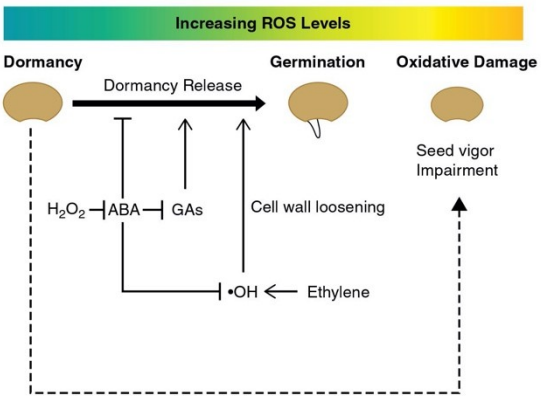

Figure 11.2 Crosstalk between ROS and hormones. ABA inhibits hydroxyl radical (•OH) production and dormancy release, counteracting the effects of GA. Instead, GA and ethylene promote dormancy release. Hydrogen peroxide (H_2O_2) inhibits the biosynthesis of ABA, whereas •OH promotes dormancy release contributing also to the cell wall loosening required for germination. ABA, Abscisic acid; GAs, gibberellins.

Experimental evidence of the interplay between ROS and hormones was provided by administration of exogenous H_2O_2 to stimulate dormancy release and germination in the seeds of many species, including barley (*Hordeum vulgare*) (Fontaine et al. 1994; Wang et al. 1995, 1998), rice (Naredo et al. 1998), apple (*Malus domestica*) (Bogatek et al. 2003), and *Zinnia elegans* (Ogawa and Iwabuchi 2001). In barley seeds, treatment with H_2O_2 reduced endogenous ABA levels (Wang et al. 1995, 1998) by inactivating two key enzymes involved in ABA signaling, namely ABI1 and ABI2 type 2C protein phosphatases (Meinhard and Grill 2001, Meinhard et al., 2002). ROS, and in particular H_2O_2 levels, appear to have positive effects on seed germination and seedling establishment (Schopfer et al. 2002; Müller et al. 2009a, b; Duan et al. 2014; Smirnova et al. 2014), as a burst in its production had been detected in embryo and seed coat during germination of radish seeds (Schopfer et al. 2001).

The specific function of H_2O_2 seems to be related to cell wall loosening to allow radicle elongation. The activity of NOX, POD, and lipoxygenases (LOX), causes a burst in the production of $O_2^{\bullet -}$ and $OH^{\bullet}$ in the extracellular environment (Kranner et al. 2010), which in turn are responsible for catalyzing the cleavage of pectins and xyloglucans of the cell wall, causing its loosening and facilitating cell wall distension and radicle elongation (Fry 1998; Schweikert et al. 2000; Fry et al. 2001, 2002; Miller and Fry 2001; Schopfer 2001; Messenger et al. 2009; Müller et al. 2009b). Cell wall loosening, caused by $OH^{\bullet}$ released in the apoplast, facilitates the rupture of the endosperm cap by the elongating radicle, as observed in *Lepidium sativum* seeds (Müller et al. 2009b). Furthermore, other processes, such as root hair and pollen tube elongation, may involve ROS production by NOX (Potocky et al. 2012), demonstrating the extent of ROS participation in plant physiological processes. Seed aging and loss of vigor appear to be

related to an impairment in $O_2^{\bullet-}$ production, normally observed in a successful germination (Kranner et al. 2010). $OH^{\bullet}$ and $O_2^{\bullet-}$ production in the apoplast and the subsequent cell wall plasticity are under hormonal regulation. ABA promotes the maintenance of dormancy by inhibiting the production of $OH^{\bullet}$, and so, the weakening of the endosperm cap (Barba-Espin et al. 2011). Conversely, ethylene promotes $OH^{\bullet}$ accumulation during the radicle protrusion stage, contrasting the effects of ABA (Graeber et al. 2010). At specific concentrations, also auxin participates in controlling the levels of $OH^{\bullet}$, as observed in maize coleoptiles (Schopfer 2001; Schopfer et al. 2002). Furthermore, it has been suggested that ROS production in seeds may not only facilitate radicle protrusion but also protect the embryos against pathogens (Schopfer et al. 2001; Bailly 2004). These data highlight a set of specific functions for ROS in the seed signaling network. Nonetheless, their accumulation above certain thresholds leads to their typical detrimental effects on membranes, proteins, and nucleic acids, responsible for embryo death, loss of vigor, and decreased viability. Therefore, the concept of an "oxidative window of germination" has been proposed to define the critical range of ROS production, sufficient to act as signal molecules for dormancy release but without the negative effects on seed viability (Bailly et al. 2008).

11.7 Which Are the Roles of ROS in Seed Priming and Seed Longevity?

Besides the implication of ROS in seed dormancy, other levels of action for these intriguing molecules can touch seed longevity and seed priming. During both of these processes, it is essential to balance the activity of antioxidant enzymes to control ROS accumulation and avoid severe oxidative damage in the seeds (Bailly et al. 2000).

11.7.1 ROS vs. Seed Priming

Priming is defined as a well-established class of treatments which allow the seeds to undergo the main physiologic and metabolic changes typical of pre-germinative phase (the so-called pre-germinative metabolism). It is highly important for priming treatments to be stopped before the loss of desiccation tolerance occurs. These techniques are usually applied to commercial seed lots in order to enhance seed quality; however, existing protocols are based on empirical observations and still require the establishment of dedicated methods to identify the best time point to stop the delivery of treatments (Paparella et al. 2015). Under these circumstances, being able to measure and identify the peaks of ROS production during seed imbibition, can provide the means to reach this goal.

Among the many types of priming, we are going to cite just a few. Hydropriming is one of the most used due to its simplicity. It is practically a procedure in which seeds are soaked in water, with or without aeration, under optimal temperature conditions, followed by subsequent drying. This initiates germination without the radicle emergence (Taylor et al. 1998). Hydropriming allows the seeds to quickly reach a high level of moisture with a constant supply of O_2, thus increasing the level of metabolites and enzymes associated with the germination process; in turn, this stimulates a uniform

seed germination (Paparella et al. 2015). On the other hand, osmopriming consists of treatments with osmotic solutions (at low water potential) delivered in order to control the water uptake (Bray 1995). Hence, the main advantage of osmopriming is to limit the ROS-mediated oxidative injury by delaying water entry (Michel and Kauffmann 1973; Heydecker and Coolbear 1977). In the case of biopriming, the solution used for priming is integrated with beneficial microorganisms, bioactive molecules, secondary metabolites or phytohormones, and these treatments are designed to improve the antioxidant response (Radhakrishnan et al. 2013). As for thermopriming, as the name indicates, seeds are treated at different temperatures before sowing. This technique was shown to improve germination efficiency under adverse environmental conditions by reducing the thermo-inhibition of seed germination (Huang et al. 2002).

All these procedures have in common the fact that they act at the level of seed transition from dormancy toward full germination, touching processes like the activation of DNA repair and antioxidant mechanisms, essential to obtain seeds with improved quality (Paparella et al. 2015). When considering the antioxidant response, enhanced enzymatic activity or increased expression of genes encoding these antioxidant enzymes (e.g. SOD, APX, CAT, GR), were evidenced during seed germination (Balestrazzi et al. 2011a,c; Lee et al. 2010; Wojtyla et al. 2006; Macovei et al. 2010) and priming treatments (Macovei et al. 2014). Another indicator of the antioxidant response in germinating seeds is the expression of metallothionein (MT) genes encoding different isoforms of a potent ROS scavenger protein (Balestrazzi et al. 2011b), that is indicative for the activation of the antioxidant defense (Macovei et al. 2014).

11.7.2 ROS vs. Seed Longevity

Seed longevity is defined in terms of seed viability after dry storage and it describes the total seed lifespan (Rajjou and Debeaujon 2008). This is also related to the rate at which the germination potential deteriorates over time, hence seed aging. The decrease in seed quality is manifested as a decline in the rapidity and uniformity of germination, where a progressive delay of radicle emergence eventually leads to the loss of seed viability (Waterworth et al. 2015).

Seed longevity varies notably both intra- and inter-specifically, being highly affected by storage conditions, including temperature and humidity (related to seed moisture content). It has been shown that both low temperature and low seed moisture content can prolong seed lifespan during storage (Walters 1998; Groot et al. 2012). Seed longevity is strongly determined by genetic components. For instance, Quantitative Trait Loci (QTLs) for seed longevity were identified in *A. thaliana*, barley, lettuce, oilseed rape, rice, and wheat (Waterworth et al. 2015). These along with other types of "omics" studies are starting to reveal the importance of repair processes and mechanisms that promote seed longevity and safeguard the seed against deterioration (Rajjou et al. 2012). For example, 12 GAAS (germination ability after storage) loci controlling seed longevity after natural aging were identified in *Arabidopsis*. Differently, other proteome analyses revealed that loss of two major SSPs, cruciferins and napins, resulted in a significant decrease in seed longevity (Nguyen et al. 2015). Alongside, this particular study was also identified the role of cruciferins in buffering oxidation during aging.

The seed response to oxidative stress also relies on different environmental factors, as highlighted in a recent study on different *Silene acaulis* ecotypes (Donà et al. 2013).

In this case, upregulation of antioxidant genes (e.g. *SOD*, *MT2*) along with higher ROS-scavenging activity were observed in imbibed seeds of low-altitude varieties in comparison with the high-altitude taxa. Moreover, artificial aging techniques have been used to demonstrate how these different responses to oxidative stress and DNA damage affect seed longevity in these *S. acaulis* ecotypes (Donà et al. 2013).

11.8 Concluding Remarks

Considering the impact that seeds have on both agriculture and environment preservation, and hence both on nature- and anthropologic-related activities, it would be trivial to try to explain why it is so important to study them. In relation to ROS, dual molecules with both negative and positive outcomes, seeds are well studied so far, but these complex associations are not quite yet understood. As we showed in this Q and A session, it is well known that the activation of cellular metabolism and the subsequent production of ROS along with the maintenance of DNA integrity are crucial for a successful seed germination. However, the mechanisms of how the balance between ROS production and ROS scavenging is preserved during seed imbibition, the step of enhanced water uptake before the start of germination, are still somewhat elusive. The implications of hormones, oxidants and antioxidants, passive and active mechanisms of defense against ROS overaccumulation, had been evidenced by many. In addition, ways to exploit ROS for breaking the seed dormancy or promoting a more effective and uniform germination are envisioned as a means of translation from basic to applied research. Further research concentrating on methodologies to detect and potentiate the positive impact of ROS on seed germination while combating the negative effects are still required to propel the transition to more effective and economical applications.

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Chapter 5

Genome editing in the context of seed research: How these novel biotechnology tools can change the future face of agricultural crop development

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1 Introduction

Genome editing is a powerful technique that must be used to accelerate crop improvement. Different from traditional genetic engineering, where genes are inserted randomly into the genome, this set of techniques harnesses site-specific nucleases (SSNs) combined with DNA/RNA recognition domains to insert designated modifications at predefined sites in the genome (Bogdanove and Voytas, 2011; Chen and Gao, 2014; Sun et al., 2016). This has implicit significance for human health, agriculture, and the environment. Thanks to genome editing, it is now possible to accelerate basic research using model organisms for the study of genes of interest and related mutations. By modifying the plant genome through targeted insertions or deletions, it is possible to evaluate how these mutations influence the phenotype (Liu and Moschou, 2018). This allows more in-depth knowledge of gene functions and relative regulatory mechanisms. Moreover, genome editing makes it possible to modify targeted cellular metabolic pathways that allow the development of new varieties of plants of agricultural interest characterized, for instance, by greater tolerance to biotic and abiotic stresses (Baltes and Voytas, 2015; Shih et al., 2016; Li et al., 2018; Sedeek et al., 2019; Debbarma et al., 2019).

So far, the main tools available for genome editing encompass meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat-associated proteins (CRISPR-Cas). Among these, the CRISPR-Cas system is the most used due to its facility for target design (20 nucleotides long single-guide RNAs), high efficiency, and relatively low cost (Ran et al., 2013; Khatodia et al., 2016; Jaganathan et al., 2018; Chen et al., 2019). This is also reflected by the high number of articles published since the discovery of this technique by Jennifer Doudna and Emmanuelle Carpentier (Jinek et al., 2012). For instance, a search carried out on PubMed at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/pubmed, on March 8, 2019) using the keywords "((CRISPR) AND plant) NOT review" identified more than 1100 published papers so far. All the available genome editing tools work in a similar manner by generating double-strand breaks (or nicks) at targeted sites, whereas the repair of the break is performed by the endogenous DNA repair systems (Bogdanove and Voytas, 2011; Zhang et al., 2017; Puchta, 2017; Shan and Voytas, 2018). As many comprehensive reviews were dedicated to explaining the structure, functionality, and use of these tools in plants (Chen and Gao, 2014; Baltes and Voytas, 2015; Khatodia et al., 2016; Malzhan et al., 2017; Baltes et al., 2017; Shan and Voytas, 2018; Chen et al., 2019; Kausch et al., 2019), this chapter mainly focuses on their relevance for seed research. Hence, this work convenes the application of genome editing techniques with regard to seed production (intended as plant productivity or yield), the nutritional and biochemical composition of the seed, and other quality traits that include aspects related to seed vigor, dormancy, and germination.

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2 Seeds: The protagonists of agricultural productivity

Seeds are the main propagating vectors in higher plants, as they guarantee plant reproduction. From an evolutionary point of view, seeds represent the fundamental step in the transition from nonvascular plants to seed plants (Spermatophyta). The first fossil evidence of seed-like morphological structures refers to the Progymnosperm, a monophyletic taxon dated to the late Devonian period, about 370 million years ago. This correlates a taxon of extinct "seed ferns" to the first gymnosperms and to the ancestors of all the modern Spermatophyta (Niklas, 1997; Linkies et al., 2010). Whereas a monophyletic origin for angiosperms and gymnosperms was ascribed, the precise evolutionary connections between the two clades have still to be defined (Linkies et al., 2010).

From seed to seed across their entire lifecycle, plants are unable to physically escape the deleterious fluctuations present in variable environments. Consequently, evolution has endowed plants with a variegated set of mechanisms and strategies that allows them to face and survive stress conditions (Haak et al., 2017). Because adaptation for survival is a major driving force in evolution, the seed itself is regarded as an adaptive trait to enhance plant survival and diffusion under fluctuating environmental conditions through the production of a quiescent and diffusible form able to wait for optimal germination parameters to occur. In strict interconnection with their role in plant reproduction, seeds encapsulate plant versatility and adaptability to different kinds of biotic (e.g., pathogens) and abiotic (e.g., drought, flooding, extreme temperatures) stresses (Haak et al., 2017). Among these, salinity stress and soil contaminants were shown to decrease the protein content due to nitrogen depletion and altered metabolism; drought stress alters the oleic/linoleic acid ratios in legume seeds; and heat stress induces protein depletion and oil content increase along with alterations in fatty acid composition (Farooq et al., 2018). It has been estimated that the average crop yield losses caused by drought, cold, and salinity pass 50% (Mahajan and Tuteja, 2005). Moreover, abiotic stresses are reported to induce notable changes in seed content composition and quality (Farooq et al., 2018). Such considerations have relevant outcomes not only on seed survival, but also on agricultural production and human nutrition.

From an anthropological perspective, seeds are essential for several aspects (e.g., nutrition, biodiversity, maintenance of ecosystem niches, landscape, etc.). Mainly, the nutritional property of seeds is linked to major alimentary resources for all the populations across different cultures, with cereals, fruits, vegetables, and oilseed crops representing the big portions of the seed market. The relevance of seeds is reflected by the increase in the global seed market (from $30 billion in 1996 to $36 billion in 2007), and this trend was mainly observed in emerging economies and developing countries (FAO, n.d.). Moreover, while facing the food demand of a growing population, the global seed industry was valued at more than $54 billion in 2016 and constant growth is predicted for the next few years (2017–2023) (Allied Market Research, n.d.).

In this context, seed industries, breeders, and biotechnologists have identified the main features of interest to optimize the quality of a seed lot at many steps of the production process, including storage, transporting, sowing, plant development, and seed maturation. Thus, seed quality is defined and quantified by taking into account a set of measurable parameters such as germination speed and rates, the long-term and after-storage viability, the homogeneity and purity of the seed lot, resistance to mechanical damage, and the absence of biological and chemical contaminants (Finch-Savage and Bassel, 2016). Many of these characteristics are defined and can be improved according to the requirements of the seed industry and market. Nonetheless, plant and human interests converge in regard to the capacity of a seed to produce a healthy and resistant seedling able to withstand biotic and abiotic stresses, thus ensuring plant reproduction along with abundant harvests even under variable environmental parameters, seasonal cycles, and climate changes. Many traits in the genetic background of each seed lot contribute to these properties that are collectively referred to as "seed vigor" (Finch-Savage and Bassel, 2016), broadly defined as "the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments" (ISTA, 2018).

Seed quality and vigor are variable not only intrinsically as well as among different crop species, cultivars, and seed lots, but also in response to the different cultivation conditions and schedules. Maturation and germination timings are important limiting factors. Indeed, most crop seeds reach their optimal vigor at their physiological maturity, which can be difficult to synchronize, while strict temporal windows are often required for harvesting, sowing, germination, and growing. Furthermore, as a general tendency, field cultivation leads to poorer and more variable germination performances than laboratory-controlled conditions, making standardization even more difficult (Finch-Savage and Bassel, 2016).

The improvement of seed quality and vigor is a challenge that has been addressed since ancient times. The notion entails that seed germination can be enhanced through specific treatments, and the first attempts in this sense are historically attested throughout time, from Theophrastus (371–287 BCE), Gaius Plinius Secundus (23–79 CE), Oliver de Serres (1539–1619), up to Charles Darwin (1809–1882). Since the first effective approaches to be discovered, a common element was a water-soaking step before sowing while more recent protocols also implemented solutions containing salts or osmotic agents (Everari, 1984; Paparella et al., 2015). The development of techniques to improve the germination rate, speed,

consistency, and viability under stress conditions evolved in the modern concept of "seed priming," broadly defined as "any treatment that improves seed quality" (Osburn and Schroth, 1989; Paparella et al., 2015). Although different priming protocols have been devised according to the characteristics and requirements of various species of interest, certain common elements can be identified.

3 Crop yield: A complex trait that can be undertaken with the use of genome editing

Crop yield and quality are quantitative traits generally controlled by multiple genes localized in specific quantitative trait loci (QTLs). Within this context, seeds are considered among the main vectors conditioning crop yield. Traditional breeding, the original method used to improve yield, relies on the production of various QTL combinations and subsequent selection of the most favorable breeds (Zuo and Li, 2014; Shen et al., 2018), but it is quite a time-consuming process. Hence, crop yield improvements need to be quickened in order to avoid an eventual food insecurity situation. To do so, genetically modified (GM) crops represent a solid alternative, already proven to be effective when considering the much-studied examples related to the production of herbicide- and insect-resistant crops (Brookes and Barfoot, 2017; Paul et al., 2018).

With the advent of genome editing techniques, researchers have further plunged into finding more rapid and easier ways to address these complex traits. Some examples of crops with improved yields developed mainly through the use of the CRISPR/Cas9 system are given in Table 1 and discussed here. The considered traits span seed size and number to plant productivity and heterosis. Starting from the last, heterosis (also known as hybrid vigor) has been extensively applied in agriculture to improve plant productivity, although hybrid seed production is quite difficult and expensive for many crops (Schnable and Springer, 2013). Manipulating heterosis was achieved by synthetic apomixes, defined as an asexual reproductive strategy in which offspring are generated through seeds in the absence of meiosis or fertilization (Spillane et al., 2004). A study published this year reported that targeted mutations in multiple genes played roles in meiosis to produce hybrid rice (*Oryza sativa*) lines with increased heterosis (Wang et al., 2019). Here, a multiplex CRISPR/Cas9 vector was

TABLE 1 Examples of genome editing used to improve crop yield.

Trait	Gene	Species	Tool	References
Seed size	*GW2*	*O. sativa* *T. aestivum*	CRISPR/Cas9	Xu et al. (2016) Wang et al. (2018a,b)
	GW5	*O. sativa*		Xu et al. (2016)
	TGW6	*O. sativa*		Xu et al. (2016)
	RGG2	*O. sativa*		Miao et al. (2019)
	gs3	*O. sativa*		Li et al. (2016)
	GASR7	*T. aestivum*		Zhang et al. (2016)
Seed number	*Gn1a*	*O. sativa*	CRISPR/Cas9	Li et al. (2016)
	ARGOS8	*Z. mays*		Shi et al. (2017)
	CLVTA3	*B. napus*		Yang et al. (2018)
Plant architecture and productivity	*WUS*	*S. lycopersicum*	CRISPR/Cas9	Rodríguez-Leal et al. (2017)
	SP	*S. lycopersicum*		Rodríguez-Leal et al. (2017)
	SFT	*S. lycopersicum*		Rodríguez-Leal et al. (2017)
	DEP1	*O. sativa*		Li et al. (2016)
	IPA1	*O. sativa*		Li et al. (2016)
Heterosis	*REC8*	*O. sativa*	CRISPR/Cas9	Wang et al. (2019)
	PAIR1	*O. sativa*		Wang et al. (2019)
	OSD1	*O. sativa*		Wang et al. (2019)
	MTL	*O. sativa*		Wang et al. (2019)

TABLE 2 Examples of genome editing used to improve seed nutritional content.

Trait	Gene	Species	Tool	References
Seed oil content	*FAD2*	*O. sativa*	CRISPR/Cas9	Abe et al. (2018)
		A. hypogeaea	TALEN	Wen et al. (2018)
		C. sativa	CRISPR/Cas9	Jiang et al. (2017) and Morineau et al. (2017)
		G. max	TALEN	Haun et al. (2014) and Demorest et al. (2016)
	DGAT1	*C. sativa*	CRISPR/Cas9	Aznar-Moreno and Durrett (2017)
	FAE1	*C. sativa*	CRISPR/Cas9	Ozseyhan et al. (2018)
Carotenoids	*CYP97A4* *DSM2* *CCD4a* *OsCCD4b* *CCD7*	*O. sativa*	CRISPR/Cas9	Yang et al. (2017)
	SGR1 *LCY-E* *Blc* *LCY-B1* *LCY-B2*	*S. lycopersicum*	CRISPR/Cas9	Li et al. (2018)
Fragrance	*BADH2*	*O. sativa*	TALEN CRISPR/Cas9	Shan et al. (2015) Shao et al. (2017)
Amylose content	*SBEIIb* *SBEI*	*O. sativa*	CRISPR/Cas9 nCas9-PBE	Sun et al. (2017) Li et al. (2017)
	Waxy	*O. sativa* *Z. mays*	CRISPR/Cas9	Zhang et al. (2018) Waltz (2016)
	GBSS	*S. tuberosum*	CRISPR/Cas9	Andersson et al. (2017)
Gluten	*α-Glandin*	*T. aestivum*	CRISPR/Cas9	Sánchez-León et al. (2018)
Cadmium	*NRAMP5*	*O. sativa*	CRISPR/Cpf1	Tang et al. (2017)
Acrylamide	*Vlnv*	*S. tuberosum*	TALEN	Clasen et al. (2016)
Phytic acid	*IPK*	*Z. mays*	ZFN TALEN CRISPR/Cas9	Shukla et al. (2009) Liang et al. (2014)
	PAPhy	*H. vulgare*	TALEN CRISPR/Cas9	Holme et al. (2017)

Brassicaceae family, for its short growing season and high productivity in diverse regions. *C. sativa* was engineered through the use of the CRISPR/Cas9 system by targeting several genes involved in the fatty acid biosynthetic pathway. For instance, aside from *FAD2* (Jiang et al., 2017; Morineau et al., 2017), also the *FAE1* (fatty acid elongase) and *DGAT1* (diacylglycerol acyltransferase) genes were targeted to produce plants with altered seed oil composition (Ozseyhan et al., 2018; Aznar-Moreno and Durrett, 2017). Moreover, ample field trials were also conducted to assess the productivity and safety of the generated lines while the authors strongly argue against the GM regulation of genome-edited crops in Europe (Faure and Napier, 2018).

Prodigious work was carried out to enhance the lycopene (a carotenoid synthesized during fruit ripening and considered a bioactive component responsible for lowering the risk of cancer and cardiovascular diseases) content in tomatoes (Li et al., 2018). The authors used multiplex genome editing dedicated to boosting lycopene production by hindering the carotenoid biosynthetic pathway. One CRISPR/Cas9 vector was used to induce targeted mutations in five genes, namely *SGR1* (stay-green 1), *Blc* (β-lycopene cyclase), *LCY-E* (lycopene ε-cyclase), *LCY-B1* (lycopene β-cyclase 1), and *LCY-B2*, that resulted in the loss of gene function. This strategy allowed specifically increasing by 5.1-fold the lycopene content in the

fruits (Li et al., 2018). Another attempt to engineer the carotenoid pathway was performed in rice grains to enhance the β-carotene content (Yang et al., 2017). Here, five other carotenoid catabolic genes were singularly targeted for the knockout, namely two carotene hydrolases (*OsCYP97A4*, *OsDSM2*) and three carotene dioxygenases (*OsCCD4a, OsCCD4b, OsCCD7*). Several types of mutated lines were identified and characterized, but these did not accumulate more carotenoids in rice grains in either the mono- or biallelic mutants obtained by targeting the five genes. On the other hand, homozygous rice plants for mutations in the *OsCCD7* gene presented a dwarf phenotype with an enhanced tiller number, hence increased seed productivity (Yang et al., 2017).

Rice is often targeted to improve nutritional aspects mainly because it is a staple food for more than half the worlds' population while also being poor in nutrient import. For this, multiple biofortification programs, using both traditional breeding and genetic engineering approaches, are being conducted to enhance the vitamin and mineral content of rice (Slamet-Loedin et al., 2015; Trijatmiko et al., 2016; Singh et al., 2017; Descalsota et al., 2018). Additionally, genome editing tools, and precisely CRISPR/Cpf1, were used to lower the concentration of damaging minerals such as cadmium (Tang et al., 2017). This was accomplished by knocking out the *NRAMP5* (natural resistance-associated macrophage protein5) metal transporter, and the resulting lines grown under field conditions showed a significantly lower Cd concentration (less than 0.05 mg/kg) in the grains, compared to wild-type lines (0.33–2.90 mg/kg). Genome editing techniques were also used to enhance rice flavor by targeting the *BADH2* (betaine aldehyde dehydrogenase) gene, encoding for an enzyme that obstructs the synthesis of 2-acetyl-1-pyrroline (2AP) responsible for the dominant rice fragrance (Shan et al., 2015; Shao et al., 2017). A more complex trait engineered in rice through the use of genome editing relates to the production of high-amylose lines (Sun et al., 2017). This was addressed because rice grains with high amylose content are considered better sources of resistant starch and this was linked with a decreased incidence of gastrointestinal and cardiovascular disorders (Vonk et al., 2000). Hence, CRISPR/Cas9 was used to generate targeted mutagenesis in genes coding for starch branching enzymes (*SBEI* and *SBEIIb*, respectively), that catalyzes the cleavage of the α-1,4-linked glucan chain to produce branches in amylopectin (Syahariza et al., 2013). This strategy led to obtaining rice lines with an increased proportion of amylose and resistant starch only when the *SBEIIb* gene was silenced (Sun et al., 2017). In addition, in another study, the same gene was targeted using a deactivated Cas9 (nCas9-PBE) fused with a cytidine deaminase enzyme to generated specific cytidine (C) to thymine (T) base editing (point mutations) without inducing any cuts in the genome (Li et al., 2017). Another gene targeted for the same trait in rice is *Waxy*, encoding for a granule-bound starch synthase (GBSS) responsible for the synthesis of amylose specifically in the endosperm. In this case, the loss-of-function mutants presented reduced amylose, leading to the generation of low-glutinous rice without yield penalties (Zhang et al., 2018). A similar approach was also applied for maize lines developed by DuPont Pioneer (Waltz, 2016) whereas in the potato, all four *GBSS* alleles were silenced to produce tubers without amylose-containing starch (Andersson et al., 2017).

Aside from enhancing certain nutritional contents, other approaches were focused on lowering the amount of antinutritional elements, such as phytic acid or acrylamide. The development of low phytic acid maize seeds was among the first traits targeted with the use of genome editing tools. These products have a dual purpose because the *IPK1* (inositol-1,3,4,5,6-pentakisphosphate 2-kinase, involved in the final step of phytate biosynthesis) gene was disrupted while inserting within its open reading frame another gene (*PAT*, phosphinothricin *N*-acetyltransferase) that confers resistance to glufosinate herbicides (Shukla et al., 2009; Liang et al., 2014). In another study, Holme et al. (2017) used both TALEN and CRISPR/Cas9 to induce mutations in the promoter region of the barley (*Hordeum vulgare*) phytase gene (*PAPhy*, the main contributor to the phytase metabolism in mature grains). In this case, the developed lines with very low phytate content were also associated with a significant delay in germination. Acrylamide, a potential carcinogen formed as a byproduct of the Maillard reaction, is another example of antinutritional qualities in processed (mainly heated) foods. To decrease the sugar content (the main substrate for the Maillard reaction) in potato (*Solanum tuberosum*) tubers, Clasen et al. (2016) used TALEN to knock out the *VInv* (vacuolar invertase) gene, responsible for the breakdown of sucrose into glucose and fructose, and succeeded in producing acrylamide-free potatoes. An interesting application of genome editing for nutritional purposes was related to the development of low-gluten wheat, designated for patients suffering from coeliac disease, an autoimmune disorder triggered by the ingestion of gluten. In this case, the *α-gliadin* gene was targeted by CRISPR/Cas9 and the produced lines had a decrease of up to 85% in the gluten content (Sánchez-León et al., 2018).

5 Tackling seed physiology and development through genome editing applications

Due to their importance for both natural biodiversity and agricultural purposes, seed development and related features are being targeted to optimize their quality at several levels spanning the production process from storage to seed maturation to germination. However, because many basic molecular aspects related to seed quality are still meagerly understood (Macovei et al., 2017), engineering such complex traits is still a big challenge. Nonetheless, some studies where genome

editing approaches were used to develop better products or to understand basic molecular functions were recently published (Table 3). Among the targeted traits, hybrid seed production has intrinsic value due to its relation to heterosis vigor, hence substantial yield enhancement. For this, approaches that lead to male pollen sterility are used to develop hybrid seed (Wu et al., 2016). CRISPR/Cas9 was used to target genes involved in pollen development to generate male sterile lines in two important cereal crops, maize and bread wheat (Chen et al., 2018; Singh et al., 2018). In the first case, the *MS8* (male sterility 8) gene, encoding a β-1,3-galactosyltransferase enzyme involved in anther development (Chen et al., 2018), was targeted while in the second case, *Ms45* (male sterile 45), coding for a strictosidine synthase-like enzyme, was investigated (Singh et al., 2018). Because wheat is a hexaploidy crop, all three gene homologs had to be knocked out, and only the triple mutants were able to achieve the desired trait. On the other hand, rice continues to remain a species often used in genome editing approaches related to seed development. Among the most known examples are the SWEET sugar transporters that have roles both in grain filling (Ma et al., 2017) and bacterial blight disease resistance (Zhou et al., 2015). In this case, however, silencing of the SWEET11 gene resulted in deficient rice grain filling due to a decline of sucrose release from the maternal tissue (Ma et al., 2017). A base editing approach through the CRISPR/Cas9-APOBEC1 cytidine deaminase was used to create point mutations in the rice *SLR1* gene, encoding a DELLA protein with known roles in seed germination and plant development (Lu and Zhu, 2017). Differently, a multiplex CRISPR/Cas 9 was used to target the *Hd2*, *Hd4*, and *Hd5* genes that negatively affect the heading date in rice (Li et al., 2017), resulting in the development of lines with shortened heading dates.

Seed preservation during processing and storage is a key trait to promote sustainable agricultural practices without additional economic losses. However, the molecular characterization of seed preservation-related traits is still ongoing. Nevertheless, two studies were conducted to target genes that resulted in reduced seed shattering during harvest and enhanced resistance during storage in rapeseed (*B. napus*) and rice, respectively (Ma et al., 2015; Braatz et al., 2017). In the first case, a CRISPR-Cas9 construct was designed to target the two *ALC* (ALCATRAZ) alleles in rapeseed plants. This gene, known to be involved in seed valve margin development, is responsible for seed shattering from mature fruits; hence, by silencing the *ALC* alleles, an enhanced shatter resistance trait was developed, which permitted averting seed loss during mechanical harvest (Braatz et al., 2017). In another study, TALEN constructs were used to target the *LOX3* (lipoxygenase 3, involved in the deoxygenation of polyunsaturated fatty acids) gene in rice, leading to the development of lines showing improved seed storability (Ma et al., 2015).

Plant phytohormones have multiple roles in plant growth and development. Among them, abscisic acid (ABA) and gibberellins (GAs) are involved in maintaining the equilibrium between seed dormancy and germination (Rodríguez-Gacio et al., 2009). Within this context, the *NCED4* (9-cis-epoxycarotenoid dioxygenase 4) gene encodes a key enzyme in the ABA biosynthesis (Huo et al., 2013). CRISPR/Cas9 targeting the *NCED4* gene in lettuce (*Lactuca sativa*) resulted in the development of lines with an enhanced range of temperatures that conditions seed germination. This study showed that more than 70% of the seeds were able to germinate at temperatures up to 37°C, when the usual germination temperature in

TABLE 3 Examples of genome editing used to address seed physiology and development.

Trait	Gene	Species	Tool	References
Male sterility	*MS8*	*Z. mays*	CRISPR/Cas9	Chen et al. (2018)
	Ms45	*T. aestivum*	CRISPR/Cas9	Singh et al. (2018)
Seed development	*SWEET11*	*O. sativa*	CRISPR/Cas9	Zhou et al. (2015) and Ma et al. (2017)
	SLR1	*O. sativa*	CRISPR/Cas9-APOBEC1	Lu and Zhu (2017)
	Hd2,4,5	*O. sativa*	CRISPR/Cas9	Li et al. (2017)
Seed storability and processing	*LOX*	*O. sativa*	TALEN	Ma et al. (2015)
	ALC	*B. napus*	CRISPR/Cas9	Braatz et al. (2017)
Seed germination and dormancy	*NCED4*	*L. sativa*	CRISPR/Cas9	Bertier et al. (2018)
	PYL1–13	*O. sativa*	CRISPR/Cas9	Miao et al. (2018)
Seedling development	*CDF*	*A. thaliana*	CRISPR/Cas9	Zhao et al. (2016)

this particular case of lettuce ranged between 15°C and 25°C (Bertier et al., 2018). Still, related to ABA biosynthesis, the pyrabactin resistance 1 (PYR1)/PYR1-like (PYL) genes encode for regulatory components of the ABA receptor (RCAR) family of proteins, involved in ABA sensing. CRISPR/Cas9 editing of the *PYL1-13* genes in rice resulted in the generation of multiple lines with modified plant growth and seed dormancy (Miao et al., 2018). However, within the obtained mutations in single lines, the *pyl1* and *pyl12* mutants presented significant defects in seed dormancy whereas the *pyl1/4/6* triple mutant lines maintained normal seed dormancy and showed improved plant growth and grain productivity under field conditions. When addressing seedling development, a transcription factor known to regulate the cold acclimation in *Arabidopsis thaliana* was shown to be involved in other aspects of the plant's life. This is the case of the C-repeat binding factors (CBF1, CBF2, and CBF3), coding proteins that are involved in the regulation of the lipid and carbohydrate metabolisms, gene transcription, and cell wall modification. When all three genes were targeted by a CRISPR/Cas9 construct, the *cbf* triple mutants were defective in seedling development and tolerance to salt stress (Zhao et al., 2016). Hence, to produce improved lines, a knock-in or transcription activation approach should be used instead of gene knockout.

6 Computational analysis and technological advancements

Due to the site-specificity of custom-designed nucleases, computational tools are required to identify the genomic locations of highest interest. To the greatest extent, this is the case of the CRISPR/Cas system, where the in silico design of sgRNA is a decisive step for the success of planned experiments. Hence, extended efforts are directed to refine the in silico sgRNA design in order to have high on-target efficacy and reduced off-target effects (Khatodia et al., 2016; Bradford and Perrin, 2018; Adli, 2018). So far, many sgRNA design tools are at hand (e.g., CRISPRdirect, CasFinder, CRISPR-P, CRISPR-Target, E-CRISP, CHOPCHOP, CRISPRscan), although scrupulous estimation of their applicability and performance are required to enhance the precision of these valuable techniques (Chuai et al., 2017). Moreover, even specific tools dedicated for plant CRISPR sgRNA analysis, namely CRISPR-PLANT (Xie et al., 2014), were developed and are being constantly updated (CRISPR-PLANT v2, Minkenberg et al., 2019). In addition to in silico analyses, high-throughput sequencing (NGS) methods are being used for screening genome-edited lines, leading to a startling amount of big data accumulation (Shalem et al., 2015). As a consequence, many bioinformatics tools (e.g., GenomeCRISPR, CRISPRcloud, CRISPResso, CRISPR-GA) have been developed to analyze the CRISPR-generated NGS data (Guell et al., 2014; Pinello et al., 2016; Rauscher et al., 2017; Jeong et al., 2017).

Likewise, the continuous technological improvements of custom-designed nucleases, ranging from cloning and vector design (Čermák et al., 2015, 2017) to the so far bountiful variety of applications, represent an endless source of beneficial outcomes for the genome editing revolution (Wolter and Puchta, 2018; Chen et al., 2019). Specifically, the use of the CRISPR/Cas system has expanded from the usual gene knockout or gene replacement to transcriptional regulation (deactivated dCas9), epigenome editing (dCas9 fused with effectors that can modulate DNA methylation), base editing (dCas9 used with cytidine deaminases), RNA editing (Cas13, c2c2 systems), and even imaging techniques (dCas fused to fluorescent molecules) (Dreissig et al., 2017; Malzhan et al., 2017; Wolter and Puchta, 2018).

All these, along with future advancements of these technologies, can be put to good use to perpetually improve the plant agricultural system and develop the crops of the future, following sustainable seed-to-seed applications.

7 Conclusions and future perspectives

In conclusion, this chapter emphasizes the implementation of genome editing tools to improve seed quality traits, covering aspects spanning from seed productivity and nutritional content to seed germination and dormancy. Further advances in both technical development and basal research will bring to light more applications of these amazing tools to expand the number of targeted traits and frame the future of sustainable agriculture.

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