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Calcium redistribution contributes to the hard-to-cook phenotype and increases PHA-L lectin thermal stability in common bean *low phytic acid 1* mutant seeds

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ABSTRACT

Seed phytic acid reduces mineral bioavailability by chelating minerals. Consumption of common bean seeds with the *low phytic acid 1* (*lpa1*) mutation improved iron status in human trials but caused adverse gastrointestinal effects, presumably due to increased stability of lectin phytohemagglutinin L (PHA-L) compared to the wild type (wt). A hard-to-cook (HTC) defect observed in *lpa1* seeds intensified this problem. We quantified the HTC phenotype of *lpa1* common beans with three genetic backgrounds. The HTC phenotype in the *lpa1* black bean line correlated with the redistribution of calcium particularly in the cell walls, providing support for the "phytase-phytate-pectin" theory of the HTC mechanism. Furthermore, the excess of free cations in the *lpa1* mutation in combination with different PHA alleles affected the stability of PHA-L lectin.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) seeds are a good source of energy, as they are rich in proteins and carbohydrates, making them highly valuable nutritionally. They are also a good source of minerals and vitamins (such as iron, zinc, B-vitamin) as well as bioactive compounds, such as polyphenols (Cominelli, Rodiño, De Ron, & Sparvoli, 2019). Different studies have shown that including common beans in the diet is linked to reduced risks of obesity, diabetes, cardiovascular diseases, and some cancers (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014). Nevertheless, the presence of some antinutritional compounds, such as phytic acid (PA), which decreases mineral bioavailability (Petry, Egli, Campion, Nielsen, & Hurrell, 2013), can limit the nutritional value of common beans.

Phytic acid (PA, *myo*-inositol hexa*kis*phosphate) is the main form of storage of seed phosphorus, accounting for an average of 75% total phosphorus. Moreover, in vegetative tissues, PA has an important role in responses to environmental stress and hormonal changes (Sparvoli & Cominelli, 2015). PA is stored in specialized vacuoles, protein storage vacuoles, forming spherical inclusions called globoids (Lin, Ockenden, & Lott, 2005; Pilu, Landoni, Cassani, Doria, & Nielsen, 2005). Typically, it is present as phytate salts of mono- and di-valent cations, in particular zinc (Zn^{2+}), calcium (Ca^{2+}), and iron (Fe^{2+}), which are bound tightly by negatively charged phosphate groups. For this reason, PA reduces mineral bioavailability strongly, contributing to "hidden hunger" (Murgia, Arosio, Tarantino, & Soave, 2012).

Lectins are another antinutritional compound present in beans. Lectin phytohemagglutinin (PHA) is the second most abundant seed storage protein after phaseolin (Phsl), a 7S globulin. PHA consists of two types of polypeptide chains, E and L subunits (PHA-E and PHA-L,

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respectively), which preferentially bind to erythrocytes and leukocytes, respectively, and are responsible for the erythroagglutinating and leukoagglutinating activities of beans' PHAs (Fig. S1). PHA is a tetramer of about 120 kDa, in which E and L subunits are assembled randomly in five possible combinations: L_4, L_3E, L_2E_2, LE_3, E_4 (Felsted, Leavitt, Chen, Bachur, & Dale, 1981).

Bean lectins are antinutritional compounds because, if not properly inactivated during processing, they can exert toxic effects on the digestive tract, leading to vomiting, diarrhoea, bloating and nausea, which interfere with nutrient absorption, decreasing their nutritional value (Vasconcelos & Oliveira, 2004). Many plant lectins, including bean PHA, have been found to be resistant to degradation by proteases *in vitro* and in the gastro-intestinal tract (GI-tract) (Lajolo & Genovese, 2002; Morari, Stepurina, & Rotari, 2008). Partially undigested PHA may bind to a wide variety of cell membranes glycoconjugates in the intestinal mucosa and cause dramatic changes in cellular morphology and GI-tract metabolism (Bardocz, Grant, & Pusztai, 1996).

A range of efforts have been applied to improve the nutritional quality of bean seeds through selection of varieties with low or specific types of storage proteins (lectins, phaseolin) (Confalonieri, Bollini, Berardo, Vitale, & Allavena, 1992; Pandurangan et al., 2016) as well as *lpa* mutants (Campion et al., 2009; Cominelli et al., 2018).

The common bean *lpa1* mutant, originally isolated from an ethyl methanesulfonate-mutagenized population, affects the PvMRP1 PA transporter (Panzeri et al., 2011). It is also characterized by a 25% reduction in raffinosaccharides content, a 30% reduction in *myo*-inositol, and a 7-fold increase in free Fe cations (Campion et al., 2009; Panzeri et al., 2011). Nevertheless, *lpa1* plants do not exhibit negative pleiotropic effects, described in mutants affected by the orthologous gene (Sparvoli and Cominelli, 2015), due to the presence of the *PvMRP2* paralog gene, which is able to complement *PvMRP1* function in organs other than the seed (Cominelli et al., 2018). Lines harbouring the *lpa1* mutation were used for a stable-Fe-isotope absorption study in Swiss women that showed markedly improved Fe bioavailability (6.1%) from *lpa1* beans compared to the parent beans (3.8%) (Petry et al., 2013). An allelic *lpa1* mutant was recently isolated, characterization of which is still on-going (Cominelli et al., 2018).

In some cases, the nutritional effects are not dependent on the content of specific compounds but may be linked more closely to storage conditions. This is the case for the so-called hard-to-cook (HTC) defect, which arises when bean seeds are stored for long periods at high temperatures (≥ 25 °C) and high relative humidity ($\geq 65\%$). The HTC phenomenon consists of a progressive hardening of beans during storage, which means that the cotyledons do not soften during cooking. This leads to extended cooking times, greater costs associated with cooking and a reduction in acceptability by consumers (Coelho, Prudencio, Christ, & Sampaio, 2011; Yi et al., 2016).

Several hypotheses have been proposed to explain the HTC defect in legumes, but the most widely accepted hypothesis is the "phytase-phytate-pectin" theory, which suggests storage at high temperature and high relative humidity increases phytase activity, leading to phytate dephosphorylation and reduced capacity to chelate divalent cations (Ca^{2+}, Fe^{2+}, Zn^{2+}, and Mg^{2+}) (Galiotou-Panayotou, Kyriakidis, & Margaris, 2008). These divalent cations, especially Ca^{2+}, eventually migrate to the cell-wall-middle lamella of parenchyma cells where they bind to carboxyl groups in pectin, forming insoluble pectates, thus hardening the cell walls (Kruger, Minnis-Ndimba, Mtshali, & Minnaar, 2015). The involvement of PA in the HTC defect is supported by observations of a negative correlation between phytate contents and cooking times: seeds with long cooking times have low PA content, implying that most of it has been hydrolysed, according to the "phytase-phytate-pectin" theory (Galiotou-Panayotou et al., 2008). Besides being a deterrent to bean consumption, the HTC defect also lowers nutritional quality. Beans with the HTC defect have decreased protein digestibility *in vitro* (Nyakuni et al., 2008).

Interaction of PA with proteins and carbohydrates has been proposed in the development of the HTC defect. Bean storage proteins are thought to have a role in water absorption and increased cooking time of HTC beans (Nyakuni et al., 2008). It has been suggested that high storage temperatures may induce protein denaturation, followed by coagulation, reducing their solubility. Furthermore, the coagulated proteins may form a physical barrier preventing water absorption during cooking (Coelho et al., 2011).

In a recent study, Petry and colleagues (2016) provided *lpa1* common beans, with only the PHA-L isoform at the APA locus, to Rwandese women with low Fe status in a composite double-meal design study, also including biofortified high Fe (BBs) and control beans. However, no statistical differences in Fe bioavailability from the *lpa1* beans and BBs, either fractional Fe absorption or total Fe absorbed, were observed. However, the *lpa1* beans exhibited the HTC phenotype, and their consumption caused GI-tract symptoms in the majority of participants. It was hypothesized that the problems arose from the presence of PHA-L residues, which were poorly hydrolysed during cooking, possibly because cooking temperatures did not exceed 95 °C, due to the high altitude of the site (Petry et al., 2016). The conclusion of this study was that the *lpa1* bean line needs to be improved to achieve comparable cooking quality and eliminate side effects and intolerability/toxicity.

We aimed to determine the cooking times of *lpa1* mutants with different genetic backgrounds (black PHA-L, cream PHA-E, cranberry PHA-E, L) to understand more about the mechanisms involved in the expression of the HTC phenotype, and factors affecting PHA-L and PHA-E stability. We hypothesised that, in *lpa1* seed tissues, there is an excess of free Ca^{2+} due to reduced PA levels. This free Ca^{2+} chelates to pectin in cell walls forming insoluble pectates, which leads to cell wall hardening and manifests as the HTC phenotype. Furthermore, free Ca^{2+} increases the stability of PHA-L.

2. Materials and methods

2.1. Plant materials

The common bean (*Phaseolus vulgaris* L.) genotypes used in this study were:

- Black-seeded 905 line (wt-B), containing only PHA-L at the APA locus (hereinafter PHA-L allele) from a common bean breeding population (Campion et al., 2009);
- Cream-seeded Mesoamerican BAT 93 genotype (wt-C), containing only PHA-E and α-AI (PHA-E allele), kindly provided by the International Center for Tropical Agriculture (CIAT, Cali, Colombia);
- Borlotto (cranberry type) variety Mercato (Blumen; wt-Cr), containing both PHA-E and PHA-L and α-AI (PHA-E,L allele);
- Black-seeded *lpa*-280-10 mutant (*lpa*-B), with the PHA-L allele, isolated through mutagenesis of the 905 genotype (Campion et al., 2009), affecting the *PvMRP1* gene (Panzeri et al., 2011);
- Cream-seeded *lpa1* line (*lpa*-C), with the PHA-E allele, derived from the introgression of the original *lpa*-280-10 into the BAT 93 genotype (Cominelli et al., 2018);
- Borlotto (cranberry type) BC_2F_3 line (*lpa*-Cr), containing both PHA-E and PHA-L and α-AI, derived from the introgression of the original *lpa*-280-10 mutant with the Mercato variety (Blumen), using the CAPS molecular marker previously described (Cominelli et al., 2018) (data not shown).

The main characteristics of these genetic materials are summarized in Supplemental Table S1.

2.2. Cooking time measurements

Seeds were moisture equilibrated to approximately 10% by storing them at 4 °C and 75% relative humidity for two weeks prior to cooking.

A sample of 25 seeds was soaked for 12 h in distilled water at room temperature. A Mattson pin drop cooker (Michigan State University Department of Physics and Astronomy Machine Shop) was used to measure cooking times of soaked seeds (Wang & Daun, 2005). The base plate of the cooker contains 25 wells and each well holds an individual bean seed. A 70 g piercing rod rests on the centre of each bean. The cooker containing 25 seeds from a single sample was placed in a metal beaker with boiling distilled water set on a hot plate. Individual beans were considered cooked when the piercing rod had passed through a seed. A sample's cooking time was recorded when 80% of the pins pierced the beans. Two technical replicates were evaluated for each sample.

2.3. Sample preparation for biochemical analyses

Samples were divided into a number of falcon tubes (15 ml) according to the different cooking time they were submitted to. Each tube contained 4 seeds and 10 ml of soaking solution.

Experiment 1. Each sample was soaked in distilled water for 7 h at room temperature. During soaking, seeds were weighed at one hour intervals. Then, for each genotype a sample was removed (time 0) while the others were cooked for different times (1, 2.5 and 5 h) at two different cooking temperatures: 100 °C, considered a normal condition (high temperature, HT) or 95 °C considered a sub-optimal condition (low temperature, LT) in order to reproduce the working conditions of Petry et al. (2016).

Experiment 2. Samples of genotypes containing only PHA-L, both *lpa1* and wt, were soaked for 4 h at room temperature and cooked at 95 °C for different times, as in Experiment 1, in distilled water or solutions with different concentrations of $CaCl_2$ (10 mM, 35 mM, 50 mM) or EDTA (20 mM, 55 mM, 100 mM).

At the end of the cooking period, or imbibition only, seeds from each sample were lyophilized at −40 °C, 400 Pa for 16 h.

2.4. Protein extraction, SDS-PAGE and immunoblot analyses

Lyophilized seeds were ground in a mortar until flour was obtained. Then, 50 mg of flour for each sample were extracted with 20 volumes of 20 mM NaBorate solution, pH 9. Samples were vortexed and shaken at room temperature for 2 h, then centrifuged for 5 min at 16,200 × g and the supernatant was stored at −20 °C until use. Equal amounts of extracts of each sample were analysed and total proteins were separated by electrophoresis on a 15% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, Missouri, USA) or blotted on a supported nitrocellulose membrane (Hybond-C, GE-Healthcare, Chicago, Illinois, USA). Immunoblot analysis was performed using rabbit antibodies raised against common bean PHA (Vitale, Ceriotti, & Bollini, 1985) at 1:1000 dilution. Goat IRDye680 Anti-Rabbit IgG (LI-COR Biosciences Lincoln, Nebraska, USA) was used as the secondary antibody.

2.5. Histological analysis of globoids

For histological analysis ten wt-B, wt-C, *lpa*-B and *lpa*-C seeds were collected 18 and 24 days after flowering (DAF) and fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol). Paraffin sections were prepared and stained with toluidine blue O, as described by Pilu et al. (2005).

2.6. Concentration and localisation of Ca in common bean seeds

For bulk analysis, the wt-B, wt-C, *lpa*-B and *lpa*-C seeds were soaked in bidistilled water overnight at 4 °C. Soaking for shorter times prevented optimal sample preparation. The seed coat was separated from cotyledons and intact seeds, seed coats and cotyledons were frozen in liquid nitrogen and freeze dried for three days at −25 °C and 0.112 mbar (Alpha, Christ, Osterode, Germany). All material was ground separately in an agate mortar, pressed into pellets and analysed using ED-XRF as described by Nečemer et al. (2008). The measurements were performed by a table-top X-ray fluorescence spectrometer PEDUZO T02 (Jožef Stefan Institute, Slovenia) with Rh-anode X-ray tube. X-ray fluorescence was detected by a silicon drift diode detector (Amptek, Inc., Bedford, Massachusetts, USA). The energy resolution of the spectrometer at count rates below 1000 cps was 140 eV at 5.9 keV. X-ray fluorescence analysis was performed in air and the samples were irradiated for 1000–5000 s to ensure sufficient statistics. The spectra were analysed by custom-developed software for XRF quantitative analysis operating in LabVIEW software (National Instruments, Austin, Texas, USA). The element analysis was validated using standard reference materials: NIST SRM 1573a (tomato leaves), and CRM 129 (hay), both in the form of pressed pellets. For localisation analyses, the wt-B, wt-C, *lpa*-B and *lpa*-C seeds were soaked in bidistilled water overnight at 4 °C, then frozen-hydrated cross-sections were prepared as described previously (Vogel-Mikuš, Pongrac, & Pelicon, 2014). Beans were cryo-fixed, cryo-sectioned to 30 μm, sandwiched between two Ultralene ® foils (each 4 μm thick, Spex Europe, Stanmore, UK) and deposited on custom made Cu holders. These frozen-hydrated samples were analysed at the ID21 beamline (ESRF, Grenoble, France) (Koren, Arčon, Kump, Nečemer, & Vogel-Mikuš, 2013) with excitation energy of 7.2 keV. Qualitative maps of Ca lateral distribution were subjected to quantitative analysis as described in Kump and Vogel-Mikuš (2018) and quantitative Ca distribution maps were generated with PyMCA software (Solé, Papillon, Cotte, Walter, & Susini, 2007).

2.7. Statistical analysis

Seed weight and cooking time data were analysed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using PROC GLM and Tukey's test was used for means' separation. Bulk element data were analysed using Duncan's post-hoc test at $p < 0.05$ after analysis of variance (ANOVA) in Statistica 6.0 (StatSoft Inc, Oklahoma, Tulsa, USA).

3. Results

3.1. Cooking time of the lpa1 seeds

lpa-B beans displayed an HTC defect since they have been shown not to reach a desirable softness even after 7 h soaking and 5 h cooking (Petry et al., 2016). Hence, we analysed cooking time of *lpa1* seeds in three different genetic backgrounds, characterized by different seed coat colours and the presence of different PHA alleles, as summarized in Table S1: cream beans harbouring the PHA-L allele (wt-C and *lpa*-C), the black ones with PHA-E allele (wt-B and *lpa*-B) and cranberry seeds harbouring PHA-E,L allele as representative of the majority of cultivated varieties (wt-Cr and *lpa*-Cr). High variability in cooking time was observed among the genetic backgrounds (Table 1). Moreover, for each

Table 1
Dry, soaked and cooked seed weight (g/25 seeds) and cooking time (min) of the wt and *lpa* mutant common bean seeds of three different genetic backgrounds: cream (C), black (B) and cranberry (Cr). Time of soaking was 12 h at room temperature. Means in a column that do not share a letter are significantly different at alpha 0.05 according to Tukey's Studentized Range (HSD) test.

Seed genotype	Dry weight (g/25 seeds)	Soak weight (g/25 seeds)	Cooked weight (g/25 seeds)	Cooking time (min)
wt-C	5.13 ± 0.00b	10.60 ± 0.46b	12.01 ± 0.17b	17 ± 3d
lpa-C	4.32 ± 0.36bc	8.33 ± 2.06bc	10.00 ± 0.37b	49 ± 9 cd
wt-B	4.11 ± 0.07c	5.44 ± 1.20c	8.24 ± 1.33b	100 ± 11c
lpa-B	3.68 ± 0.15c	7.05 ± 0.82bc	8.03 ± 0.20b	291 ± 28b
wt-Cr	14.39 ± 0.17a	29.74 ± 0.80a	34.38 ± 1.41a	47 ± 1 cd
lpa-Cr	15.00 ± 0.40a	32.22 ± 0.81a	34.01 ± 1.01a	430 ± 3a

of the three genetic backgrounds, the *lpa* lines took significantly longer to cook than the wild type. The *lpa*-C and the *lpa*-B lines took nearly three times as long to cook as the wt-C and wt-B, respectively, and the *lpa*-Cr took nine times as long as the wt-Cr to cook. The within-replication variability was highest for the black bean samples, which is likely due to the susceptibility of this genotype to hard shell. Not all the seeds uniformly took up water during soaking, thereby resulting in higher cooking time variability.

3.2. Influence of cooking conditions and bean genotype on PHAs' stability

The gastrointestinal side-effects which occurred after consuming *lpa-B* seeds were ascribed by Petry et al. (2016) to the incomplete hydrolysis of PHA-L, despite the 7 h of soaking and the following 5 h of cooking. The strong thermal resistance of this protein may be linked to the hard-to-cook (HTC) behaviour. Moreover, the cooking temperature used by Petry et al. (2016) did not exceed 95 °C, because of the high altitude. Considering that PHAs are known to be very stable proteins (Morari et al., 2008), cooking at only 95 °C could inhibit their hydrolysis.

To verify this hypothesis and assess whether this behaviour is also preserved at normal cooking conditions (100 °C), beans were cooked under normal (100 °C, HT) or sub-optimal conditions (95 °C, LT) and samples were taken at three different cooking times (1, 2.5 and 5 h) to monitor PHAs hydrolysis in the course of cooking time (Fig. 1). In order to verify if the HTC defect observed in *lpa*-B by Petry et al. (2016) was PHA-L specific or if it could be observed also for other PHA alleles, we used genotypes carrying different PHA alleles (PHA-L, PHA-E and PHA-E,L) together with the *lpa1* or wt trait.

As expected, cooking at LT inhibited thermal hydrolysis of major seed proteins (Fig. 1A, compare lanes 9–16 to 1–8 in panels a, b, c). Proteins of samples cooked at HT were more promptly hydrolysed; indeed, after 1 h of cooking only a low amount of total proteins remained. The effect of cooking temperature was particularly apparent for Phsl, which is the most abundant protein in bean seeds (Fig. 1A, red bars). At LT the Phsl of genotypes carrying PHA-E and PHA-E,L alleles was mostly hydrolysed after 2.5 h of cooking (Fig. 1A panels a and c, lanes 9, 10 and 15, 16), while it was more stable in genotypes with the PHA-L allele, and was still detectable after 2.5 h of cooking (Fig. 1 A panel b, lanes 9, 10, 15, 16). At HT, after 1 h of cooking Phsl was almost completely hydrolysed in all samples, except in the wt genotype carrying the PHA-E,L allele in which Phsl was completely hydrolysed after 2.5 h (Fig. 1A, panels a, b, lanes 3, 6 and panel c lane 3, respectively). In general, at LT the extent of hydrolysis of the major seed proteins appeared to depend on which PHA allele was present. When the PHA-L allele was present alone, protein thermal hydrolysis was strongly reduced as residual proteins were more abundant at all times of cooking, and this was particularly evident at 2.5 h of cooking (Fig. 1A, compare lanes 10, 11, 14, 15 of panel b with those of panels a and c). Furthermore, hydrolysis of minor proteins seemed less efficient in genotypes with PHA-E,L allele. In fact, some polypeptides were still detectable, even after 5 h of cooking (Fig. 1A, lanes 1, 8, 9 and 16). In all the genetic backgrounds, the presence of the *lpa1* mutation slightly reduced the hydrolysis of major seed proteins (Fig. 1A, compare lanes 1–3 with 6–8 and lanes 9–10 with 14–16 in all panels).

To further evaluate the effect of temperature (HT vs LT) and genotypes on PHA thermal stability, we conducted a western blot analysis using polyclonal antibodies specific to PHA (Fig. 1B). Although PHA represents a consistent part of the seed proteins (about 10%), we aimed to unambiguously identify PHA polypeptides (even at low concentration). We observed that the stability of PHA was highly dependent on the PHA allele. PHA-E, when present alone, was highly susceptible to thermal hydrolysis and no differences were detected between LT and HT, and the presence of the *lpa1* mutation did not affect PHA-E hydrolysis (Fig. 1B, panel a). By contrast, the presence of the *lpa1* mutation seems to increase PHA stability in genotypes with PHA-L and PHA-E,L alleles cooked both at HT and LT (Fig. 1B, panels b, c). PHA-L was strongly resistant to thermal degradation when co-occurring with the *lpa1* mutation. Furthermore, it was still detectable in a similar amount after 5 h of cooking as it was at the beginning of cooking (0 h) (Fig. 1B, panel b, lanes 5–8 and 13–16). Interestingly, PHA-L stability was also consistently affected by cooking temperature since it was detectable in wt beans cooked at LT up to 2.5 h (Fig. 1B, panel b, compare lanes 9–12 with lanes 1–4). The behaviour of PHA-E,L was intermediate between that of PHA-E and PHA-L. When cooking was performed at HT, PHA-E,L polypeptides were easily hydrolysed, however at LT small amounts of protein were still present until 1 h of cooking, with a tendency to be more stable in the *lpa1* genotype (Fig. 1B, panel c, lanes 3–6 and 11–14). A magnification of PHA-E,L polypeptides at 0 and 1 h of cooking allowed us to distinguish PHA-E and PHA-L polypeptides (Fig. 1C grey and red arrowheads, respectively). After 1 h of cooking, the ratio between PHA-E and PHA-L was different from that at 0 h, as the PHA-L subunit was more abundant than PHA-E (Fig. 1C, compare lanes 1, 4 with lanes 2, 3, respectively). This suggests that PHA-L is more stable than PHA-E and this stability is more pronounced in the *lpa1* common bean seeds (Fig. 1C, lanes 1 and 4).

3.3. Effect of $CaCl_2$ and EDTA on seed protein stability

One of the mechanisms proposed for the HTC defect involves the enzymatic dephosphorylation of phytate by phytases, which may be activated by storage conditions.

The *lpa1* mutation analysed in this work produced a strong reduction of seed PA content, which allowed an increase of free cations that may contribute to the development of the HTC defect. Increase of free cations may also cause PHA stabilization as reported by Morari et al. (2008). Therefore, we hypothesised that the long cooking times and the PHA-thermal stability observed in *lpa1* beans might be connected to the high number of free cations, especially Ca^{2+}.

Ethylenediaminetetraacetic acid (EDTA) is a well-known chelating agent that is widely used for its ability to form complexes with many divalent cations in 1:1 ratio. It can also sequester metals from metalloprotein molecules, such as PHA, destabilizing the whole protein structure, which becomes more susceptible to enzymatic digestion (Janecki & Reilly, 2005). Therefore, the effect of $CaCl_2$ and EDTA presence on PHA-L stability during imbibition and cooking of wt and *lpa1* beans was investigated.

As shown in Fig. 2, all the tested EDTA concentrations reduced PHA-L stability in *lpa1* samples and the effect was higher with increasing EDTA concentrations (Fig. 2B). PHA-L hydrolysis in *lpa1* beans cooked in 10 mM EDTA was comparable to that of wt beans cooked in water (Fig. 2A, B, red lines). Increasing concentrations of EDTA also increased PHA-L susceptibility to hydrolysis in wt samples (Fig. 2A). On the contrary, when beans were cooked in the presence of increasing concentrations of $CaCl_2$, a strong effect on PHA-L stability was observed in wt samples. In fact, a concentration of 20 mM $CaCl_2$ was enough to mimic an *lpa1* phenotype in wt samples (Fig. 2C red lines). No significant effect of $CaCl_2$ was detected on PHA-L hydrolysis through time in *lpa1* samples (Fig. 2D).

The effect of $CaCl_2$ and EDTA on protein hydrolysis/stability was also observed for Phsl, the most abundant seed protein (Fig. S2, red arrow). Presence of EDTA during cooking increased protein susceptibility to hydrolysis and the effect was marked at concentrations of 55 and 100 mM at which Phsl showed significantly decreased stability after 2.5 h of cooking compared to samples cooked in water (Fig. 2A lanes 9, 10, 12, 13 and B lanes 22, 23, 25, 26 compared to lanes 3, 4 and 16, 17, respectively). This effect of EDTA was more evident in *lpa1* than in wt (Fig. 2, compare A lanes 9, 10, 12, 13 with B lanes 22, 23, 25, 26).

Cooking in the presence of $CaCl_2$ had an apparent impact on many seed proteins and particularly on Phsl stability, even at the lowest concentration (20 mM of $CaCl_2$). In fact, most of the proteins showed strong resistance to hydrolysis and the SDS-PAGE pattern did not

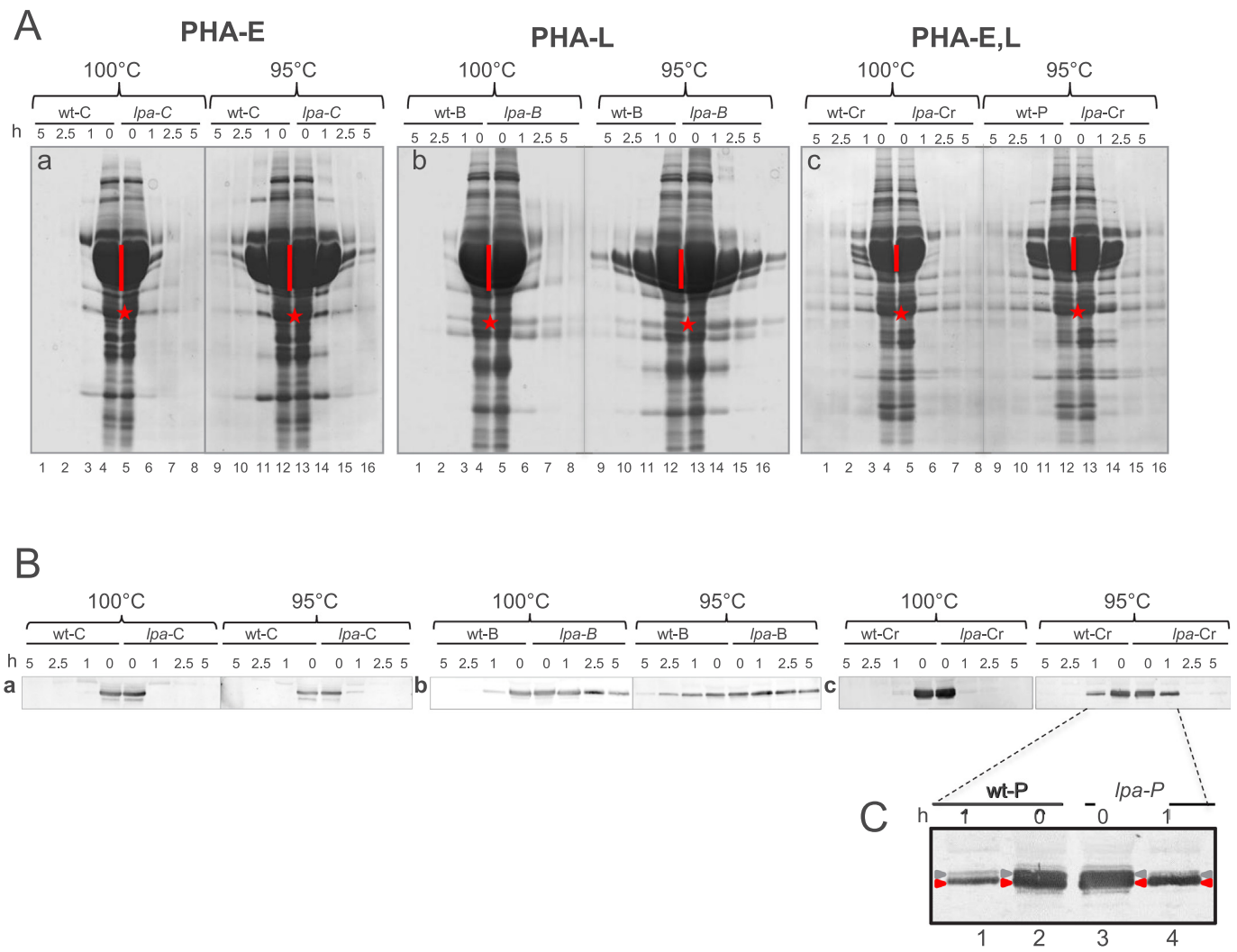

Fig. 1. Analysis of temperature and genotype effect on seed protein stability. SDS-PAGE (**A**) and western blot (**B**) analysis of seed proteins and PHAs, respectively, from bean samples (wt and *lpa*) cooked under normal conditions (100 °C) or sub-optimal conditions (95 °C). **B.** For this analysis specific antibodies against PHA were used. **a**: *lpa*-B and wt-B genotypes with PHA-L allele; **b**: *lpa*-C and wt-C genotypes with PHA-E allele; **c**: *lpa*-Cr and wt-Cr genotypes with PHA-E,L allele. **C.** Magnification of PHA-E,L polypeptides separation, detected by western blot, from wt and *lpa1* genotypes cooked at 95 °C. Grey arrowheads indicate PHA-E; red arrowheads indicate PHA-L. Red bars indicate the region in which Phsl polypeptides migrate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

change up to 2.5 h of cooking times, while at 5 h of cooking the most effective concentration was that of 50 mM $CaCl_2$ (Fig. 2 C compare lanes 3, 4 with lanes 6, 7, 9, 10 and 12, 13 and D compare lanes 16, 17 with lanes 19, 20, 22, 23, and 25, 26).

3.4. Histological analysis of lpa1 mutant developing seeds

To verify whether the *lpa1* mutation causes alteration of tissue morphology we performed histological analysis on developing seeds. Longitudinal sections of black and cream seeds at two developmental stages (18 and 24 DAF) were stained with toluidine blue (Fig. 3), as previously described by Pilu et al. (2005). Globoids, cellular structures in which phytic acid is normally accumulated, are mainly present close to the cotyledon margins in the parenchymatic tissues, close to the provasculature, and, in some cases, also inside the provascular tissue. Their number increased during development. The comparison between wt (Fig. 3 A, C, E, G, I and J) and *lpa1* (Fig. 3 B, D, F, H and K) seeds clearly illustrates a dramatic reduction in globoid number in the *lpa1* mutant in both genetic backgrounds at 18 as well as 24 DAF. Some differences were also evident between the two genetic backgrounds: the wt-C seeds (Fig. 3 E, G, I and J) accumulated more globoids than did the wt-B seeds (Fig. 3 A and C), while in the case of the *lpa1* the opposite was observed, with 18 DAF *lpa*-C seeds showing almost no globoids (Fig. 3 B).

3.5. Concentration and localisation of Ca in common bean seeds

It was previously shown that Ca distribution is crucial in the HTC defect (Kruger et al., 2015). Moreover, our biochemical data suggest that Ca also plays a major role in determining PHA-L stability. Hence, we analysed Ca concentration and localisation in our common bean samples. Only *lpa-B* had significantly more Ca in seed coat and cotyledons than did the wt-B (Table 2). By contrast, no differences were found in bulk Ca concentration between the wt-C and *lpa-C*. The higher concentration of Ca in the *lpa-B* than in the wt-B was found to be allocated to the cell walls in cotyledon parenchyma cells, as revealed by micro-XRF mapping (Fig. 4). No apparent differences were seen between the wt-C and *lpa-C*, in agreement with the bulk data.

Shown are means ± standard errors (n = 5). Different letters indicate statistically significant differences (Duncan's post hoc test at $p < 0.05$).

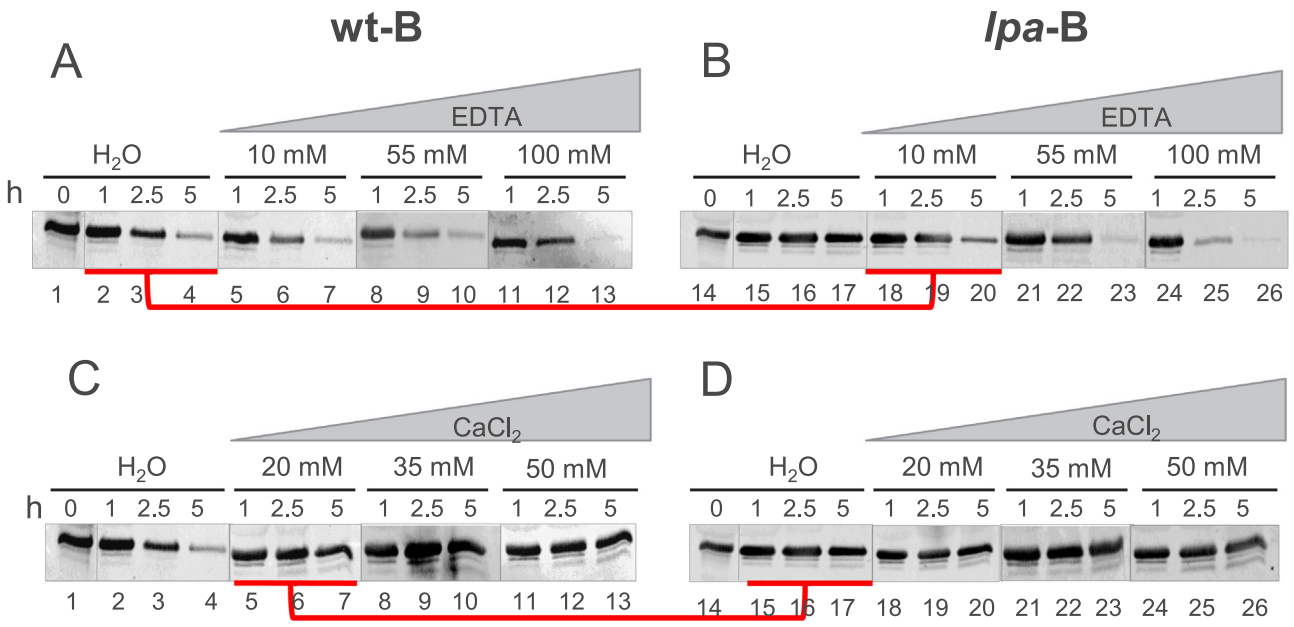

Fig. 2. Western blot analysis to evaluate the effect of different concentrations of $CaCl_2$ and EDTA on the hydrolysis of PHA-L from soaked and cooked wt-B and *lpa*-B beans carrying the PHA-L allele. Sample at 0 h (h), beans soaked for 4 h; samples at 1, 2.5, 5 h, beans cooked for 1, 2.5 and 5 h. Immunoblotting was performed with 1:1000 dilution of recombinant anti-PHA antibodies.

4. Discussion

The *lpa* trait is desirable for the development of biofortified crop varieties, since the reduced PA content results in higher mineral bioavailability. However, in the breeding process it is important to ensure that the *lpa* mutation does not cause any adverse effect on either the plant performance or on the nutritional value of the produce and consequently the health of consumers. Positive effects of the *lpa* mutation have been demonstrated in young women in whome the consumption of the *lpa1* mutant seeds of common bean increased Fe absorption (Petry et al., 2013). However, in a subsequent study, *lpa1* common bean seeds, but not wt seeds, caused gastrointestinal disorders in the majority of the young women with low Fe status participating (Petry et al., 2016). It was suggested that these side effects of *lpa1* beans were likely caused by the presence of PHA-L residues in the *lpa1* cooked beans and by their poor cooking quality (Petry et al., 2016).

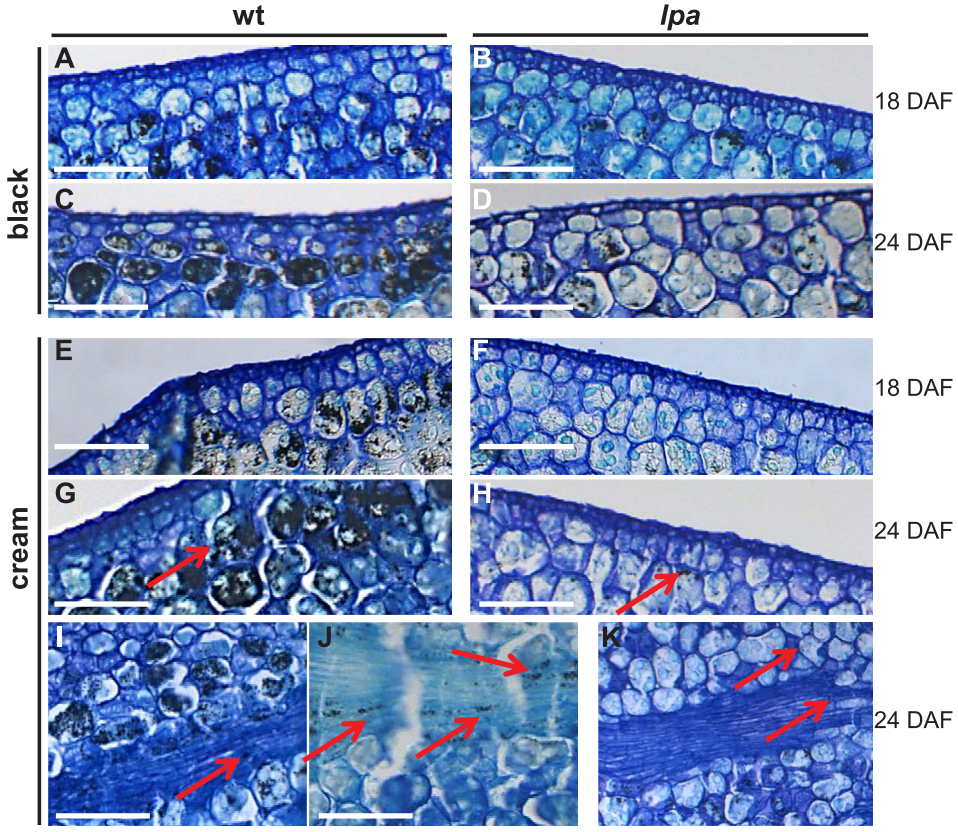

Fig. 3. Histological analysis of globoids. Longitudinal sections of wt-B (**A** and **C**), wt-C (**E**, **G**, **I-J**), *lpa*-B (**B** and **D**) and *lpa*-C (**F**, **H** and **K**) developing seeds at 18 (**A**, **B**, **E** and **F**) and 24 DAF (**C**, **D**, **G–K**), stained with toluidine blue. (**A–H**) staining at margin of cotyledons, where globoids are mainly accumulated, and (**I–K**) in proximity of vasculature. Some of the globoids are marked with red arrows. Bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Concentration of calcium (Ca) in whole seed, seed coat and cotyledons in wild type (wt) and *lpa* seeds of black (B) and cream (C) common bean genetic backgrounds.

Seed genotype	Ca (mg kg^{-1} dry weight)		
	Whole seed	Seed coat	Cotyledons
wt –B	592 ± 70.2 a	7760 ± 1230 a	67.8 ± 15.5 a
lpa-B	893 ± 106 b	10960 ± 1223 b	129 ± 30.8 b
wt –C	332 ± 39.7	5450 ± 1054	72.4 ± 36.22
lpa-C	392 ± 46.6	4580 ± 132	74.7 ± 49.01

The main objective of this work was to elucidate the role of the *lpa1* mutation in the HTC defect and to evaluate thermal stability of common bean lectins during cooking.

In their study, Petry et al. (2016) used the *lpa*-B beans, derived from the original 280–10 mutant, isolated after EMS mutagenesis of the 905 breeding population, harbouring the PHA-L allele, developed by Campion et al. (2009). The original *lpa1* mutation, causing 90% reduction in seed PA content, was introgressed in other genetic backgrounds, characterized by different phenolic compound content in the seed coat (cream, black and cranberry-coloured) and by the presence of different PHA alleles (PHA-A, PHA-E, PHA-E,L), as summarized in Table S1. In the present study we compared the cooking time of the different *lpa1* beans to their corresponding wt. Differences were evident between wt seeds belonging to the different genetic backgrounds with the darkest (wt-B) and the lightest (wt-C) ones, showing the longest and the shortest cooking times, respectively. These data correlate with the conclusions of a recent study performed on different easy-to-cook and HTC kidney bean accessions, showing that dark colour accessions had greater tendency to develop the HTC defect (Parmar, Singh, Kaur, &

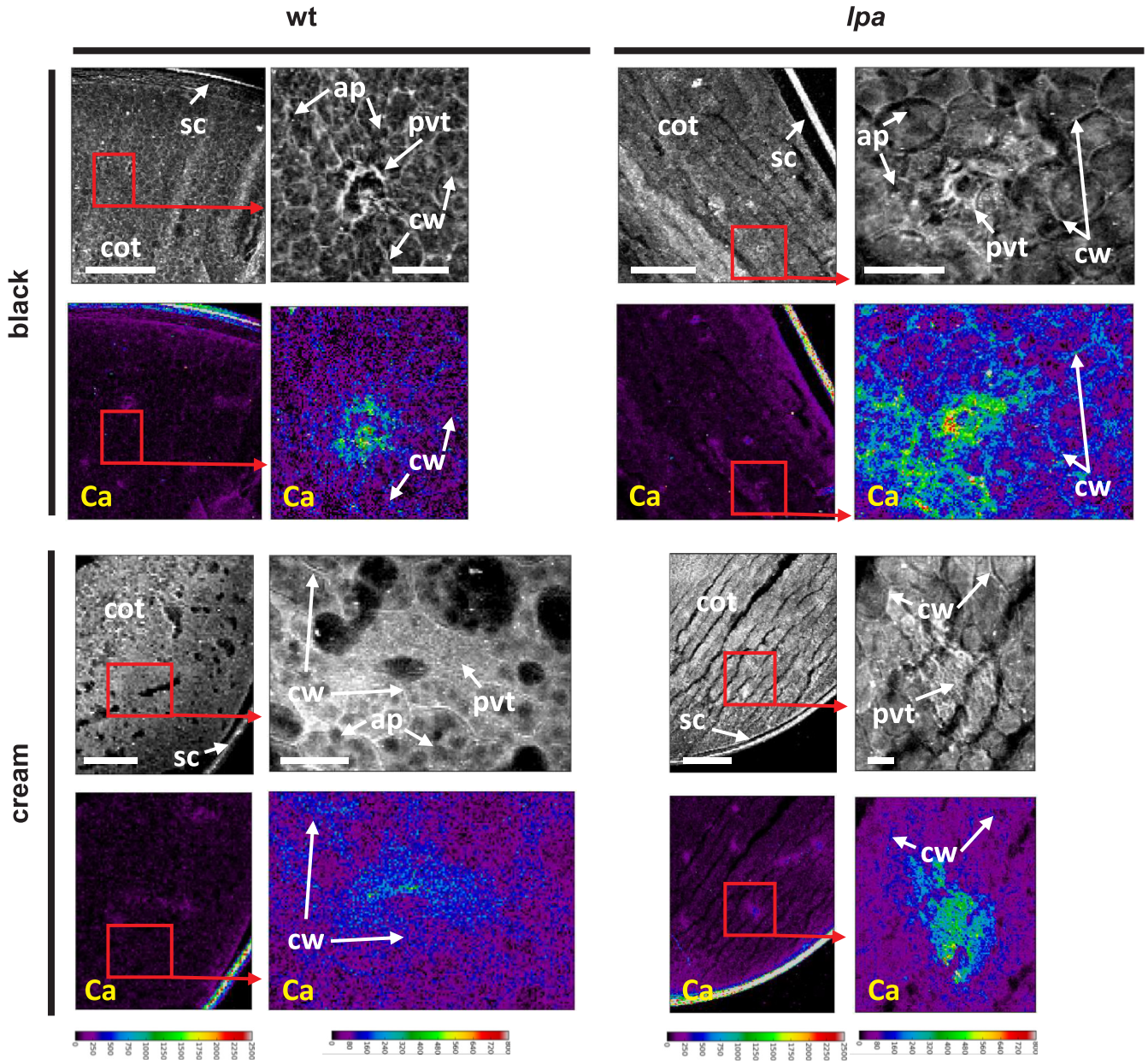

Fig. 4. X-ray absorption image (black and white) and distribution of calcium (Ca) in wt and *lpa1* common bean seeds of the black and cream genetic backgrounds. Red squares on the left-hand-side panels (scale bars are 500 μm) indicate zooms on the provascular tissue (pvt) shown on the right-hand side-panels (scale bars are 100 μm) for each type of the seed. Colour legends at the bottom of the figure are in mg Ca kg^{-1} dry weight for all distribution maps above them. sc, seed coat; cot, cotyledon; cw, cell walls; ap, amyloplasts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Thakur, 2017). In all the genetic backgrounds the *lpa1* seeds had longer cooking times than their wt counterparts, whereas in the cream and black seeds, a nearly threefold increase was observed, while in the cranberry coloured common bean a nine fold increase was recorded. Differences in the structure, composition and extractability of non-starch polysaccharides were observed between easy-to-cook and HTC common beans (Yi et al., 2016). Further studies will be necessary to verify whether the different genetic backgrounds used in this study may differ in their non-starch component and consequently require different cooking times. Moreover, the interaction of the different polyphenol components present in the different genetic backgrounds with the *lpa1* mutation will be investigated. To our knowledge, our data provide the first genetic demonstration of the "phytase-phytate-pectin" theory, proposed to explain the HTC defect in legumes. Histological analysis showed a dramatic decrease in globoid number in the *lpa*-B and *lpa*-C developing seeds compared to their wt counterparts, in accordance to reports in rice and maize *lpa* mutants (Lin et al., 2005; Liu, Ockenden, Truax, & Lott, 2004; Pilu et al., 2005). These results suggested that in the *lpa1* seeds there is an increase in the content of free cations, or they could be less strongly bound to other ligands (Kumar, Sinha, Makkar, & Becker, 2010). These cations, of which Ca being a macroelement is presumably one of the most abundant, can eventually migrate to the cell-wall-middle lamella area of parenchyma cells, as shown for the cowpea with defective HTC for Ca and Mg, where they can react with pectic acid and phenolic compounds, forming insoluble substances (Kruger et al., 2015; Yi et al., 2016). In agreement with these reports is our observation of higher Ca concentration in all seed parts in the *lpa1* beans than in the wt, depending on the genetic background, with significant differences found only in the black beans (Table 2). Furthermore, at the cell level, more Ca was allocated to the cell walls in the *lpa-B* mutant than in the wt-B (Fig. 4) supporting the hypothesis that the low PA phenotype is accompanied by a different cation allocation in the seed. Similar findings have been reported for P, K, Zn and Cu in rice, in which the redistribution was attributed to the *lpa* mutation (Sakai et al., 2015). Compared to tissue-specific effects, many more data have been compiled for the bulk Ca concentrations in crops for which *lpa* mutants have been developed. In soybean, higher Ca concentrations were found for some *lpa* mutants only, compared to the wt, with the growing location and season having minor effects on the total Ca concentrations (Frank, Nörenberg, & Engel, 2009). Contradictory results were found in cereals as well. In maize, the bulk Ca concentration was found to be higher (Landoni et al., 2013) or lower (Lin et al., 2005) in the *lpa1-1* mutant compared to the wt. In rice, either only negligible differences in Ca concentrations were found in whole grain or in different grain tissues (Liu et al., 2004) and in differently milled grain with small effects due to the growing seasons (Bryant, Dorsch, Peterson, Rutger, & Raboy, 2005), or significantly more Ca was found in at least some grain tissues with significant effects due to the growing location and season (Ren, Liu, Fu, Wu, & Shu, 2007) in *lpa1* mutants compared to parent lines. In wheat, no effect of the *lpa* mutation was seen for the Ca concentrations in different milling fractions of the grain (Guttieri, Bowen, Dorsch, Raboy, & Souza, 2004). In barley, where two types of *lpa* mutants have been identified, namely strong and moderate, more Ca was measured in the strong *lpa* mutants compared to wt, while moderate *lpa* mutants had intermediate Ca concentration (Hatzack, Johansen, & Rasmussen, 2000). Regardless of the differences in Ca concentrations, estimating the bioavailability of Ca in the *lpa* mutants will be fundamental. Feeding trials in animals already suggest improved bioavailability of Ca. For example, in rainbow trout fed with low PA barley grain, increased Ca bioavailability was reported (Overturf, Raboy, Cheng, & Hardy, 2003), as was shown for pigs, fed with low PA barley meal, where better Ca utilisation was reported at the same grain bulk Ca concentrations (Veum, Ledoux, Bollinger, Raboy, & Cook, 2002). Based on our observations and in accordance with the contradictory results reported, it appears that different genetic backgrounds may result in unpredictable phenotype(s) in different crops. Further mechanism-oriented studies should be able to solve the phenomenon of the interaction between the *lpa* mutation and the genetic background.

The analysis we performed on the thermal hydrolysis of different PHAs' forms (PHA-L, PHA-E or PHA-E,L) by using *lpa1* and wt genotypes carrying different PHA alleles, showed that seed proteins are generally less susceptible to thermal hydrolysis when cooking was performed at 95 °C compared to 100°, as expected. Furthermore, we demonstrated that, among PHA polypeptides, the PHA-L, when present alone, showed the highest thermal stability, especially in *lpa1* beans. On the contrary, PHA-E was easily hydrolysed by cooking, even at LT, and the presence of the *lpa1* mutation did not affect its susceptibility to thermal hydrolysis. An intermediate behaviour was observed in beans containing both PHA-E and PHA-L (Fig. 1B). We compared the thermal stability of homotetramers of the E_4 (the only PHA form present in the wt-C and *lpa*-C genotypes) or L_4 (present in the wt-B, *lpa*-B) type and of different heterotetramers (E_3L_1, E_2L_2, E_1L_3, present together with E_4 and L_4 in the wt-Cr and *lpa-Cr*) (Felsted et al., 1981; Miller, HSU, Heinrikson, & Yachnin, 1975). Overall, our results indicate that the resistance to thermal hydrolysis of the different PHAs present in our genotypes is PHA-L > PHA-E,L > PHA-E, suggesting for PHA-E,L $L_4 > E_1L_3 > E_2L_2 > E_3L_1 > E_4$. The different behaviour in response to thermal hydrolysis displayed by PHA-L and PHA-E is quite striking, considering that the two polypeptides share a very high sequence homology. The analysis of a multiple alignment of the amino acid sequences of known PHA-E and PHA-L isoforms (data not shown), including those used in this study, did not reveal any significant differences in critical amino acid residues among each type of PHA, suggesting that the specific PHA-L allele present in the black genotype (wt and *lpa*) used in the present study may confer a particularly high stability. Some differences between PHA-L and PHA-E subunits are present in the region involved in the metal binding (Hamelryck et al., 1996), suggesting that some of these residues may play an important role in conferring a higher thermal stability to PHA-L compared to PHA-E.

The PHA-L subunit stability further increased in presence of the *lpa1* mutation, as shown by the comparison of the western blot results between *lpa*-B and wt-B samples, while the stability of the E subunit is not affected. PHA-L, like other lectins, is a metalloprotein with two bound metal ions (one Ca^{2+} ion and one Mn^{2+}, a transition metal ion) per monomer in the vicinity of the sugar binding site (Hamelryck et al., 1996). The mechanism by which an increase of free cations alters the PHA-L stability is not clear, however it has been reported that Ca^{2+} plays a role in modulating native PHA susceptibility to *in vitro* proteolysis by trypsin. EDTA enhances and $CaCl_2$ inhibits PHA hydrolysis by trypsin. It has been hypothesized that, in the absence of $CaCl_2$, PHA loses some of the bound Ca^{2+}, and this results in conformational changes that make PHA susceptible to trypsin proteolysis. In the absence of Ca^{2+}, the PHA protein changes conformation, mainly the loops that form the Ca-binding site, and perhaps this makes these sites more accessible and susceptible to proteolytic attack (Morari et al., 2008). A similar mechanism can explain the increased stability of PHA-L, when present alone in the *lpa*-B common bean seed, where most cations could not be complexed as phytate salts, due to the reduced content of PA, (Kumar et al., 2010), and would be available to form salts with other compounds more prone than PA to dissociation. Indeed, we showed that, when beans carrying the PHA-L allele are cooked at LT in presence of EDTA, the PHA-L stability decreased as EDTA concentration increased (Fig. 2B). On the contrary, cooking of wt beans carrying the PHA-L allele in presence of an excess of Ca^{2+} cations strongly increased PHA-L stability (Fig. 2C).

Considering that the *lpa1* mutation was developed with the purpose of increasing bean nutritional value, the finding that PHA-L may become very stable when co-occurring with the *lpa1* mutation is of great concern. In addition, considering that the PHA-L thermal stability was also observed in wt beans cooked at LT, we cannot exclude the possibility that this particular isoform, if not properly cooked, could cause

health problems regardless from the *lpa1* mutation. However, we showed that the association of the *lpa1* mutation with different PHA alleles, such as PHA-E or PHA-E,L does not significantly increase the thermal stability of these other PHAs, indicating that these genotype combinations are suitable candidates for breeding programmes aimed at improving bean nutritional value by exploiting the *lpa1* mutation.

5. Conclusions

The results of this work have produced important knowledge on how, unexpectedly, the *lpa1* mutation may affect important cooking and nutritional traits. The susceptibility of the *lpa1* beans to develop the HTC defect should be taken into account by breeders, because it is detrimental to the bean nutritional quality, as well as to their acceptability. Moreover, we demonstrated that the PHA-L allele should be avoided when attempting the development of safe *lpa1* mutants. As there is an interaction between the *lpa1* mutation and the genetic background, it is desirable that through breeding efforts it will be possible to avoid these negative pleiotropic effects.

Other common bean *lpa* mutants, recently isolated (Cominelli et al., 2018) and with milder effects on PA reduction than the *lpa1*, can be assayed for their cooking and nutritional properties in order to develop useful biofortified beans devoid of negative traits.

In conclusion, our results provide important information that will help to select the best traits to be used in breeding programmes aimed at improving the nutritional value of common bean. This is of great importance considering that common beans are consumed as a staple food by most of the developing countries, where they represent the primary source of proteins that complements their carbohydrate rich diets based mainly on the consumption of rice and maize.

CRediT authorship contribution statement

Eleonora Cominelli: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Visualization. **Michela Galimberti:** Investigation. **Paula Pongrac:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision. **Michela Landoni:** Investigation, Resources, Writing - review & editing. **Alessia Losa:** Investigation. **Dario Paolo:** Investigation. **Maria Gloria Daminati:** Investigation. **Roberto Bollini:** Conceptualization, Methodology, Validation, Writing - review & editing. **Karen A. Cichy:** Methodology, Validation, Investigation, Resources, Writing - review & editing. **Katarina Vogel-Mikuš:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing. **Francesca Sparvoli:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126680.

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