A defective ABC transporter of the MRP family, responsible for the bean lpa1 mutation, affects the regulation of the phytic acid pathway, reduces seed myo-inositol and alters ABA sensitivity

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Summary

• We previously identified the lpa1 (low phytic acid) 280-10 line that carries a mutation conferring a 90% reduction in phytic acid (InsP6) content. In contrast to other lpa mutants, lpa1 (280-10) does not display negative pleiotropic effects. In the present paper, we have identified the mutated gene and analysed its impact on the phytic acid pathway.
• Here, we mapped the lpa1 (280-10) mutation by bulk analysis on a segregating F2 population, and then, by comparison with the soybean genome, we identified and sequenced a candidate gene. The InsP6 pathway was analysed by gene expression and quantification of metabolites.
• The mutated Pvmrp1 (280-10) cosegregates with the lpa1 (280-10) mutation, and the expression level of several genes of the InsP6 pathway are reduced in the lpa1 (280-10) mutant as well as the inositol and raffinosaccharide content. Pvmrp2, a very similar parologue of Pvmrp1 was also mapped and sequenced.
• The lpa1 mutation in beans is likely the result of a defective Mrp1 gene (ortho-logous to the lpa genes AtMRP5 and ZmMRP4), while its Mrp2 paralog is not able to complement the mutant phenotype in the seed. This mutation appears to down-regulate the InsP6 pathway at the transcriptional level, as well as altering inositol-related metabolism and affecting ABA sensitivity.

Abbreviations: ABC, ATP-binding cassette; MRP, multidrug resistance-associated protein; InsP6, myo-inositol-1,2,3,4,5,6-hexakisphosphate; IMP, myo-inositol-phosphate monophosphatase; MIPS, myo-inositol-3-phosphate synthase; MIK, myo-inositol kinase; IPK2, inositol 1,4,5-tris-phosphate kinases; ITPK, inositol 1,3,4-trisphosphate 5/6-kinase; IPK1, inositol 1,3,4,5,6 pentakisphosphate 2-kinase.

Introduction

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate; InsP6) is the main storage form for phosphate in plant seeds which accumulates in the protein storage vacuole in inclusions called globoids. Phytic acid is poorly digested by monogastric animals and humans and decreases the nutritional value of the seeds by trapping phosphate and chelating nutritionally important minerals, such as iron, zinc, magnesium, and calcium. Therefore, the isolation of low phytic acid (lpa) mutants is considered a highly desirable objective in the genetic improvement of the nutritional quality of grain crops (Raboy, 2006), also considering the correlation between InsP6 reduction in lpa seeds and increased phosphate and mineral cation bioavailability demonstrated in nutritional trials (Mendoza et al., 1998;
Hambidge et al., 2004, 2005). These mutants have been isolated in important crops such as maize, barley, rice, wheat, soybean (reviewed by Cichy & Raboy, 2009) and, by our research group, common bean (Campion et al., 2009), a legume entirely used for human consumption which represents a very important source of nutrients for resource-poor people worldwide.

The lpa mutations characterized so far affect the partitioning of P into InsP₆, inorganic phosphorus (Pᵢ) and inositol phosphates (InsPs) with five or fewer P esters (Raboy, 2002), and fall into three classes: mutations affecting the early part of the phytic acid biosynthetic pathway, with decreased InsP₆ and a molar equivalent increase in inorganic Pᵢ; mutations in the late part of the pathway, with decreased InsP₆ matched by increases in both Pᵢ and lower InsPs; and mutations affecting the transport of phytic acid to the vacuole. Mutants in the early pathway carry defective structural genes needed for myo-inositol synthesis and subsequent phosphorylation. These include myo-inositol-3-phosphate synthase, myo-inositol kinase and 2-phosphoglycerate kinase (Hitz et al., 2002; Shi et al., 2005; Kim et al., 2008). Conversely, mutants of the late part of the pathway are altered in one of the genes coding for inositol kinases involved in the sequential phosphorylation steps of InsP₃, InsP₄ and/or InsP₅ intermediates (reviewed by Cichy & Raboy, 2009). Mutants in phytic acid vacuolar storage have a nonfunctional multidrug resistance-associated protein (MRP) type ATP-binding cassette (ABC) transporter which is a high-affinity InsP₆ transporter (ABCC5) (Shi et al., 2007; Nagy et al., 2009; Xu et al., 2009).

From an agronomical point of view, lpa mutants are often associated with negative effects on seed and plant performance, such as compromised germination and emergence, lower stress tolerance and poor seed filling (Meis et al., 2003; Pilu et al., 2005; Bregitzer & Raboy, 2006; Guttieri et al., 2006). Although there are some exceptions, like the Arabidopsis mutant AtMrp5 (bearing a mutation in the InsP₆ transporter), which is drought-tolerant (Klein et al., 2003), or the barley lpa1-l, which showed a good agronomic performance in both irrigated and nonirrigated environments (Bregitzer & Raboy, 2006), projects aimed at obtaining lpa crops should take into account aspects regarding agronomic potential.

Recent papers suggest a correlation between myo-inositol content and response to ABA during seed germination. In fact, Arabidopsis mps1 and vcv4 mutants (mutated in myo-inositol-3-phosphate synthase and inositol-phosphate monophosphatase, respectively), have impaired germination in response to ABA, a phenotype which correlates with reduced myo-inositol seed content (Torabinejad et al., 2009; Donahue et al., 2010). Conversely, seeds of Arabidopsis lines overexpressing the AtPAP15 gene, coding for a purple acid phosphatase with a phytase activity, have been shown to be ABA-insensitive (Zhang et al., 2008). These plants show a 20–30% decrease in foliar phytate and a twofold increase in foliar ascorbic acid, possibly because of an increase in myo-inositol that is also a substrate for ascorbic acid biosynthesis (Lorence et al., 2004). Interestingly, an altered ABA phenotype during germination was also reported for the Arabidopsis mrp5 mutant which was shown to be ABA-insensitive (Klein et al., 2003). Most likely, all the above pleiotropic effects could be explained by the fact that metabolites of the phytic acid pathway, such as myo-inositol, Ins(1,4,5)P₃ and InsP₆, have been shown to play a key regulatory role in signal transduction and in the control of plant response to environmental stresses (Munnik & Vermeer, 2010).

We previously (Campion et al., 2009) presented data on the isolation and initial characterization of the common bean lpa-280-10 mutant, which, in accordance with the Phaseolus nomenclature guidelines, is hereafter referred to as lpa1(280-10). This mutant shows several nutritionally important characteristics, such as a 90% reduction in InsP₆, a 25% reduction in raffinosaccharide content and sevenfold increase of free iron cations in the seeds. We also provided evidence that, unlike other `strong' lpa mutants (having up to 90% of phytic acid reduction), lpa1(280-10) shows no negative pleiotropic effects on traits of agronomic relevance. In this study we have mapped the lpa1(280-10) mutation to the bean chromosome 1 and demonstrated that it is associated with a defective MRP type ABC transporter gene (Pomrp1), orthologous to Arabidopsis AtMRP5/AtABCC5 and maize ZmMRP4 (lpa1). Investigations at biochemical and molecular levels aimed at assessing the effects of the lpa1(280-10) mutation on the amounts of some metabolites and on the expression of genes involved in the phytic acid pathway showed that the Pomrp1 mutation determines a general repression of the pathway, indicating that transient accumulation of InsP₆, or products derived from its degradation, may reduce its biosynthesis through a negative feedback mechanism. Moreover, we detected increased ABA sensitivity during germination of the mutant that apparently correlates with the lower myo-inositol content observed in the in lpa1(280-10) seeds, a finding supported by data on Arabidopsis mutants with reduced myo-inositol content (Zhang et al., 2008; Torabinejad et al., 2009; Donahue et al., 2010).

Materials and Methods

Plant material

Plants (Phaseolus vulgaris L.) of lpa1(280-10) mutant line or parental line 905 (Campion et al., 2009) were grown in phytotron under a 16 : 8 h light : dark photoperiod, with temperatures set at 25 : 18°C day : night and a relative humidity of c. 65%. Seeds and pods were collected on different days after flowering (DAF). At 4–6 DAF only pods

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could be collected, while after 12 DAF cotyledons could be dissected from the tegument and embryo. Leaves and roots were collected from adult plants. All samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Labelling of leaf tissue and developing cotyledons

Leaf discs were cut from wild-type (wt) and lpa1(280-10) leaves. Six discs were incubated on 3 ml of 10 mM KCl, 0.1 mM CaCl2, 50 mM MES2-(N-morpholino)ethanesulfonic acid, pH 5.6, containing 1.85 MBq of myo-[2-3H]inositol, specific activity 752 GBq mmol⁻¹ (PerkinElmer, Boston, MA, USA). For analysis, two discs were washed in water, blotted dry and extracted. Developing seeds were labelled for periods up to 72 h with 1.85 Mq (50 µl) of myo-[2-3H]inositol applied directly to the inner faces of half cotyledons. For analysis, three cotyledons were washed briefly in water, blotted dry and extracted.

High-performance liquid chromatography (HPLC) analyses of inositol phosphates

Mature seed flours were extracted with 40 vol of 0.4 M HCl, overnight at 4°C under shaking. Samples were centrifuged 10 min at 12 000 g and the supernatant was filtered on a Millipore 0.45 µm filter. Quantification of InsPs was carried out by metal-dye detection (MDD)-HPLC analysis as described in Bohn et al. (2007).

Extracts from developing seeds and leaves were analysed on a 25 cm × 4 mm Dionex (Sunnyvale, CA, USA) AS11 column fitted with AG11 guard cartridge eluted at a flow rate of 1 ml min⁻¹. Samples (100 µl) were loaded and eluted with a NaOH gradient generated by mixing solvents from buffer reservoirs (A, water; B, 150 mM NaOH), as follows (time (min), %B): 0, 3.3; 5, 3.3; 22.5, 35; 22.6,100; 27, 100; 27.1, 3.3; 0; 40, 3.3. Peaks of ions were detected by suppressed ion conductivity on the ED40 detector of a Dionex DX500 chromatography system fitted with an anion micromembrane suppressor operated at 100 mA suppressor current. In some experiments, eluent from the suppressor was analysed for radioactivity after admixture of scintillation fluid at a flow rate of 1 ml min⁻¹. Radiolabelled InsPs extracts were analysed according to Nagy et al. (2009). All data were exported and redrawn in Delta Graph 4.0.

GC-MS analyses of myo-inositol

Seed flours were extracted with 10 vol of 50% aqueous ethanol. After 1 h at room temperature the extract was decanted through a 0.45 µm nylon syringe filter attached to a 1 ml syringe barrel. Residue was re-extracted with 1 ml of fresh 50% aqueous ethanol and the second extract was filtered as before. The combined filtrates were evaporated to dryness. The myo-inositol derivative was produced by redissolving the residues in 50 µl of pyridine and 50 µl of trimethylsilylimidazole:trimethylchlorosilane (100:1). After 15 min at 60°C, 1 ml of 2,2,4-trimethylpentane and 0.5 ml of distilled water were added, the sample was vortexed and centrifuged for 5 min, and the upper organic layer was transferred into 2 ml glass vial.

Myo-inositol was quantified as a hexa-trimethylsilyl ether derivative by GC-MS (G1800C Series II instrument; Hewlett Packard, Palo Alto, CA, USA). Samples were injected split mode (split ratio 10) with the injector temperature at 250°C and the oven at 70°C. After 2 min, the oven temperature was ramped at 25°C min⁻¹ to 170°C, then continued at 5°C min⁻¹ to 215°C and finally increased at 25°C min⁻¹ to 250°C and returned to the initial temperature. Electron impact mass spectra from m/z 50–560 were acquired at −70 eV after a 5 min solvent delay. Myo-inositol hexa-trimethylsilyl ether was identified by comparing the mass fragmentation pattern with the database library NIST05 (MS Library Software Varian, Palo Alto, CA, USA). Authentic myo-inositol standards in aqueous solutions were dried, derivatized and analysed at the same time. Concentrations of myo-inositol were calculated from the standard curve, obtained using the external standard method.

lpa1(280-10) mapping

To map the recessive lpa1(280-10) mutation, we produced an F2-segregating population by selfing hybrid plants Jalo EEP558 × lpa1(280-10). Flours of F2 seeds were assayed for the high inorganic phosphate (HIP) phenotype as in Campion et al. (2009). Samples of the same flours were also used for DNA extraction using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma, St. Louis, MO, USA).

We used bulk segregant analysis to identify the approximate chromosomal region where the lpa1(280-10) mutation is located. Two DNA bulks were prepared: bulk A, obtained from a pool of 15 wt F2 seeds, and bulk B, obtained from a pool of 15 HIP F2 seeds. Both samples were amplified using the 19 simple sequence repeat (SSR) markers that were polymorphic on the parental lines (Supporting Information, Table S1) among 47 markers distributed over the entire genome (Grisi et al., 2007). A map was then generated by testing the entire F2 population using the linked SSR markers.

Polymerase chain reaction were performed in a final volume of 10 µl, containing 10 ng DNA, 0.1 µM of each primer, 200 mM of each dNTP, 2.5 mM MgCl2 and one unit of Taq DNA polymerase (Promega, Madison, WI, USA). The reactions were carried out as follows: 94°C for 1 min, 35 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final step at 72°C for 7 min. The amplified fragments were resolved on 4% agarose gels. Recombinant values were converted to map distances using MAPMAKER 3 (Lander et al., 1987).
PvMrp1 and PvMrp2 gene sequence analysis and marker development

PaMRP4-F and PaMRP4-R primers (Tables S2) were designed on the conserved regions among the soybean MRP sequence (CS229850) described by Shi et al. (2007), the maize ZmMRP4 sequence and a Phaseolus acutifolius expressed sequence tag (EST) sequence (CX129901), which shares c. 80% of sequence identity with the soybean MRP. PCR reactions were carried out as described earlier (melting temperature \( T_m = 50^\circ \text{C} \)). The amplified fragment was sequenced and then used to screen a bean Bacterial Artificial Chromosome (BAC) library (Galasso et al., 2009) for PvMrp-containing clones.

The partial bean Mrp sequence was aligned to Gm03g32500, Gm19g35230 and Gm13g18960 MRP genes and the multiple alignment was used for further primer design (Table S3). To discriminate between the two homologous PvMrp genes, primers were always tested for amplification on PvMrp1- and PvMrp2-specific BAC clones, and then used for cDNA and genomic DNA amplification from both wt and \( lpa1(280-10) \) plants. Raw sequences of the amplified fragments (Table S2) were assembled and managed using the Vector NTI Advance 9.0 software (Invitrogen, Paisley, UK). Sequences have been deposited in GenBank database under ID FR694187 (PvMrp1), FR694188 (PvMrp2).

Cosegregation analysis

Cosegregation analysis between \( lpa \) phenotype and \( PvMrp1 \) sequence was performed using the primers pol_19.03f and pol_19.04r (Table S3) to obtain allele-specific amplified products. PCR reactions were carried out as above (\( T_m = 52^\circ \text{C} \)).

PvMrp2 sequence mapping

In order to map the \( PvMrp2 \) sequence, we amplified a portion of this gene using the primers pol1F and pol1R (Table S3) to obtain allele-specific products of 122 bp for the \( PvMrp2 \)-[Jalo EEP558] allele and 131 bp for the \( PvMrp2 \)-[280-10] allele. PCR reactions were carried out as described earlier (\( T_m = 52^\circ \text{C} \)).

Samples from flour bulk A and B (see earlier in the \( lpa1(280-10) \) mapping section) yielded 122 bp and 131 bp amplified products, respectively. Both samples were amplified using the 19 polymorphic SSR markers and the map was created testing the entire \( F_2 \) population with the linked SSR markers as described earlier for the \( lpa1(280-10) \) mapping.

Northern blot analysis and semiquantitative RT-PCR

Total RNA was isolated from leaves, roots, immature pods or cotyledons as described by van Tunen et al. (1988). Ten micrograms from each sample were resolved on 1.2% agarose/formaldehyde/3-[N-Morpholino]-propanesulfonic acid (MOPS) gel and transferred to a nylon membrane (Hybond™ N+; GE Healthcare, Uppsala, Sweden). The isolated cDNA sequences of genes for phytic acid synthesis (Filetti et al., 2010) and the one coding for phaseolin storage protein (Slightom et al., 1985) were labelled with \( \alpha-^{[32P]} \text{-dCTP} \) using a random primer DNA labelling kit (Fermentas, Burlington, Ontario, Canada) and used for membrane hybridizations (Sparvoli et al., 1994). The membrane was washed at 0.2 \( \times \) saline-sodium citrate buffer (SSC), 0.5% (w/v) sodium dodecyl sulfate (SDS) at \( 65^\circ \text{C} \).

Semiquantitative reverse transcription (RT)-PCR and RT-PCR analysis were performed according to Filetti et al. (2010). \( PvMrp1 \) and \( PvMrp2 \) were amplified using primers 42f, 41r (\( T_m = 54^\circ \text{C} \)) and PaMRP4-F, pol1R (\( T_m = 50^\circ \text{C} \)) (Table S3); the PCR amplification programme for both transcripts was: 37 cycles at \( 94^\circ \text{C} \) for 30 s, \( T_m \) for 30 s, 72°C for 50 s, and a final step at 72°C for 7 min.

Seed germination assay

One hundred seeds of wt or \( lpa1(280-10) \) lines were imbibed in 70 ml water or solutions of different ABA concentrations (2.5, 5, 25, 50, 100, 250 \( \mu \text{M} \)) in 20-cm-diameter Petri dishes at \( 25^\circ \text{C} \) in the dark. Counts of germinating seeds were made once a day, starting on the first day and until the maximum of germination was achieved.

Results

Metabolic characterization of the mutant line \( lpa1(280-10) \)

To gain information on the \( lpa \) mutation, we first performed a suppressed anion conductivity HPLC analysis (Fig. 1a,b). On the AS11 column used here, D/Ins(1,2,4,5,6)P\(_5\) and Ins(1,3,4,5,6)P\(_5\) elute after InsP\(_6\) (Casaravilla et al., 2006), as do some InsP\(_4\)s (C. Brearley, unpublished). A peak co-eluting precisely with InsP\(_6\) was detected in developing seeds of wt, but was absent from \( lpa1(280-10) \). In parallel HPLC runs to that shown in the figure, we have shown that the principal component in a commercial sample of Ins1P eluted at 11.2 min, while Ins(1,4)P\(_2\) gave a peak at 14.7 min. While there are peaks in the chromatograms (Fig. 1a,b) with similar retention times, we are unable to conclude that they are InsPs (stereoisomerism unknown) set against the likely presence of many anions in the extracts. Further analysis of flours from mature seeds was performed by MDD-HPLC (Fig. 1c,d). Contrary to what was found in the case of \( lpa \) mutants affected in \( myo \)-inositol phosphate kinases acting in the late pathway, no peaks corresponding to lower InsPs were visible in the chromatograms of wt or mutant flours.
indicate InsP5 peaks. At maturity, low concentrations of InsP6 while InsP5 is almost undetectable.

A consequence of the myo-inositol-3-phosphate synthase (MIPS) gene mutation in the soybean L33 mutant is a significant reduction in the total raffinosaccharide synthesis (Hitz et al., 2002). As myo-inositol-3-phosphate, the product of the MIPS, is the only known precursor for the de novo synthesis of myo-inositol, we reasoned that a strong reduction in the amount of this latter important metabolite might be detected in lpa1(280-10) seeds if the mutation had affected MIPS activity. Data obtained by GC-MS analysis showed that the amount of myo-inositol detectable in lpa1(280-10) grains is reduced (30% decrease) compared with wt grains (Fig. 2).

**lpa1(280-10) mutation mapping**

To map lpa1(280-10) mutation, we used a DNA bulks strategy to reduce the number of PCR reactions required. We selected 19 polymorphic SSR markers out of 47 (28 SSRs proved monomorphic) to establish linkage. The molecular markers PV31, PV218, PV133 and BM53 showed a clear bias towards a lpa1(280-10)-specific band in the mutant pool as compared with the wt pool, indicating that the mutation maps on chromosome 1. Using these markers, an F2 mapping population of 198 individuals was screened for recombinants for each SSR marker and the values obtained were converted to map distances using MAPMAKER 3 (Lander et al., 1987). Mapping data established the position of the mutation at a distance of c. 2.8 cm from PV31 and PV218, 5.4 cm from PV133 and 4.8 cm from BM53 (Fig. 3a).

To identify the gene responsible of the lpa1(280-10) mutation, we took advantage of the high synteny between soybean and common bean genomes (Choi et al., 2004). We used an *in silico* approach to identify which region of soybean genome corresponded to the one identified by the SSR markers closer to the lpa1(280-10) mutation. The results of the comparative mapping analysis allowed the identification of two syntenic regions of c. 3.3 Mbp, delimited by the PV31 and PV133 homologues on soybean chromosomes 3 and 19 (Table 1). These two regions contain two putative MRP proteins that we refer to as GmMRP03 and GmMRP19 (coded by Gm03g32500 and Gm19g35230), which have recently been reported to be mutated in the soybean lpa1(280-10) mutation. The results of the comparative mapping analysis allowed the identification of two syntenic regions of c. 3.3 Mbp, delimited by the PV31 and PV133 homologues on soybean chromosomes 3 and 19 (Table 1).

The three soybean MRP genes share a high degree of homology (93% between Gm03g32500 and Gm19g35230 and c. 86% between Gm13g18960 and Gm19g35230) and are located in syntenic regions (Fig. S1).

**Identification of PvMrp1 and PvMrp2, cosegregation analysis and mapping**

To verify if the lpa1(280-10) mutant was linked to a defective orthologue of one of the soybean MRP genes described...
earlier, we needed to isolate common bean MRP orthologous sequences from which to design allele-specific primers. To this purpose, we screened a BAC library (Galasso et al., 2009) and found four positive clones, containing two types of MRP sequences: one named \( \text{PvMrp1} \), with greatest similarity to \( \text{Gm03g32500} \) and \( \text{Gm19g35230} \), and another named \( \text{PvMrp2} \), with higher homology to \( \text{Gm13g18960} \).

Cosegregation analysis was performed using allele-specific primers designed for both \( \text{PvMrp1} \) and \( \text{PvMrp2} \) to verify if one of the two genes is associated with the \( \text{lpa} \) phenotype. For \( \text{PvMrp1} \), gene-specific amplifications of 140 bp for the \( \text{Mrp1\,(280-10)} \) allele and 125 bp for the \( \text{wt \,Mrp1\,(Jalo EEP558)} \) allele were obtained using pol_19.03f and pol_19.04r primers. For the \( \text{PvMrp2} \) gene, gene-specific amplifications of 131 bp for the \( \text{Mrp2\,(280-10)} \) allele and 122 bp for the \( \text{Mrp2\,(Jalo EEP558)} \) allele were obtained using pol1F and pol1R primers. The genotyping, performed on 173 \( \text{lpa} \) mutants, from the described \( \text{F}_2 \) mapping population, showed that all the samples analysed were homozygous for the \( \text{Mrp1\,(280-10)} \) allele, thus indicating the \( \text{PvMrp1} \) gene as a likely candidate for the \( \text{lpa} \) mutation (Fig. 3a). Conversely, in the same mutant individuals, the \( \text{PvMrp2} \) alleles showed independent assortment.

To further confirm this finding, the same strategy used for mapping the \( \text{lpa1} \) mutation was used for the \( \text{PvMrp2} \) sequence. Both bulks were amplified using the 19 polymorphic SSRs (Table S1). Since the \( \text{BM210} \) and \( \text{PV35} \) markers showed a clear bias toward a sequence-specific band in bulk B (homozygous for the 131 bp band) as compared with bulk A (homozygous for the 122 bp band), the \( \text{PvMrp2} \) sequence could be located on chromosome 7. \( \text{PvMrp2} \) was then mapped by analysis of the distribution of \( \text{BM210} \) and \( \text{PV35} \) markers in 145 individuals from the \( \text{F}_2 \) segregating population, and its position was estimated at a distance of 3 cM from \( \text{BM210} \) and 21.9 cM from \( \text{PV35} \) (Fig. 3b).

\( \text{PvMrp1} \) (\( \text{Lpa1} \)) and \( \text{PvMrp2} \) genes codes for highly orthologous proteins

Based on sequence data of soybean \( \text{Gm03g32500} \), \( \text{Gm19g35230} \) and \( \text{Gm13g18960} \) MRP genes, the bean \( \text{PvMrp1} \) and \( \text{PvMrp2} \) genes and their complementary DNA (cDNA) were isolated from both \( \text{lpa1\,(280-10)} \) and \( \text{wt} \) plants. Genomic and cDNA sequencing showed that \( \text{PvMrp1} \) is composed of 11 exons and 10 introns, and according to the deduced open reading frame, encodes a protein of 1538 amino acids (Fig. 4a). Sequencing of \( \text{PvMrp2} \) is not complete yet, but, according to results obtained thus far, it shares the same intron-exon structure with \( \text{PvMrp1} \) (Fig. 4a). Both genes have the same intron-exon structure of their soybean homologues and the main difference is the length of corresponding introns (Fig. 4a,
compare \( \text{PvMrp1} \) with \( \text{Gm03g32500} \) and \( \text{Gm19g35230} \) and \( \text{PvMrp2} \) with \( \text{Gm13g18960} \).

\textit{In silico} conserved domain analysis (Marchler-Bauer et al., 2009) confirmed the presence of typical MRP modular structures, comprising two transmembrane domains (\text{ABC_transmembrane}) each followed by a nucleotide-binding domain (\text{ABCC_MRP domain}) (Fig. 4b) (Rea, 2007).

Sequence comparison between the candidate Mrp1 (280-10) and wt \( \text{MrpI} \) (905) alleles showed a single base pair change (G/C-A/T transition) in the sixth exon (Fig. 4a), causing an amino acid change from Glu to Lys at position 1155. In order to confirm this point mutation, the surrounding sequence (amplicon 19.R, Table S2) was amplified and sequenced from 50 different bean genotypes of our collection; no polymorphisms were found (data not shown). Moreover, a BLASTp search and subsequent multiple alignment using the translated \( \text{PvMrp1} \) protein against the green plants database, showed that the Glu\(_{1155} \) residue is highly conserved, present in 99 out of the 100 most similar hits (in the other it was replaced by Asp).
Interrogation of the Conserved Domain Database tool at the National Center for Biotechnology Information (NCBI) located the mutation in the second ABC_transmembrane domain. Moreover, alignment of the peptide sequence of \textit{PvMrp1} with the three-dimensional structure of the model ABC transporter mouse P-glycoprotein (PDB ID: 3G5U_A) suggests that Glu1155 locates on the inside face of the transmembrane domain, towards the cytosolic side, close to the second ATP binding domain (Fig. S2).

The discovery of the highly similar paralogue \textit{PvMrp2} gene raised the question whether it was expressed in developing seeds. Semiquantitative RT-PCR of \textit{PvMrp1} and \textit{PvMrp2} expression in wt and \textit{lpa1}(280-10) developing seeds as well as in leaves and roots showed that both genes are expressed during seed development, roots, leaves (Fig. 5) and other green tissues (data not shown), suggesting that both \textit{PvMRP1} and \textit{PvMRP2} proteins are synthesized in all the tissues and developmental conditions analysed. Interestingly, \textit{Pvmrp1} and \textit{PvMrp2} expression appears to be differently regulated in leaves and roots of \textit{lpa1} plants, compared with the wt, indicating that further investigation is needed to address this point.

The \textit{lpa1} mutation causes a negative feedback regulation on the phytic acid pathway

Analysis of phytate content in developing seeds revealed that \textit{InsP6} was not detected at any time during seed development in \textit{lpa1}(280-10), while, by contrast, phytate accumulation was already evident in the wt at 18 DAF (Fig. 1a,b). Suppressed ion conductivity is a much less sensitive technique than radiolabelling, and thus, while we did not detect accumulation of phytate, even in storage tissue, the \textit{lpa1}(280-10) mutant still retains the ability to synthesize \textit{InsP6}, as shown by \textit{in vivo} labelling of mature leaves and developing seeds with \textit{myo-[2-3H]inositol} (Fig. 6). The low concentrations of radiolabelled \textit{InsPs} achieved precluded their stereoisomeric analysis. We think it possible that the major peak of labelling evident at the retention time of 26 min for developing beans of both wt and \textit{lpa1}(280-10) is an \textit{InsP2}.

The lack of substantial accumulation of phytate in seeds of \textit{lpa1}(280-10) indicates the existence of regulatory mechanisms to control the accumulation of cytosolic phytate that might involve negative feedback, whereby \textit{InsP6} might function as a feedback signal to repress its own biosynthetic pathway, as has been suggested by Shi \textit{et al.} (2007).

To confirm or deny this hypothesis we performed a transcriptional analysis of a set of structural genes, shown to be involved in \textit{InsP6} synthesis in various plants, during wt and \textit{lpa1}(280-10) seed development (Fileppi \textit{et al.}, 2010). We also undertook a protein blot analysis for MIPS using antibodies developed against recombinant \textit{PvMIPSs} protein.
In addition, each considered gene was sequenced as both cDNA and genomic DNA in order to exclude the occurrence of ethane methyl sulfonate (EMS)–induced point mutations in the \( \text{lpa1}(280-10) \) mutant. None of the analysed genes showed sequence differences between wt and \( \text{lpa1}(280-10) \) line (data not shown). However, at the transcriptional level there was a broad effect of the mutation on key genes for the synthesis of \( \text{InsP}_6 \). Some genes (\( \text{PvMIPSs} \), \( \text{PvIMP} \), \( \text{PvIPK2} \) and \( \text{PvIPK1} \)) were downregulated in the mutant compared with the wt, although to different extents. In fact, while \( \text{PvMIPSs} \) and \( \text{PvIPK1} \) expression was slightly reduced in the mutant, \( \text{PvIMP} \) and \( \text{PvIPK2} \) were more repressed (Fig. 7). In the absence of detailed analysis of metabolite pool sizes and of the pathway flux in mutant and wt, it is particularly difficult to predict the influence of the \( \text{lpa1}(280-10) \) mutation on individual reactions catalysed by enzymes encoded by genes with reduced transcripts. For instance, protein blot analysis of \( \text{PvMIPS} \) expression showed that, although the encoding gene is slightly down-regulated in the \( \text{lpa1}(280-10) \) mutant, this does not hold true for the protein, which was detectable at the same level in both mutant and wt developing seeds (Fig. 3Sb). Our interpretation is further hampered by limited evidence of the physiological substrate(s) of these enzymes, particularly in the case of inositol phosphate kinases.

Enhanced sensitivity of \( \text{lpa1}(280-10) \) seeds toward ABA-mediated germination

To verify if the low amount of \( \text{myo}-\text{inositol} \) found in the \( \text{lpa1}(280-10) \) seeds was correlated to their response to ABA during seed germination, we carried out germination tests. Our results indicate that after 3 d of germination, the \( \text{lpa1}(280-10) \) mutant displayed 35% inhibition of germination at 50 \( \mu \text{M} \) of ABA, compared with a figure of 84% for the wt, thus indicating an increased sensitivity to ABA of the mutant (Fig. 8).

Discussion

Several \( \text{lpa} \) mutants have been isolated in many crops and for a number of them the mutated gene has been identified.

\( \text{Fig. 7} \) Expression analysis of genes of the phytic acid pathway during wild-type (wt) and \( \text{lpa1}(280-10) \) seed development. For Northern blot analyses, total RNAs (10 \( \mu \text{g} \) for each sample) from wt and \( \text{lpa1}(280-10) \) developing seeds (from 6 to 24 d after flowering (DAF)) were loaded on to the same gel and blotted on the same membrane. This membrane was then hybridized with the different probes as follows: \( \text{PvMIPSs} \), \( \text{PvIMP} \), \( \text{PvMIK} \), \( \text{PvIPK2} \) and \( \text{PvPHSL} \). The latter one, coding for phaseolin storage protein, was used as an additional control. In fact, we assumed that phaseolin expression would not be affected by the \( \text{lpa1}(280-10) \) mutation. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed on \( \text{PvITPK}\alpha \), \( \text{PvITPK}\beta \), \( \text{PvIPK1} \) and 18S rRNA. For each gene the upper panel refers to the wt, while the lower one refers to the \( \text{lpa1}(280-10) \) mutant.

\( \text{Fig. 8} \) Abscisic acid (ABA) dose–response curve for germination inhibition of wild-type (wt, black line) and \( \text{lpa1}(280-10) \) (grey line) seeds. Reported percentages of germination were recorded after 3 d of germination in the presence of different ABA concentrations (2.5, 5, 25, 50, 100, 250 \( \mu \text{M} \)). Bars indicate standard errors of the means from three independent experiments, each consisting of 100 seeds.
Among the stronger lpa mutants are those carrying a defective InsP₆ transporter; however, the effects of each mutation on plant physiology may be quite different.

Here, we provide evidence that the bean lpa1(280-10) mutant, which is viable and agronomically robust, carries a defective PvMrp1 gene. This mutation was previously reported to result in an almost complete block of InsP₆ accumulation and a 25% reduction in seed raffinosaccharide content (Campion et al., 2009). Furthermore, we detected a 30% reduction of myo-inositol content that may underlie the ABA hypersensitive response found during lpa1(280-10) seed germination. These data, together with the results of the expression analyses of genes involved in InsP₆ biosynthesis, demonstrate that the mrp1 mutation accompanies a general repression of its pathway. We propose that failure to accumulate phytate in storage organelles has consequences for cytosolic InsP₆ that, directly or indirectly, reduces its biosynthesis through a negative feedback mechanism which manifests in altered myo-inositol content of mature seeds.

Common bean and soybean contain two MRP genes orthologous to known InsP₆ transporters

Because of the high degree of synteny existing between soybean and common bean, we could map and identify the mutated gene. Furthermore, we demonstrated the existence of a second highly conserved MRP orthologous gene (Gm13g18960 and PvMrp2) in these two crops. The proteins coded by these two orthologues share > 80% similarity with PvMRP1 orthologues, a much higher similarity than that shared with AtMRP5 and ZmMRP4 proteins (Fig. S4). This finding strongly suggests that the MRPs protein might be an InsP₆ transporter too. However, despite the coexpression of PvMrp1 and PvMrp2 in developing seeds, mutation of PvMrp1 is sufficient to confer a lpa phenotype on the lpa1(280-10) mutant. Although PvMrp2 does not complement the lpa1(280-10) mutation in seeds, even if it shares to some extent the expression pattern with PvMrp1, a hypothetical functional similarity cannot be excluded. A different post-transcriptional regulation or intracellular localization could still explain the lack of functional complementation between the two MRP paralogues in seeds. As for other tissues, a detectable phenotype of the mutated Pvmrp1 could not be found. In this regard, the lack of negative pleiotropic effects that lpa mutants often displayed in other species could be explained by a PvMrp2 functional complementation. In fact, according to the phylogenetic analysis of the MRPs homologues (Fig. S4), the paralogous duplication that generated PvMrp1 and PvMrp2 occurred in a common ancestor of both common bean and soybean, and not in other species, such as the cereals, which display impairing pleiotropic effects when their single MRP homolog is mutated. Similar conclusions can be drawn for the soybean mutant CX1834, in which it has been shown that mutations in Gma03g32500 and Gma19g35230 genes, orthologous of PvMrp1, are sufficient to confer a lpa phenotype. However, we cannot further compare the two species, since no expression data of Gma13g18960, the PeMrp2 orthologue, are available in soybean. Moreover, while we have no data on PvMRP2 protein synthesis in developing seeds, further characterization of this second gene may yet reveal novel functions for InsP₆ transporters and thus this gene deserves much attention.

Sequencing data showed that the PeMrp1 gene underwent a nucleotide substitution that predicts an amino acid change from Glu₁₁₅₅ to Lys₁₁₅₅ in a highly conserved position in plant MRPs proteins. Based on the alignment with the structure of the mouse P-glycoprotein, a multiple drug resistance (MDR) transporter of the ABC superfamily (Aller et al., 2009), this amino acid substitution is presumably located in the inner face of the second transmembrane domain toward the cytosolic side. Considering that the effect of this mutation is an almost complete disappearance of phytate in lpa1(280-10) bean seeds, we may speculate that the dramatic substitution, from an acidic to a basic residue, has occurred in a position that might be involved in ligand interaction, relevant for the recognition and/or transport of InsP₆. Detailed studies on the structure of PvMRP1 (or orthologous proteins from other species) are needed to address the role of Glu₁₁₅₅ in the transport of InsP₆.

Effects of PvMrp1 mutation on inositol phosphate metabolism and the role of myo-inositol

The evidence collected demonstrates that in lpa1(280-10) seeds and leaves, InsP₆ is still synthesized. In the absence of transport we would expect InsP₆ to accumulate in the cytosolic compartment. We were, however, unable to detect InsP₆ at any stage of seed development by suppressed ion conductivity (Figs 1, 6). It is worth noting that, while others (Bentsink et al., 2003) have reported measurement of InsP₆ in nonstorage tissues (e.g. leaves by suppressed ion conductivity), our own analysis of leaf tissue of Atmrp5 and bean lpa1(280-10) did not allow us to distinguish phytate from interferences.

Considering cytosolic phytate, Veiga et al. (2006) made a theoretical estimate of phytate solubility and set the limiting value at a concentration of 49 μM, above which any InsP₆ precipitates as magnesium phytate. This concentration is well below the one present in the globoids of mature seeds, the vacuolar subcompartment in which InsP₆ is normally stored. Assuming that the plant cell cannot accumulate and/or manage large amounts of phytate salts in the cytosol, it is likely that cytosolic phytate concentrations are regulated, in part, by degradative processes, mediated by the action of phosphatases and phytases, which, together with
the synthetic process, modulates turnover. This is evident in
the stereoisomeric profile of InsPs identified, for example, in
aleurone tissue of imbibing de-embryonated barley grains
(Brearley & Hanke, 1996) in which the radiolabelled inter-
mediates were identical to the products of in vitro phytate
degradation by bran phytases (reviewed in Irving, 1980). It
is therefore possible to speculate that any regulation of turn-
over of cytosolic phytate, by InsP₆ dephosphorylation and/or
negative feedback on InsP₆ synthesis, is already operable in
the micromolar range.

Coelho et al. (2005) showed that InsP₆ synthesis in
developing bean seeds is maximal at 22 d after pollination,
suggesting that InsP₆ synthesis begins early in seed develop-
ment. Newly synthesized InsP₆ is expected to increase with
the progression of seed development and if it cannot be
stored in the cytosol as phytate salts, it may be degraded to
myo-inositol and free phosphate. We speculate that this
could cause a transient increase in myo-inositol that will
negatively regulate the expression of genes and/or enzymes
controlling InsP₆ biosynthesis as well as the myo-inositol
cellular pool, as reported to occur in Catharanthus roseus
(Mitsuhashi et al., 2005). A 31–34% reduction in magnesium
content in Arabidopsis Atmrs5 mutants has been
reported by Nagy et al. (2009). Since Mg²⁺ is known to be
an essential activator of myo-inositol-phosphate mono-
phosphatase (IMP) (Atack et al., 1995; Islas-Flores &
Villanueva, 2007), it is possible that a decrease of the intra-
cellular concentration of Mg²⁺ may further affect IMP
activity/expression.

One interpretation of our data is that InsP₆, or a product
of its turnover, regulates the transcription of genes coding
for key enzymes of phytic acid pathway, as already suggested
by Shi et al. (2007). In maize lpa1-1 and its allelic mutant
lpa1-241, a consistent reduction of MIPS gene expression
was reported (Raboy et al., 2000; Pilu et al., 2003). We
observed a slight reduction of PvMIPS transcript and no
changes in MIPS protein expression (Fig. 3S). Conversely,
we found a strong inhibition of PmIMP transcription that
would be expected to strongly reduce myo-inositol content.
The fact that the myo-inositol content of mature lpa1(280-
10) seeds is only 30% lower than in the wt, may be
explained if myo-inositol concentrations are raised by a
futile cycle in which InsP₆ is continuously synthesized and
dephosphorylated to myo-inositol (Fig. 9). In this con-
text, the presence of lower InsPs which retain the 2-
phosphate are diagnostic markers of InsP₆ turnover
(Brearley & Hanke, 1996). The limited extent of labelling
of lower InsPs that we observed, however, precluded stere-
omic analysis.

Reduction of phytate accumulation in many lpa mutants
is often correlated with pleiotropic effects which frequently
affect seed germination and field emergence, seed filling,
biotic and abiotic stress responses and protection from ox-
dative stress (Keller et al., 1998; Klein et al., 2003; Meis
et al., 2003; Pilu et al., 2005; Bregitzer & Raboy, 2006;
Guttieri et al., 2006; Murphy et al., 2008; Doria et al.,
2009; Meng et al., 2009). Changes in root development
and root hair growth, phosphate sensing, ABA and IAA

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**Fig. 9** Proposed model for myo-inositol recycling in the lpa1(280-10) mutant and effect of the lpa1(280-10) mutation on phytic acid and raffinosaccharides pathways. The lpa1(280-10) mutant has a
Ins(1,2,3,4,5,6)P₆ defective transporter (indicated with a black cross). However, InsP₆ is still synthesized, but, since it cannot be stored correctly in the vacuole, it is
degraded by phytases back to myo-inositol, giving rise to a futile cycle (grey dotted line). This
causes a negative feedback regulation of key genes of phytic acid pathway and a
consequent decrease of myo-inositol and raffinosaccharides (grey boxes). Light and
dark grey boxes indicate lower and higher transcriptional repression, respectively. PtdlS,
phosphatidyl inositol phosphate synthase; Ptdl4K, phosphatidyl inositol 4-kinase;
Ptdl5K, phosphatidyl inositol 5-kinase; PtdlInS, phosphatidyl inositol; PtdIns(4)P₁,
phosphatidyl inositol 4-phosphate; PtdIns(4,5)P₂, phosphatidyl inositol 4,5-
biphosphate.
response have also been reported (Gaedeke et al., 2001; Klein et al., 2003; Stevenson-Paulik et al., 2005). Nagy et al. (2009) showed that, as for wt, phytate synthesis in Admrp5 seedlings, recorded as incorporation of myo-[³H]-inositol, was reduced under P₃-limiting conditions; while Murphy et al. (2008) showed the converse, that phytate accumulation in potato tubers was enhanced by supplementation of myo-inositol. Mitsuhashi et al. (2005) showed that vacuolar phytate accumulation is enhanced in suspension cultures of both Catharanthus roseus and Arabidopsis in conditions of elevated external phosphate supply. All these pleiotropic effects reveal complex integration of inositol, phytate and phosphate status. They are not unexpected considering that myo-inositol, its phosphorylated derivatives and InsP₆ itself play a central role in several metabolic processes and in signal transduction (Loewus & Murthy, 2000). Indeed, myo-inositol biosynthesis and conversion must be a highly controlled and regulated process in order to meet the requirements of the different pathways in which this compound is involved.

The expression and protein blot data for PeMIPS do not suggest major differences in myo-inositol-3-phosphate content of mature wt and lpa1(280-10), while the strong repression observed for PeMIP gene seems to be in agreement with the reduction in myo-inositol detected in mature lpa1(280-10) seeds (Fig. 2). This reduction is similar to that observed in maize lpa1-241 (Pilu et al., 2003). Interestingly, the maize lpa1-1 mutant, allelic to lpa1-241, accumulates more myo-inositol than wt seeds (Shi et al., 2007). A similar behaviour was found for the two allelic mrp5 mutants of rice, Os-lpa-XS110-2 and Os-lpa-XS110-3, which have lower and higher myo-inositol than wt, respectively (Xu et al., 2009). Alterations in myo-inositol content have also been reported in other lpa mutants: mkk mutants, such as maize lpa3 and rice Os-lpa-XS110-1 and lpa N15-186, contain higher seed myo-inositol (Shi et al., 2005; Kim et al., 2008; Xu et al., 2009); conversely decreased myo-inositol is typically found in mips transgenics; mips and vtc4 mutants (Keller et al., 1998; Hitz et al., 2002; Torabinejad et al., 2009; Donahue et al., 2010). These findings indicate complex regulation of seed myo-inositol content, which, presumably, reflects the integration of a number of metabolic pathways which draw on myo-inositol and myo-inositol-3-phosphate. We conclude that the different content of seed myo-inositol does not seem to correlate exclusively with the strength of the lpa phenotype, suggesting that the regulation of myo-inositol content might be more complicated than expected and should involve several as yet unknown factors.

What is clear from our results and recently published papers on Arabidopsis mips1 and vtc4 mutants and AtPAP15 overexpressing lines, is that the concentration of myo-inositol found in mature seeds correlates with the response to ABA during seed germination (Zhang et al., 2008; Torabinejad et al., 2009; Donahue et al., 2010). In these mutants, myo-inositol changes are directly correlated with changes in ascorbic acid content and the displayed altered response to ABA is purported to depend on the capacity of the mutants to cope with reactive oxygen species (ROS). In agreement with this hypothesis is the finding that the maize lpa1-241, which has a 40% decrease in seed myo-inositol, also shows increased sensitivity to oxidative stress (Doria et al., 2009). We do not have specific data on the response of bean lpa1(280-10) to oxidative stress; however, we showed that this mutant is viable, shows no negative pleiotropic effects affecting traits of agronomic relevance and does not differ from wt plants in the response to drought stress (D. Panzera et al., unpublished). Experiments are in progress aiming to assay myo-inositol, myo-inositol monophosphate and ascorbic acid content both in leaves and during the entire course of seed maturation.

In conclusion, our results provide evidence that contrasting pleiotropic effects of lpa mutants carrying a defective InsP₆ transporter are linked to the myo-inositol content.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Representation of the synteny in genomic regions between PV31 and PV133 microsatellites and surrounding Glyma03g32500, Glyma13g18960 and Glyma19g35230 MRP genes.

Fig. S2 Molecular structure of the model ABC transporter mouse P-glycoprotein (PDP ID: 3G5U_A) showing the topology of the protein.

Fig. S3 Expression analysis of PvMIPS transcript (a) and protein (b) during wt (upper panel) and lpa1(280-10) (lower panel) seed development (4–26 DAF).

Fig. S4 Rooted neighbour-joining tree showing the phylogenetic relationships of MRP proteins and known phytic acid transporters.

Table S1 Description of the set of 47 SSR markers used in the genetic mapping

Table S2 List of overlapping amplicons of the PvMrp1 and PvMrp2 genomic sequences

Table S3 Name and 5′-3′ sequence of the primers used for sequencing and cosegregation analysis of PvMrp1 and PvMrp2

Methods S1 Production of anti-PvMIPSs antibodies and protein blot analysis.

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