Theor Appl Genet (2008) 117:769–779
DOI 10.1007/s00122-008-0818-z

ORIGINAL PAPER

The rice *OsLpa*1 gene encodes a novel protein involved in phytic acid metabolism

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Received: 28 December 2007 / Accepted: 30 May 2008 / Published online: 20 June 2008

Abstract The rice low phytic acid 1 (*lpa*1) mutant was originally identified using a forward genetics approach. This mutant exhibits a 45% reduction in rice seed phytic acid with a molar-equivalent increase in inorganic phosphorus; however, it does not appear to differ significantly in productivity from its wild-type progenitor. A second *lpa*1 mutant was identified from additional screening for high seed inorganic phosphorus phenotypes. Using a positional cloning strategy, we identified a single candidate gene at the rice *Lpa*1 locus. Sequence analysis of the candidate gene from the *lpa*1 mutants revealed two independent mutations (a single base pair substitution and a single base pair deletion) that confirmed the identification of this candidate as the rice low phytic acid 1 gene, *OsLpa*1. The *OsLpa*1 gene has three splice variants. The location and nature of the two mutations suggests that these lesions only affect the translation of the predicted protein derived from the longest transcript. The proteins encoded by *OsLpa*1 do not have homology to any of the inositol phosphate metabolism genes recently characterized in plants, although there is homology to 2-phosphoglycerate kinase, an enzyme found in hyperthermophilic methanogens that catalyzes the formation of 2,3-bisphosphoglycerate from 2-phosphoglycerate. *OsLpa*1 represents a novel gene involved in phytic acid metabolism.

Introduction

Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate; $InsP_6$) is the primary storage form of phosphorus (P) in seeds where it is found in the form of a mixed salt called phytate and accounts for 65–85% of the total seed P (Raboy 1997). During germination, seed phytases break down phytate into its constituents (myo-inositol, inorganic P, and mineral cations) which are then mobilized to support seedling growth and development (Lott et al. 1995). In yeast, $InsP_6$ regulates of a number of nuclear processes including mRNA export, DNA repair, and RNA editing (York 2006). While $InsP_6$ appears to act primarily as a nutrient reserve in seeds, its function in other plant cells has not been well characterized although recently it has been shown to be involved in calcium signaling in guard cells (Lemtiri-Chlieh et al. 2000, 2003).

Despite its very high concentration in seeds, relatively little is known about the biosynthesis of phytic acid in plants. During the past decade, however, several groups have reported the identification of mutants that exhibit low seed phytic acid (Raboy 2007). This work has been primarily driven by the interest in developing low phytate seed crops since phytic acid cannot be digested by non-ruminant livestock and humans. This property of phytic acid requires the costly supplementation of grain-based animal feeds with phytases and/or P (Brinch-Pedersen et al. 2002) and

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S.I. Kim and C.B. Andaya contributed equally to this work. The mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Communicated by Y. Xu.

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the phytic acid P that is excreted in animal waste can cause pollution of water resources (Sharpley et al. 1994; Raboy 2001). Another attribute of phytic acid that can negatively impact nutrition is its ability to strongly bind mineral cations which prevents their absorption and reduces the nutritional value of unprocessed cereal grains to consumers (Torre et al. 1991; Lopez et al. 2002).

Low phytic acid mutants have been identified in several crop species including maize (Raboy et al. 2000; Pilu et al. 2003; Shi et al. 2003, 2005), rice (Larson et al. 2000; Liu et al. 2007), wheat (Guttieri et al. 2004), barley (Larson et al. 1998; Rasmussen and Hatzack 1998; Dorsch et al. 2003; Bregitzer and Raboy 2006) and soybean (Wilcox et al. 2000; Hitz et al. 2002; Yuan et al. 2007). Although *lpa* mutants are characterized primarily by reduction in seed phytic acid and increases in inorganic phosphorus, some mutants have also exhibited significant accumulation of inositol phosphate intermediates (Shi et al. 2003) or myo-inositol (Shi et al. 2005).

In maize, genes corresponding to three low phytic acid mutants have been identified. The first of these to be cloned was the *Lpa*2 gene which encodes an inositol phosphate kinase belonging to the Ins(1,3,4)P_3 5/6-kinase gene family (Shi et al. 2003). The maize *Lpa*3 gene encodes a myo-inositol kinase (Shi et al. 2005) and, like maize *Lpa*2, is directly involved in the inositol polyphosphate biosynthetic pathway. More recently, isolation of the maize *Lpa*1 gene was reported (Shi et al. 2007). Unlike the *Lpa*2 and *Lpa*3 genes, the *Lpa*1 gene encodes a multidrug resistance protein (MRP) ATP-binding cassette (ABC) transporter and appears to be involved in the transport or accumulation of phytic acid in the seed rather than its biosynthesis.

About 70% of the total seed P in rice is found in the form of phytic acid. Larson et al. (2000) identified the first rice *lpa*1 mutant by screening a population of gamma-irradiation induced mutants derived from the *japonica* cultivar Kaybonnet (KBNT). The homozygous mutant line, KBNT *lpa*1-1, exhibits a 45% reduction in seed phytic acid with a molar-equivalent increase in inorganic P and little change in total seed P, suggesting that the *Lpa*1 gene is involved in phytic acid biosynthesis. Despite a reduced amount of phytic acid, KBNT *lpa*1-1 appears to be comparable to KBNT under standard production conditions (Rutger et al. 2004) suggesting that the rice *Lpa*1 gene may be a good target for modifying seed phytic acid without affecting seed or plant performance. The mutation was initially mapped to the long arm of rice chromosome 2 (Larson et al. 2000) and, subsequently, fine mapped to a region of less than 150 kb using microsatellite and sequence tagged site markers (Andaya and Tai 2005).

We report here the isolation and characterization of a second rice *lpa*1 mutant (DR1331-2). Using a positional cloning approach, we have identified a single candidate at the *OsLpa*1 locus. Sequence analysis of the KBNT *lpa*1-1 and DR1331-2 mutants has revealed mutations in this candidate gene, indicating that the gene is *OsLpa1*. *OsLpa1* encodes three expressed splice variants. Both mutations appear to affect the protein encoded by the longest transcript. The predicted proteins encoded by *OsLpa1* do not have homology to any of the inositol phosphate metabolism genes recently characterized in plants (Shi et al. 2003, 2005, 2007; Stevenson-Paulik et al. 2005; Sun et al. 2007) although there is homology to 2-phosphoglycerate kinase, an enzyme found in hyperthermophilic methanogens (Lehmacher and Hensel 1994). Identification and characterization of the *OsLpa1* gene will contribute to understanding phytic acid metabolism in rice and other plants and provide a foundation for the development low phytate crops.

Materials and methods

Plant materials, phenotyping, and DNA extraction

The Kaybonnet *lpa*1-1 (KBNT *lpa*1-1, also known as KB 1-1) mutant was crossed with the indica cultivar Zhe 733 (*Lpa*1/*Lpa*1) to generate an F_2 mapping population (K/Z F_2) consisting of 576 progeny. F_2 seeds were cut in half and the embryo halves were surface sterilized and germinated on media [0.22% (w/v) Murashige-Skoog basal salts (Sigma-Aldrich, St. Louis, MO), 1% sucrose, and 0.25% Gelrite® (Sigma-Aldrich), pH 5.8]. Seedlings were transferred to the greenhouse for seed production and leaf tissue was collected for DNA extraction. F_2 progeny were genotyped with the microsatellite markers RM3542 and RM482, which flank the *OsLpa*1 locus (Andaya and Tai 2005). The non-embryo half seeds of the recombinant F_2 progeny were then phenotyped using the high inorganic phosphate (HIP) assay (Larson et al. 2000). F_3 seeds were collected from the recombinants and used to confirm the F_2 phenotyping. The K/Z F_2 recombinants were used to fine map the *OsLpa*1 locus.

M_2 seeds from 1,790 M_1 plants derived from gamma-irradiation mutagenesis of the *japonic*a cultivar Drew seeds (provided courtesy of J.N. Rutger) were screened using the HIP assay. Following the initial screening to identify putative mutant lines, the HIP assay was conducted using half-seeds to avoid destroying the embryos of seeds exhibiting the HIP phenotype. DR1331-2, a homozygous Drew mutant line exhibiting a HIP phenotype, was crossed with KBNT *lpa*1-1 (KBNT *lpa*1-1/DR-1331-2) to examine the relationship between the two mutants. The resulting F_1 progeny were confirmed using microsatellite markers and F_2 seeds were phenotyped using the HIP assay. Genomic DNA samples for use in mapping, sequencing, and gel blot analysis were extracted from leaf tissues as described previously except tissues were frozen and mechanically ground (Tai and Tanksley 1990). Chi-square (χ^2) tests were performed to examine the goodness-of-fit between the expected Mendelian ratios for the M_2 and F_2 populations and the segregation data for the *lpa* phenotype and the DNA markers.

Determination of phytic acid and inorganic P content

In order to ascertain if the HIP phenotype observed in DR1331-2 was indicative of a reduction in phytic acid, mature seeds (i.e. brown rice) of DR1331-2 and Drew were analyzed to determine the total phosphorus (total P), phytic acid phosphorus (phytic acid P), and inorganic P (P_i) contents as described by Raboy et al. (1984) with minor modification. Following wet-ashing of 100 mg of ground brown rice, total P was determined by colorimetric assay (Chen et al. 1956). For phytic acid P and P_i, single grains were weighed and ten volumes of 0.4 N HCl were added. Extraction was performed by homogenization for 15 min in a Minibeadbeater-96™ (Biospec Products, Bartlesville, OK) with stainless steel dowel pins (Small Parts, Inc., Miami Lakes, FL). Seed extracts were centrifuged and supernatants were transferred into fresh tubes for both phytic acid and P_i determinations. The total P, phytic acid-P, and P_i of each sample were expressed as P content on a dry weight basis. For seed dry weight, 32 mature seeds per line were weighed to determine the means and standard deviation. All assays were performed multiple times and data were expressed as an average with standard deviation. Statistical significance was evaluated using Student's *t* test ($P = 0.05$). Seeds of KBNT *lpa*1-1 and KBNT were also analyzed for comparison.

High-performance liquid chromatography (HPLC) was performed using a Dionex DX-500 ion chromatography system (Dionex, Sunnyvale, CA) as described previously with minor modifications (Mitsuhashi et al. 2005). Supernatants from seed extracts were passed through 0.2 micron filters prior to loading 30 μL aliquots onto a Dionex IonPac AS11 analytical column (2 × 250 mm) and an IonPac AG11 guard column (2 × 50 mm). A Dionex IonPac ATC-1 column (4 × 35 mm) was used to remove carbon dioxide and carbonate. The inositol phosphates were eluted with a linear gradient from 0 to 60 mM NaOH using a flow rate of 1 ml min^{-1} at room temperature. A Dionex conductivity detector was used with an anion self-regenerating suppressor (ASRS-Ultra II) in an external water mode operating with a current of 300 mA. All standards were purchased from Sigma-Aldrich. The retention times for inorganic phosphate (K_2HPO_4) and the various inositol phosphates were: Ins(2)P_1, 9.5 min; P_i, 15.5 min; Ins(1,4)P_2, 21 min; Ins(1,3,4)P_3, 32 min; Ins(13,4,5)P_4, 33 min; Ins(1,3,4,5,6)P_5, 35 min; and Ins(1,2,3,4,5,6)P_6, 29 min.

Marker development and analysis of recombinants

Single nucleotide polymorphism (SNP) markers were identified by comparing sequence data from the Nipponbare (*japonica*) and 93-11 (*indica*) reference genomes (http://www.gramene.org). Regions (500 bp to 1 kb in length) containing potential SNPs were amplified from genomic DNA of parental lines (KBNT *lpa*1-1 and Zhe733) using primer designed from Nipponbare sequence. Sequence data were obtained by direct sequencing of PCR products that were amplified in 50 μL reactions consisting of 50 ng of genomic DNA template, 0.25 μM primer, 1X PCR buffer (20 mM Tri-HCl pH8.0, 100 mM KCl, 25 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol), 4 μL of 25 mM dNTPs, and 1 U of ExTaq polymerase (TaKaRa Bio USA, Madison, WI). PCR amplifications were performed using the following conditions: denaturation at 94°C for 5 min, 35 amplification cycles of 94°C for 20 s, 55°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 5 min. Four independent PCR reactions for each line were combined and then purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) prior to sequencing. SNP genotyping of recombinants was performed in the same manner. The SNP-7 marker was amplified with the primers SNP-7F: 5′-ATTCATGGTCCCACC GATT-3′ and SNP-7R: 5′-TCGGACACTAAACACAC TTTCC-3′. SNP-14 marker was amplified with the primers SNP-14F: 5′-GATTAGCTCGCCATGAATCACC-3′ and SNP-14R: 5′-CCCAATATTCGCG TACACATTAGC-3′.

DNA sequencing was performed by the UC Davis Sequencing Facility. Sequences were analyzed using the Contig Express program of Vector NTI Advance 10 (Invitrogen, Carlsbad, CA). All primers used in this study were designed manually or using the Primer 3 program (http://www.genome.wi.mit.edu/) and were purchased from Integrated DNA Technologies (Coralville, IA).

Sequence analysis of the *OsLpa*1 candidate gene

Alleles of the *OsLpa*1 candidate gene from both *lpa*1 mutants (KBNT *lpa*1-1 and DR1331-2) and the wild-type cultivars (KBNT and Drew) were analyzed by sequencing genomic DNA. For the analysis of genomic DNA, primers were designed to amplify overlapping, 1 kb fragments using Nipponbare sequence. The overlap between fragment ends was about 250 bp to ensure that any differences between Nipponbare sequences and those of the parental lines were not mistaken for polymorphisms. PCR amplifications were performed using the same conditions as described for SNP marker analysis except annealing temperatures were optimized for each primer pair. To analyze the segregation of the mutant alleles of the *OsLpa*1 gene in selected recombinants, two primers (forward 5′-TGCAAA

CTGTTGCCTAGCTGATGAGA-3′ and reverse 5′ GCATTCACTCATCCGGAATTGTATGG-3′) were designed to amplify a 1 kb fragment of *OsLpa*1 that spans both the mutations identified in KBNT *lpa*1-1 and DR1331-2. Genotyping of the recombinants was performed as described for the other SNP markers. All of the PCR products used for sequencing were purified using QIAquick PCR purification kit (Qiagen) and directly sequenced two to four times using the appropriate primers. Sequences were analyzed as described earlier.

Gene structure of *OsLpa*1

Gene structure of *OsLpa*1 (Os02g57400 locus) was obtained using the FGenesh and GeneMarkHMM prediction algorithms and data from the TIGR rice gene expression database (http://www.tigr.org/). The presence of alternative splicing variants was verified experimentally by reverse transcriptase (RT)-PCR using One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instruction and exon-intron boundaries were verified by sequencing. All the RT-PCR experiments were carried out using total RNA samples isolated from 7-day old seedlings of mutant and wild-type genotypes using TRIzol® reagent (Invitrogen). Specific primers were designed for *OsLpa*1.1 (forward: 5′-ATGGCGGAGGAGGCGCCGCCGC-3′ and reverse: 5′-CTATGCACACGGCAGTTCTGTG-3′; 2,058 bp product), *OsLpa*1.2 (forward: 5′-GTGCATTTTCCAATTGTTCTGTTCAGAA-3′ and reverse: 5′-CTATGCACACGGCAGTTCTGTG-3′; 1,927 bp product), and *OsLpa*1.3 (forward: 5′-GTGCATTTCCAATTGTTCTGTTCAGAA-3′ and reverse: 5′-TCATACTGTAAACACACCAATG-3′; 1,540 bp product).

The full-length cDNA sequence of *OsLpa*1.1 was obtained by carrying out the 5′ and 3′ rapid amplification of cDNA ends (RACE) experiments using 5′ and 3′ RACE kit (Gibco-BRL, Gaithersburg, MD). The first strand of the 5′ RACE was synthesized from total RNA by using a gene specific primer (5′-CTTGAAGGCATGGCGAGCC-3′) and a poly(dC) tail was added according to the manufacturer's instructions. The RACE product was amplified by using a nested primer (5′-GCGGCGGCGGCGCCTCCTCCGCCAT-3′) and an anchor primer. The first strand of the 3′ RACE was synthesized by using total RNA with the oligo(dT) primer according to the manufacturer's instructions. Two successive PCR were carried out to amplify the 3′ RACE products; the first amplification was based on the anchor primer to the poly(A) + tail and a gene specific primer (5′-CTCATGCTGCAGTTCACCAAAG-3′), and the second amplification was performed with a nested primer (5′-AGTCCGACGAGGAATACGACGATCT-3′). The PCR products of 5′ and 3′ RACE were purified from a gel and sequenced directly.

Sequence identity searches were performed using the Basic Local Alignment Search Tool (BLASTX) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Additional homologous proteins were identified from the Gramene database (http://www.gramene.org/). Multiple sequence alignments were generated using the ClustalW program (http://www.ebi.ac.uk/clustalw).

*OsLpa*1.1 gene expression analysis

For the analysis of expression pattern of *OsLpa*1.1, total RNA samples were prepared from the shoots (leaf blade and leaf sheath) and roots of 2 week old seedlings of the variety Nipponbare. Total RNA samples of reproductive tissues were prepared from panicle tissue harvested during grain filling. First-strand cDNA was synthesized by random priming using SuperScript™ III reverse transcriptase according to the manufacturer's instructions (Invitrogen). Specific primer pairs were designed for *OsLpa*1.1 (forward 5′-CTTGCTTGCAGAATTAAGGAGAG-3′ and reverse 5′-GATCAACTCCGTTCTAGGAGGCA-3′; 372 bp product). The rice myo-inositol phosphate synthase (MIPS) (forward 5′-GAGGCTGCCGAGGCCGAGCAGC-3′ and reverse 5′-GAATTGGTTATGTTACAAGGCGAGA-3′; 326 bp product) and actin (forward 5′-GAAGATCACTGCCTTGCTCC-3′ and reverse 5′-CGATAACAGCTCCTCTTGGC-3′; 249 bp product) genes were also analyzed. A sum of 5 μL of single strand cDNA was used in 50 μL PCR reactions as described above and amplifications were performed using the following conditions: denaturation at 94°C for 5 min, 35 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, followed by a final extension of 72°C for 5 min. RT-PCR products were separated and visualized using 1% agarose/1X TAE gels containing ethidium bromide.

Accession numbers

Sequence data from this article can be found in the Genbank Database under the following accession numbers: *OsLpa*1.1 (EU366951), *OsLpa*1.2 (EU366953), and *OsLpa*1.3 (EU366954).

Results

Identification and characterization of a second *lpa*1 mutant

In order to identify additional *lpa*1 mutants, a forward genetic screen was carried out using the HIP assay. Eight M_2 seeds from each of 1,790 gamma-irradiation induced M_1 lines were initially tested. One line was identified as segregating for the HIP phenotype (three mutants to five wild-types). Additional M_2 seeds of this line were tested using half-seeds to facilitate recovery of putative homozygous mutant plants. In total, 47 M_2 seeds of this line, designated DR1331, were assayed and the ratio of mutant to wild-type was 10:37, indicating the HIP phenotype is consistent with a single, recessive gene mutation ($\chi^2 = 0.348$, $df = 1$, P value = 0.5555). The embryo halves of the DR1331 M_2 half-seeds that exhibited the HIP phenotype were germinated and one plant (DR1331-2) was selected for seed increase and genetic analysis.

Comparison of DR1331-2 seed dry weight, total P, phytic acid P, and inorganic P with KBNT *lpa*1-1 and the wild-type cultivars Drew and KBNT indicated that DR1331-2, which exhibits a reduction in seed phytic acid of about 50% compared to Drew, is a low phytic acid mutant (Table 1). The total P of DR1331-2 and KBNT *lpa*1-1 seeds were not significantly different compared to their respective wild types. In addition, HPLC analysis of DR1331-2 produced the same profile as KBNT *lpa1-1* (Fig. 1). Differences in peaks corresponding to phytic acid and inorganic P were observed in comparison to the wild-type cultivars. No accumulation of inositol phosphate intermediates was observed under the conditions used.

To determine the genetic relationship between KBNT *lpa*1-1 and DR1331-2, a cross was made (KBNT *lpa*1-1/DR1331-2) resulting in three F_1 plants, which were verified as hybrids using the microsatellite markers RM164, RM228, and RM333 (data not shown). Twenty-four F_2 seeds from each of the F_1 plants were tested using the HIP assay and all exhibited the HIP phenotype indicating that the mutant gene in DR1331-2 is another *lpa*1 allele.

Fine mapping of the *OsLpa*1 locus

Previously, we reported the fine mapping of the *OsLpa*1 locus using a recombinant inbred line mapping population derived from the cross KBNT *lpa*1-1/Zhe733 (Andaya and Tai 2005). The region was delimited to approximately 47 kb between KN2, a PCR-based marker, and HK3, an RFLP marker. Sequence analysis of the candidate genes in this interval from KBNT *lpa*1-1 did not reveal any mutations (S. Kim and C. Andaya, unpublished results). Given this result, the recombinants and markers used to generate the fine map were re-analyzed. Sequence analysis of the KBNT *lpa*1-1 allele of the KN2 marker did not completely match the genomic sequence. Examination of different annealing temperatures revealed that a higher annealing

Table 1 Comparison of seed phytic acid and inorganic P contents of the rice *lpa*1 mutants and their respective wild-types

Line	Seed dry weight (mg)[a]	Phytic acid P (mg g^{-1} seed)[a, b]	Inorganic P (mg g^{-1} seed)[a, b]	Total P (mg g^{-1} seed)[b, c]
KBNT	15.9 ± 1.1	2.14 ± 0.16	0.21 ± 0.08	3.13 ± 0.46
KBNT *lpa*1-1	15.1 ± 1.8	1.54 ± 0.36*	0.87 ± 0.19*	3.32 ± 0.41
Drew	17.8 ± 1.4	2.35 ± 0.23	0.13 ± 0.06	3.10 ± 0.38
DR1331-2	16.5 ± 1.5	1.22 ± 0.28*	0.77 ± 0.15*	3.04 ± 0.43

* Indicates a significant difference from its respective wild-type by Student's *t* test at the level of $P = 0.05$

[a] Values are means and standard deviation of 32 seeds per line

[b] Total P, phytic acid P and inorganic P are expressed as phosphorus (atomic weight $P = 31$) concentration to facilitate comparison

[c] Values are means and standard deviation of four independent assays

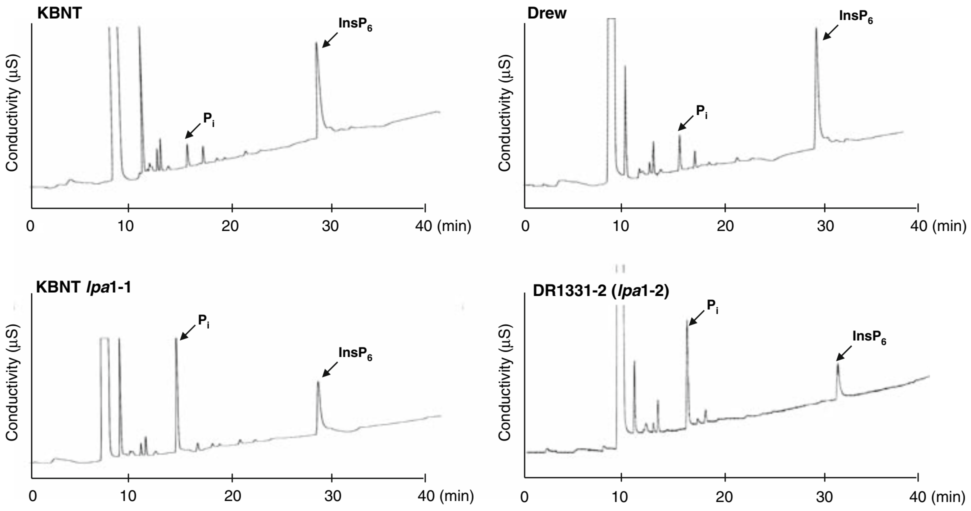


Fig. 1 HPLC analysis of inositol phosphates in the seeds of rice *lpa*1 mutants and their corresponding wild-types

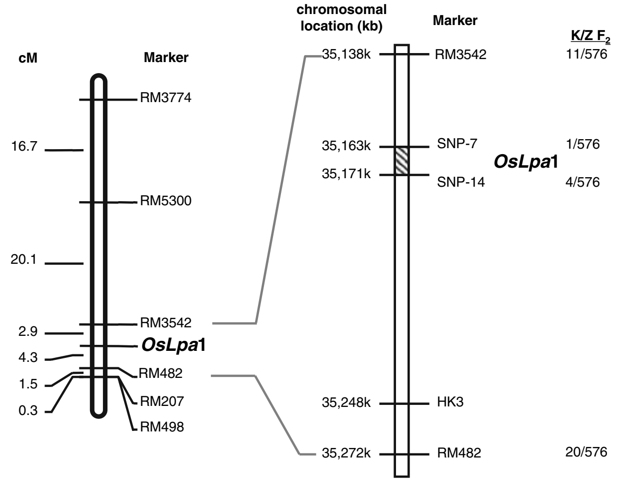


Fig. 2 Genetic and physical map of the *OsLpa*1 locus on rice chromosome 2. The number of recombinants between a marker and the locus is indicated in the far right column. A total of 576 F_2 progeny from a KBNT *lpa*1-1/Zhe 733 cross were examined to identify these recombinants

temperature (60 vs. 55°C) resulted in a shift in amplification of the polymorphic product to that of a monomorphic product that completely matched the genomic sequence at the locus (S. Kim and C. Andaya, data not shown). Based on these findings, the *OsLpa*1 locus resides in the 110 kb interval between microsatellite marker RM3542 and the RFLP marker HK3.

In order to further delimit the locus, recombinants were identified by screening the K/Z F_2 mapping population with the flanking microsatellite markers RM482 and RM3542 (Andaya and Tai 2005) and new markers were developed between RM3542 and HK3. A total of 31 recombinants (20 between *OsLpa*1 and RM482 and 11 between *OsLpa*1 and RM3542) were identified from the population of 576 F_2 individuals (Fig. 2). SNP markers were developed by comparing DNA sequences from KBNT *lpa*1-1 and Zhe733. Two markers, SNP-7 and SNP-14, were identified from this analysis. The SNP-7 marker amplifies a fragment containing one SNP whereas the SNP-14 marker amplifies a fragment with three SNPs. SNP genotyping of the 31 recombinants indicated that the *OsLpa*1 gene is located between SN-7 (one recombinant) and SNP-14 (four recombinants).

Identification and characterization of *OsLpa*1

Based on the Nipponbare genome, the region containing the *OsLpa*1 locus is about 8 kb (Fig. 2) and the available annotation indicates the presence of a single candidate gene, LOC_Os02g57400, which is predicted to have at least three splice variants that encode three different proteins (Fig. 3a). To determine if Os02g57400 is the *OsLpa*1 gene, a 5 kb genomic region spanning the candidate gene was sequenced from both *lpa*1 mutants and their wild-type progenitors. Comparison of KBNT and KBNT *lpa*1-1 sequences revealed a single base pair change (C/G to T/A). Sequencing of the DR1331-2 allele uncovered a single base pair deletion (T/A) compared to the wild-type Drew. Both mutations are located in the predicted coding region of the

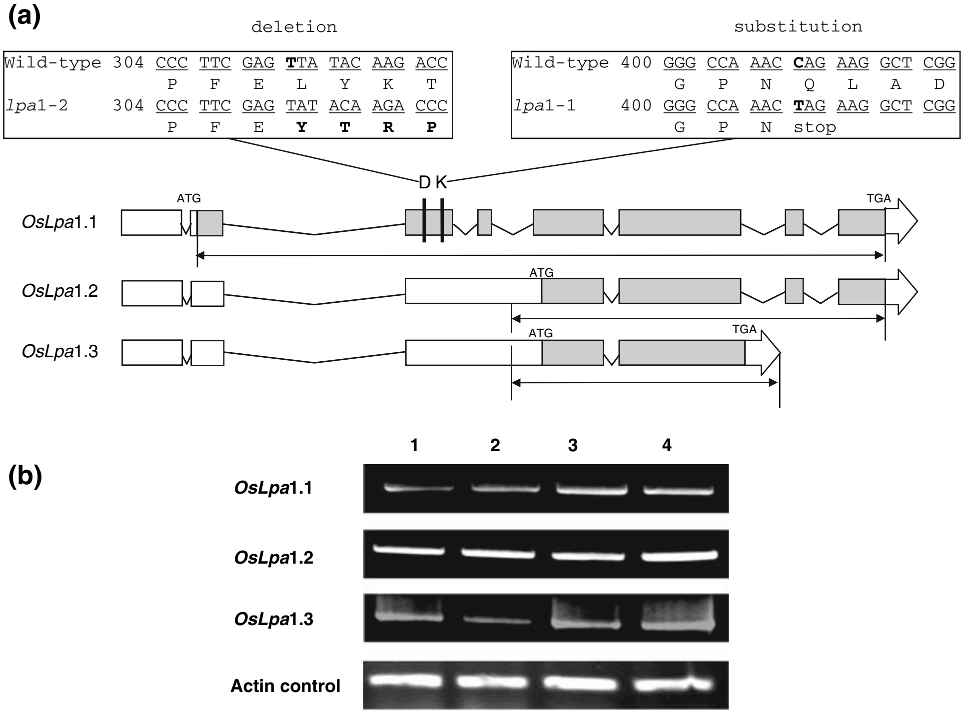
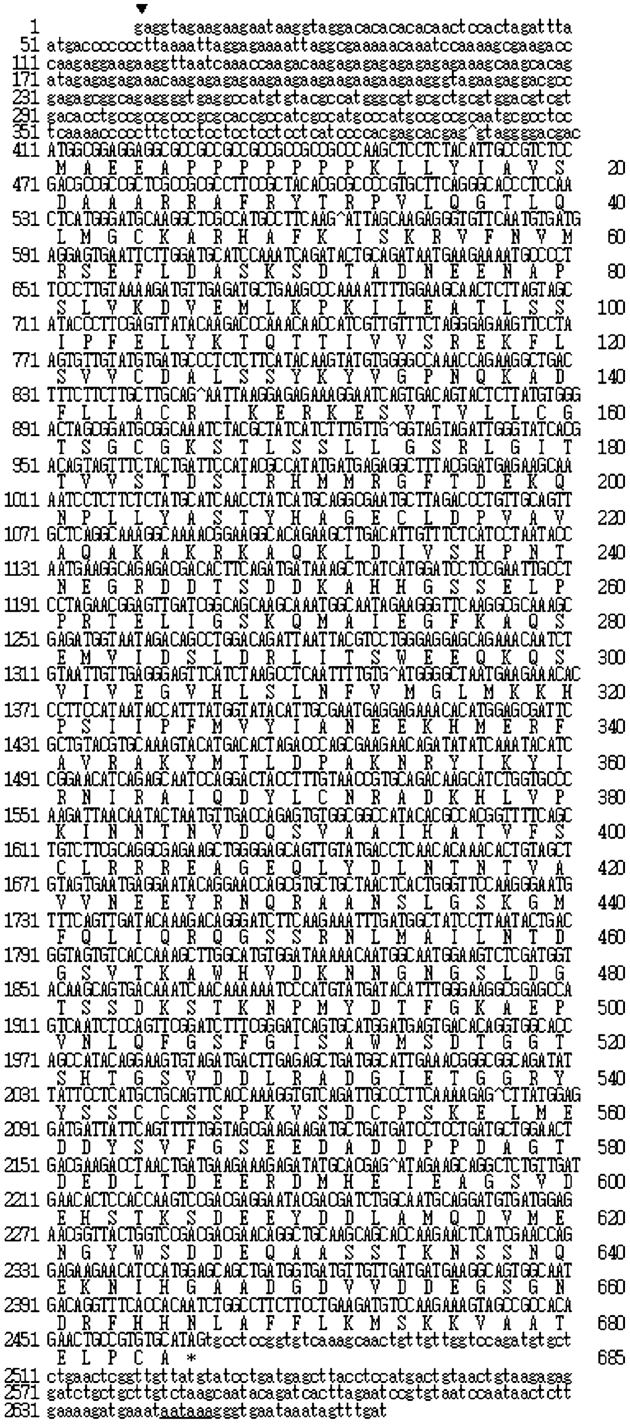


Fig. 3 Gene structure of *OsLpa*1 and evidence for the presence of its transcript variants. **a** Gene structure of *OsLpa*1. *Open boxes* represent 5′ and 3′ UTR, *filled boxes* represent the coding regions, and the *lines* between boxes indicate represent introns. The Kaybonnet *lpa*1-1 (K) and DR1331-2 (D) mutations are in the second exon. Start and stop codons are as indicated. **b** RT-PCR analysis indicates that all three transcripts are expressed (*1* = KBNT, *2* = KBNT *lpa*1-1, *3* = Drew, *4* = DR1331-2). RT-PCR products are indicated (*double headed arrow*) above in (**a**)

largest transcript designated *OsLpa*1.1 (Fig. 3a). The *lpa*1-1 mutation is located at position 409 (relative to the predicted ATG). This single base pair change results in a nonsense mutation. The DR1331-2 mutation is located at position 313 (relative to the predicted ATG), and therefore, is predicted to result in a frame shift that truncates the protein.

To verify the gene structure of *OsLpa*1.1, full-length cDNA sequence was obtained by sequencing an *OsLpa*1.1-specific RT-PCR product and the 5′ and 3′ RACE products. Sequence analysis confirmed the predicted gene structure. The 5′ UTR region of *OsLpa*1.1 is 410 bp long and a conserved poly A signal was detected in the 3′ UTR (200 bp) followed by a poly A tail (Fig. 4). The *OsLpa*1.1 open reading frame is predicted to encode a protein of 685 amino acids (aa). RT-PCR analysis using primers specific to each of the two shorter splice variants confirmed their expression in addition to *OsLpa*1.1 (Fig. 3b) and expressed sequence tags (EST) specific to each of the three transcripts were also identified from publicly available rice expression data (http://www.tigr.org/). No significant difference was observed in expression of each of the transcripts in seedlings of the *lpa*1 mutants and their respective wild-types (Fig. 3b). According to the annotation and gene prediction algorithms, the predicted start codon encoded by the two shorter splice variants, *OsLpa*1.2 and *Os.Lpa*1.3, is located at position 571 from the predicted *OsLpa*1.1 start codon and is in the same frame. Given the location of the *lpa*1 mutations, neither OsLPA1.2 nor OsLPA1.3 should be affected.

Sequence identity searches against the NCBI protein database revealed that OsLPA1.1 has homology to the P-loop kinase domain of 2-phosphoglycerate kinase (2-PGK) found in hyperthermophilic methanogens (Lehmacher and Hensel 1994; Aravind et al. 2000). The alignment of OsLPA1.1 and 2-PGK from *Methanothermus fervidus* is shown in Fig. 5. The homology to 2-PGK extends from aa 146 to aa 409 of OsLPA1.1, which contains both Walker A and B motifs. OsLPA1.2 and OsLPA1.3 are identical to OsLPA1.1 from aa 191 to aa 409 and neither has the Walker A motif. BLASTP analysis detected another rice protein, encoded by LOC_Os09g39870, which has 60% amino acid identity to OsLPA1.1. Os09g39870 is predicted to encode two OsLPA1.1-like proteins as a result of alternative splicing. OsLPA1.1 was also found to be a highly conserved protein among higher plants (Fig. 6). Two proteins in *Arabidopsis* exhibit significant homology to OsLPA1.1. The *Arabidopsis* protein encoded by the At3g45090 gene is 62% identical and the At5g60760 protein shows 60% identity. We recently obtained two *Arabidopsis* lines containing T-DNA insertions in the two homologues. One of these lines containing an insertion in the At5g60760 exhibits a low seed phytic acid phenotype as determined by the HIP assay and HPLC analysis, indicating that the function of OsLPA1.1 is important for seed phytic acid in dicots as well (S. Kim and T. Tai, manuscript in preparation).



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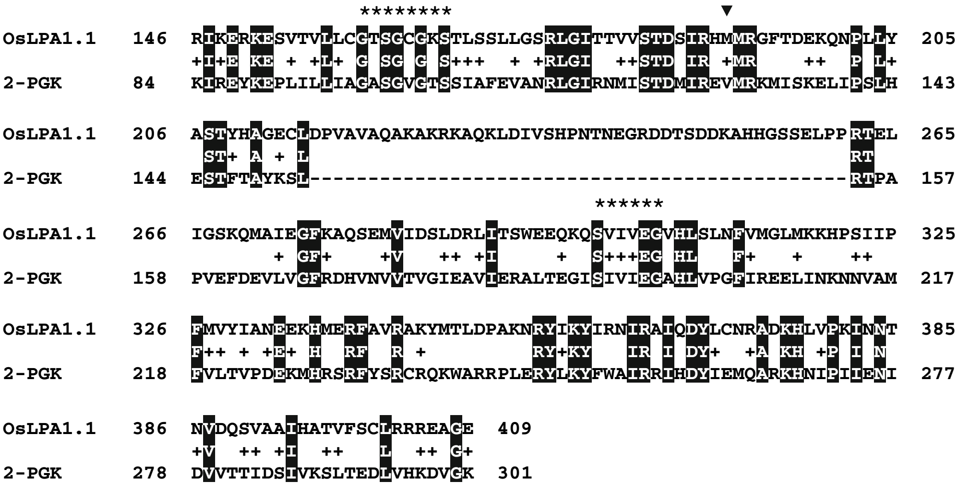
                   ▼
   1            gaggtagaagaagaataaggtaggacacacacacaactccactagattta
  51 atgaccccccccttaaaattaggagaaaattaggcgaaaaacaaatccaaaagcgaagacc
 111 caagaggaagaaggttaatcaaaccaagacaagagagagagagagagaaagcaagcacag
 171 atagagagagaaacaagagagagaagaagaagaagaagaagaagggtagaagaggacgcc
 231 gagagcggcagagggggtgaggccatgtgtacgccatgggcgtgcgctgcgtggacgtcgt
 291 gacacctgccgccgcccgcgcaccgccatcgccatgcccatgccgccgcaatgcgcctcc
 351 tcaaaacccccttctcctcctcctcctcctcatccccacgagcacgag^gtaggggacgac
 411 ATGGCGGAGGAGGCGCCGCCGCCGCCGCCGCCGCCGCCCAAGCTCCTCTACATTGCCGTCTCC
      M  A  E  E  A  P  P  P  P  P  P  P  K  L  L  Y  I  A  V  S    20
 471 GACGCCGCCGCTCGCCGCGCCTTCCGCTACACGCGCCCCGTGCTTCAGGGCACCCTCCAA
      D  A  A  A  R  R  A  F  R  Y  T  R  P  V  L  Q  G  T  L  Q    40
 531 CTCATGGGATGCAAGGCTCGCCATGCCTTCAAG^ATTAGCAAGAGGGTGTTCAATGTGATG
      L  M  G  C  K  A  R  H  A  F  K   I  S  K  R  V  F  N  V  M   60
 591 AGGAGTGAATTCTTGGATGCATCCAAATCAGATACTGCAGATAATGAAGAAAATGCCCCT
      R  S  E  F  L  D  A  S  K  S  D  T  A  D  N  E  E  N  A  P    80
 651 TCCCTTGTAAAAGATGTTGAGATGCTGAAGCCCAAAATTTTGGAAGCAACTCTTAGTAGC
      S  L  V  K  D  V  E  M  L  K  P  K  I  L  E  A  T  L  S  S   100
 711 ATACCCTTCGAGTTATACAAGACCCAAACAACCATCGTTGTTTCTAGGGAGAAGTTCCTA
      I  P  F  E  L  Y  K  T  Q  T  T  I  V  V  S  R  E  K  F  L   120
 771 AGTGTTGTATGTGATGCCCTCTCTTCATACAAGTATGTGGGGCCAAACCAGAAGGCTGAC
      S  V  V  C  D  A  L  S  S  Y  K  Y  V  G  P  N  Q  K  A  D   140
 831 TTTCTTCTTGCTTGCAG^AATTAAGGAGAGAAAGGAATCAGTGACAGTACTCTTATGTGGG
      F  L  L  A  C  R   I  K  E  R  K  E  S  V  T  V  L  L  C  G  160
 891 ACTAGCGGATGCGGCAAATCTACGCTATCATCTTTGTTG^GGTAGTAGATTGGGTATCACG
      T  S  G  C  G  K  S  T  L  S  S  L  L   G  S  R  L  G  I  T  180
 951 ACAGTAGTTTCTACTGATTCCATACGCCATATGATGAGAGGCTTTACGGATGAGAAGCAA
      T  V  V  S  T  D  S  I  R  H  M  M  R  G  F  T  D  E  K  Q   200
1011 AATCCTCTTCTCTATGCATCAACCTATCATGCAGGCGAATGCTTAGACCCTGTTGCAGTT
      N  P  L  L  Y  A  S  T  Y  H  A  G  E  C  L  D  P  V  A  V   220
1071 GCTCAGGCAAAGGCAAAACGGAAGGCACAGAAGCTTGACATTGTTTCTCATCCTAATACC
      A  Q  A  K  A  K  R  K  A  Q  K  L  D  I  V  S  H  P  N  T   240
1131 AATGAAGGCAGAGACGACACTTCAGATGATAAAGCTCATCATGGATCCTCCGAATTGCCT
      N  E  G  R  D  D  T  S  D  D  K  A  H  H  G  S  S  E  L  P   260
1191 CCTAGAACGGAGTTGATCGGCAGCAAGCAAATGGCAATAGAAGGGTTCAAGGCGCAAAGC
      P  R  T  E  L  I  G  S  K  Q  M  A  I  E  G  F  K  A  Q  S   280
1251 GAGATGGTAATAGACAGCCTGGACAGATTAATTACGTCCTGGGAGGAGCAGAAACAATCT
      E  M  V  I  D  S  L  D  R  L  I  T  S  W  E  E  Q  K  Q  S   300
1311 GTAATTGTTGAGGGAGTTCATCTAAGCCTCAATTTTGTG^ATGGGGCTAATGAAGAAACAC
      V  I  V  E  G  V  H  L  S  L  N  F  V   M  G  L  M  K  K  H  320
1371 CCTTCCATAATACCATTTATGGTATACATTGCGAATGAGGAGAAACACATGGAGCGATTC
      P  S  I  I  P  F  M  V  Y  I  A  N  E  E  K  H  M  E  R  F   340
1431 GCTGTACGTGCAAAGTACATGACACTAGACCCAGCGAAGAACAGATATATCAAATACATC
      A  V  R  A  K  Y  M  T  L  D  P  A  K  N  R  Y  I  K  Y  I   360
1491 CGGAACATCAGAGCAATCCAGGACTACCTTTGTAACCGTGCAGACAAGCATCTGGTGCCC
      R  N  I  R  A  I  Q  D  Y  L  C  N  R  A  D  K  H  L  V  P   380
1551 AAGATTAACAATACTAATGTTGACCAGAGTGTGGCGGCCATACACGCCACGGTTTTCAGC
      K  I  N  N  T  N  V  D  Q  S  V  A  A  I  H  A  T  V  F  S   400
1611 TGTCTTCGCAGGCGAGAAGCTGGGGAGCAGTTGTATGACCTCAACACAAACACTGTAGCT
      C  L  R  R  R  E  A  G  E  Q  L  Y  D  L  N  T  N  T  V  A   420
1671 GTAGTGAATGAGGAATACAGGAACCAGCGTGCTGCTAACTCACTGGGTTCCAAGGGAATG
      V  V  N  E  E  Y  R  N  Q  R  A  A  N  S  L  G  S  K  G  M   440
1731 TTTCAGTTGATACAAAGACAGGGATCTTCAAGAAATTTGATGGCTATCCTTAATACTGAC
      F  Q  L  I  Q  R  Q  G  S  S  R  N  L  M  A  I  L  N  T  D   460
1791 GGTAGTGTCACCAAAGCTTGGCATGTGGATAAAAACAATGGCAATGGAAGTCTCGATGGT
      G  S  V  T  K  A  W  H  V  D  K  N  N  G  N  G  S  L  D  G   480
1851 ACAAGCAGTGACAAATCAACAAAAAATCCCATGTATGATACATTTGGGAAGGCGGAGCCA
      T  S  S  D  K  S  T  K  N  P  M  Y  D  T  F  G  K  A  E  P   500
1911 GTCAATCTCCAGTTCGGATCTTTCGGGATCAGTGCATGGATGAGTGACACAGGTGGCACC
      V  N  L  Q  F  G  S  F  G  I  S  A  W  M  S  D  T  G  G  T   520
1971 AGCCATACAGGAAGTGTAGATGACTTGAGAGCTGATGGCATTGAAACGGGCGGCAGATAT
      S  H  T  G  S  V  D  D  L  R  A  D  G  I  E  T  G  G  R  Y   540
2031 TATTCCTCATGCTGCAGTTCACCAAAGGTGTCAGATTGCCCTTCAAAAGAG^CTTATGGAG
      Y  S  S  C  C  S  S  P  K  V  S  D  C  P  S  K  E   L  M  E  560
2091 GATGATTATTCAGTTTTTGGTAGCGAAGAAGATGCTGATGATCCTCCTGATGCTGGAACT
      D  D  Y  S  V  F  G  S  E  E  D  A  D  D  P  P  D  A  G  T   580
2151 GACGAAGACCTAACTGATGAAGAAAGAGATATGCACGAG^ATAGAAGCAGGCTCTGTTGAT
      D  E  D  L  T  D  E  E  R  D  M  H  E   I  E  A  G  S  V  D  600
2211 GAACACTCCACCAAGTCCGACGAGGAATACGACGATCTGGCAATGCAGGATGTGATGGAG
      E  H  S  T  K  S  D  E  E  Y  D  D  L  A  M  Q  D  V  M  E   620
2271 AACGGTTACTGGTCCGACGACGAACAGGCTGCAAGCAGCACCAAGAACTCATCGAACCAG
      N  G  Y  W  S  D  D  E  Q  A  A  S  S  T  K  N  S  S  N  Q   640
2331 GAGAAGAACATCCATGGAGCAGCTGATGGTGATGTTGTTGATGATGAAGGCAGTGGCAAT
      E  K  N  I  H  G  A  A  D  G  D  V  V  D  D  E  G  S  G  N   660
2391 GACAGGTTTCACCACAATCTGGCCTTCTTCCTGAAGATGTCCAAGAAAGTAGCCGCCACA
      D  R  F  H  H  N  L  A  F  F  L  K  M  S  K  K  V  A  A  T   680
2451 GAACTGCCGTGTGCATAGtgcctccggtgtcaaagcaactgttgttggtccagatgtgct
      E  L  P  C  A  *                                             685
2511 ctgaactcggttgttatgtatcctgatgagcttacctccatgactgtaactgtaagagag
2571 gatctgctgcttgtctaagcaatacagatcacttagaatccgtgtaatccaataactctt
2631 gaaaagatgaaataataaaggtgaataaatagtttgat
```

Fig. 4 Nucleotide and amino acid sequence of full length cDNA of *OsLpa*1.1. *Numbers* on the left refer to nucleotides and *numbers* on the right refer to amino acid residues. *Inverted triangle* indicates the transcriptional start site. Nucleotides corresponding to the coding region are shown in *uppercase*. *Wedge symbol* indicates the exon-intron borders, *Asterisk* represents stop codon, and the 3′ Poly A signal is *underlined*



```
                              ********                       ▼
OsLPA1.1   146   RIKERKESVTVLLCGTSGCGKSTLSSLLGSRLGITTVVSTDSIRHMMRGFTDEKQNPLLY   205
                 +I+E KE + +L+ G SG G S+++  + +RLGI  ++STD IR +MR    ++  P L+
2-PGK      84    KIREYKEPLILLIAGASGVGTSSIAFEVANRLGIRNMISTDMIREVMRKMISKELIPSLH   143

OsLPA1.1   206   ASTYHAGECLDPVAVAQAKAKRKAQKLDIVSHPNTNEGRDDTSDDKAHHGSSELPPRTEL   265
                  ST+ A + L                                              RT
2-PGK      144   ESTFTAYKSL----------------------------------------------RTPA   157
                                                   ******
OsLPA1.1   266   IGSKQMAIEGFKAQSEMVIDSLDRLITSWEEQKQSVIVEGVHLSLNFVMGLMKKHPSIIP   325
                        + GF+    +V   ++ +I     +  S+++EG HL   F+   +    ++
2-PGK      158   PVEFDEVLVGFRDHVNVVTVGIEAVIERALTEGISIVIEGAHLVPGFIREELINKNNVAM   217

OsLPA1.1   326   FMVYIANEEKHMERFAVRAKYMTLDPAKNRYIKYIRNIRAIQDYLCNRADKHLVPKINNT   385
                 F++ + +E+ H  RF  R +         RY+KY   IR I DY+  +A KH +P I N
2-PGK      218   FVLTVPDEKMHRSRFYSRCRQKWARRPLERYLKYFWAIRRIHDYIEMQARKHNIPIIENI   277

OsLPA1.1   386   NVDQSVAAIHATVFSCLRRREAGE   409
                 +V  ++ +I  ++   L  ++ G+
2-PGK      278   DVVTTIDSIVKSLTEDLVHKDVGK   301
```

Fig. 5 Sequence alignment of the OsLPA1.1 with the 2-phosphoglycerate kinase (2-PGK) found in *Methanothermus fervidus* (CAA50058). The conserved sequences are found in the P-loop kinase domain of 2-PGK. The P-loop is an ATP/GTP binding site motif found in many nucleotide-binding proteins (Leipe et al. 2003). The characteristic Walker A [GxxxxGK(S/T)] and Walker B (hhhhEG) motifs are indicated with *asterisks*. Identical (highlighted) and similar (+) amino acid residues are as indicated. The first amino acid of OsLPA1.2 and OsLPA1.3 is marked (*inverted triangle*)

Analysis of *OsLpa*1.1 expression

In order to further examine the expression of *OsLpa*1.1, RT-PCR analysis was conducted using total RNA from various tissues including shoot, root and, panicle. Expression of *OsLpa*1.1 was compared to myo-inositol 3-phosphate synthase (MIPS) and actin, which served as a control (Fig. 7). *OsLpa*1.1 transcripts were detected in all the tissues examined, but expression was higher in reproductive tissue than in vegetative tissue. Expression of the MIPS gene, which catalyzes the first step of myo-inositol biosynthesis, was highest in the reproductive tissues followed by leaves. No expression was detected in root tissues.

Discussion

The rice *low phytic acid* 1 (*lpa*1) mutation results in a 45–50% reduction in seed phytic acid with a molar equivalent increase in inorganic P and little change in total seed P (Larson et al. 2000). We report here the cloning of the rice *Lpa*1 (*OsLpa*1) gene by fine mapping of the locus to a region of rice chromosome 2 containing a single candidate gene. Sequence analysis of two independent *lpa*1 mutant alleles confirmed the identification of this gene as *OsLpa*1. The *OsLpa*1 gene is predicted to encode three different proteins (OsLPA1.1, OsLPA1.2, and OsLPA1.3). Based on the location and nature of the mutations found in the two *lpa1* mutant alleles, the largest of these proteins, OsLPA1.1, appears to be necessary for wild-type levels of seed phytic acid in rice. The existence of OsLPA1.2 and OsLPA1.3 and their possible involvement in phytic acid metabolism remains to be determined.

OsLPA1.1 has homology to a 2-phosphoglycerate kinase (2-PGK) found in hyperthermophilic methanogens (Lehmacher and Hensel 1994). This archaeal 2-PGK catalyzes the ATP-dependent phosphorylation of 2-phosphoglycerate to form 2,3-diphosphoglycerate (also known as 2,3-bisphosphoglycerate), which is in turn converted into cyclic 2,3-diphosphoglycerate (cDPG) by cDPG synthetase (Lehmacher et al. 1990). cDPG is the most abundant form of phosphate found in the hyperthermophilic methanogens *M. fervidus*, *Methanothermus sociabilis*, and *Methanopyrus kandleri* (Hensel and König 1988). The biological function of cDPG remains controversial, but the primary role of this compound may be as storage compound for carbohydrate and phosphate (Lehmacher et al. 1990). Another function of cDPG as a thermoadapter in response to environmental factors has also been proposed (Hensel and König 1988). The archaeal 2-PGK consists of an N-terminal ATP-cone regulatory domain and a P-loop-containing kinase domain (Aravind et al. 2000). OsLPA1.1 shares homology to the P-loop domain of 2-PGK, suggesting that this protein functions as a kinase.

OsLPA1.1. does not have homology to the proteins encoded by the maize *Lpa* genes or other genes involved in inositol phosphate metabolism (Shi et al. 2003, 2005, 2007; Stevenson-Paulik et al. 2005; Yoshida et al. 1999). Furthermore, no homologues of significance were found in yeast or humans. Within the rice genome, there is one gene, LOC_Os09g39870, on chromosome 9 that encodes a protein with homology to OsLPA1.1. The existence of this homologue suggests possible overlapping or redundant

```
Vitis           -----------------------------------------MAEVA-----KLLYIVVVD 14
rice(09g39870)  -----------------------------------------MAAVQGQGQGKLLYIVVVD 19
Populus_a       ----------------------------------------MTTEVG-----KVLYIVVVD 15
Populus_b       ------------------------------------------MEVG-----KVLYIVVVD 13
AtLPA1_a        ----------------------------------------MMTEAT-----KVLYIVVRE 15
AtLPA1_b        -----------------------------------------MTETT-----KVMYIVVVD 14
maize_a         MATPTANRRWGEGDTEAGNGSRSGGRWGEETNKAARCAGRHVDAMRGRGVDVVTTRHPPL 60
maize_b         ------------------------------------------------------------
sorghum         --------KRRQGKVGRGHRARQQVGDEAKANKAARRAGGHVDAMRGRGVDAVTTPAARR 52
OsLPA1.1        ----------------------------------------------------MAEEAPPP 8

Vitis           DA-------EKRAGKEKKSFRYTRPVLQTTLQLMG------------------------- 42
rice(09g39870)  D--------------DGATFRYTRSLLHSTLQLMG------------------------- 40
Populus_a       --EEEKRGKGKGK---EESFRYTRPVLQSTLQLMG------------------------- 45
Populus_b       --EEEKRDKGKGKGKGKDSFRYTRPVLQSTLQLMG------------------------- 46
AtLPA1_a        -----EGDDDDNNG--DDSFRYTRPVLQSTLQLMG------------------------- 43
AtLPA1_b        GTDTAETVEEDGTGTWKDSFRYTRPVLQSTLQLMG------------------------- 49
maize_a         LPARTAMAPPPPQCASKRPSAPAPALQVGDMAEEASPSPSPPKLLYIAIADGGVRRAFRY 120
maize_b         ------------------------------------------------------------
sorghum         PPGPHRHG---PAAAAMRLKTPLRASRAPGPPLAKPPSPPTP------------------ 91
OsLPA1.1        PPPPKLLYIAVSDAAARRAFRYTRPVLQGTLQLMG------------------------- 43

Vitis           -------------CKPRHAFKISRRVFEVLRSES------FVDAWRPTGMEASGVGDAEG 83
rice(09g39870)  -------------CKPRHAFEISGRVFDEIRG------------HMGGDMAMGGGG---- 71
Populus_a       -------------CKARHAFKISKRVFEVMRNEF------SNEVSLSKEVEIRVVDASKE 86
Populus_b       -------------CKARHAFKISQRVFELMR-----------SVSHSKEIEITGVDASNG 82
AtLPA1_a        -------------CKARHAFKISRRVFELIRSEG----SCNTSPENGKEPEFAKEVGGST 86
AtLPA1_b        -------------CKARHAFKISRRVFELIRSEGSLILSPSLSPSHSKESEFQKTGDASA 96
maize_a         TRPVLQSTLHLMGCKPRHAFKISKRVFNVMKSEFLA---TSKSDGATKQENYPAFGLGDG 177
maize_b         ---------------------ISKRVFDVMKSEFLA---QSKSDGAGKQENYPALGLGDG 36
sorghum         --------------------LIRPPFVGDMAEEEAP---PPPPPPPPPKLLYIAVADGGG 128
OsLPA1.1        -------------CKARHAFKISKRVFNVMRSEFLD---ASKSDTADNEENAPSLVKDVE 87
                                     *   ..  .

Vitis           CC-EEENNCCTGVSVSXGEVGDLFASQKDDECYAKPFELYKRLTTVVVGRETFFDFVCCV 142
rice(09g39870)  -----------GVQRY-----ELAADAEAASPRQFQFELYKRRTTLLIPRPLFLRLVCHA 115
Populus_a       N--SEREDGLSS--------------GEEDRNKSIPFELYKRRTTVVVRRESFLNVVCDA 130
Populus_b       N--NEKEDGLSSGVFLGKTEVGNSLVSEEDRYKSIPFELYKRRTTVVVRREAFLNFVCDA 140
AtLPA1_a        C--VEKLN--CLVVA-----------GDVDKNKSKPFEMYKRRTTVVVSREIFVDVVCDA 131
AtLPA1_b        CANVEKANKVNSLAT-----------DDVDKSKSKPFEVYKRRTTVVVSREIFVNVVCDA 145
maize_a         ----TDTPKMLERSN-----------------SSVPFELYKNQTTVVVSREEFVSVVCDA 216
maize_b         ----TDTQKMLERSD-----------------SSIPFELYKNQTTVVISREEFVSVVCDA 75
sorghum         RRAFRYTRPVLQSTLH--------------LMGCKPRHAFKNQTTIVVSREEFVSVVCDA 174
OsLPA1.1        ----MLKPKILEATL-----------------SSIPFELYKTQTTIVVSREKFLSVVCDA 126
                                                     . .*  **... *  *. .** .

Vitis           LTEYKYIGPNQRADLALACRLTSVLYCGHXGYXTHLKRTLTGIVSMDGLIKYLGRIRERE 202
rice(09g39870)  LALYKYVAPDQRSDLHRAC-----------------------------------RIRERK 140
Populus_a       LTEYKYVGPNQREDLVLAC-----------------------------------RIRERK 155
Populus_b       LTEYKYVGPNQREDLVLAC-----------------------------------RIRERK 165
AtLPA1_a        LAEYKYVGRDQRADLILAC-----------------------------------RIRERK 156
AtLPA1_b        LAEYKYVGHDQRADLILSC-----------------------------------RIRERK 170
maize_a         LSLYKYVGPNQKADLLLAC-----------------------------------RIKERK 241
maize_b         LSLYKYVGPNQKADLLLAC-----------------------------------RIKERK 100
sorghum         LSLYKYVGPNQKADLLLAC-----------------------------------RIKERK 199
OsLPA1.1        LSSYKYVGPNQKADFLLAC-----------------------------------RIKERK 151
                *. ***.. .*. *.  .*                                   **.**.

Vitis           ESVTVLLCGTSGCGKSTLSTLLAGRLGITTVISTDSIRHMMRSFVDEKQNPLLWASTYHA 262
rice(09g39870)  ESVTILLCGTSGCGKSTLSTLLGSRLGITTVVSTDSIRHMMRSFVEEKQNPLLWASTYHA 200
Populus_a       ESVTVLLCGTSGCGKSTLSALLGNRLGVTTVISTDSIRHMMRSFVDEKQNPLLWASTYHA 215
Populus_b       ESVTVLLCGTSGCGKSTLSALLGSRLGVTTVISTDSIRHMMRSFVDEKQNPLLWASTYHA 225
AtLPA1_a        ESVTVLLCGTSGCGKSTLSALLGSRLGITTVVSTDSIRHMMRSFADEKQNPLLWASTYHA 216
AtLPA1_b        ESVTVLLCGTSGCGKSTLSALLGSRLGITTVVSTDSIRHMMRSFVDEKQNPLLWASTYHA 230
maize_a         ESVAILLCGTSGCGKSTLSSLLGSRLGITTVVSTDSIRHMMRSFADEKQSPLLYASTYHA 301
maize_b         ESVTILLCGTSGCGKSTLSSLLGSRLGITTVVSTDSIRHMMRGFADQKQNPLLYASTYHA 160
sorghum         ESVTILLCGTSGCGKSTLSSLLGSRLGITTVVSTDSIRHMMRGFADEKQNPLLYASTYHA 259
OsLPA1.1        ESVTVLLCGTSGCGKSTLSSLLGSRLGITTVVSTDSIRHMMRGFTDEKQNPLLYASTYHA 211
                ***..**************.**..***.***.**********.*...**.***.******

Vitis           GEYLDPMAVAEAKAKR-KEKKLAHICHSVTK-HEVHDEIAGGKLCTHL---PLNTTELIN 317
rice(09g39870)  GECLDPVAVADAKARR-KAKKRSGISTTST--------IDFDKTRPLN---DKPDGKPIG 248
Populus_a       GEYLDPEAVAEAKAKR-KAKKLAGIANLRSK-DELSDGYTAGKSGSGAPEVTSGTAEFIS 273
Populus_b       GEFLDPVAVAEAKAKR-KAKKLAGTGTLHSK-DEVSDGFTTGKSGSGAPKVSSGATEVIS 283
AtLPA1_a        GEYLDPVAVAESKAKR-KAKKLK---------GSRGVNSNAQKTDAGS---NSSTTELLS 263
AtLPA1_b        GEYLDPVAVAESKARRRRAKKMDSIEDEKAKASEGGKAKNTQQTDVGS---TKNTPVLLS 287
maize_a         GEYLDPIAVAQAKAKR-NANKPTVVSHPNTSGDKDVTSGDKSQHGSSE---LPPRAELIG 357
maize_b         GEYLDPIAVAQAKAKH-KANKPTVVSHPNTCG--GTSSDDKSQQGSSE---LPPRAELIG 214
sorghum         GEYLDPIAVSQAKAKR-KANKPTIVSHPNTSGGKDVTSDDKAQQGSSE---LPPRAELIG 315
OsLPA1.1        GECLDPVAVAQAKAKR-KAQKLDIVSHPNTNEGRDDTSDDKAHHGSSE---LPPRTELIG 267
                ** *** **...**.. . .*                     .               ..

Vitis           VREMAIEGFKAQSEMVIDSLDQLITAWEERKESVIVEGVHLSLNFIMGLMRKHPSIIPFM 377
rice(09g39870)  KKQMAIEGYKAQSEMVIDSLDRLITAWEDRKESVVVEGVHLSLNFVMGLMRKHPSIIPFM 308
Populus_a       PKQMAIEGFKAQSEMVIDSLDRLITAWEERKESVVVEGVHLSLNFVMGLMKKHPSILPFM 333
Populus_b       PKQMAVEGFKAQSEMVIDSLDRLITAWEERKESVVVEGVHLSLNFVMGLMKKHPSIIPFM 343
AtLPA1_a        HKQMAIEGYKAQSEMVIDSLDRLITTWEERNESVVVEGVHLSLNFVMGLMKKHPSIVPFM 323
AtLPA1_b        PKQMAVEGFKAQSEMVIDNLDRLITAWEERKESVIVEGVHLSLNFVMGLMKKHPSIVPFM 347
maize_a         NKQMAVEGYKAQSEMVIDSLDRLITSWEEQKESVIVEGVHLSLNFVMGLMKKHPSIIPFM 417
maize_b         SKQMAVEGYKAQSEMVIDSLDRLITSWEEQKESVIVEGVHLSLNFVMGLMKKHPSIIPFM 274
sorghum         NKQMAVEGYKAQSEMVIDSLDRLITSWEEQKESVIVEGVHLSLNFVMGLMKKHPSIIPFM 375
OsLPA1.1        SKQMAIEGFKAQSEMVIDSLDRLITSWEEQKQSVIVEGVHLSLNFVMGLMKKHPSIIPFM 327
                 ..**.**.*********.**.***.**....**.**********.****.*****.***

Vitis           IYISNEDKHLERFAVRAKYMALDPGKNKYVKYIXNIRTIQAYLCNRADKYLVPKINNTNV 437
rice(09g39870)  IYISDEGKHTERFAVRAKYMTLDPTKNKYVKYISNIRTIQEYLCSRADKYLVPKVNNTNV 368
Populus_a       IYITNEEKHMERFAVRAKYMTLDPAKNKYVKYIRNIRTIQEYLCKRADKHLVPKINNTNV 393
Populus_b       IYITNEDKHLERFAVRAKYMTLDPAKNKYVKYIRNIRTIQDYLCKRADKHLVPKINNTNV 403
AtLPA1_a        VYIANEEKHLERFAVRAKYMTLDPAKNKYVKYIRNIRTIQDYLCKRADKHLVPKINNTNV 383
AtLPA1_b        VYIANEEKHLERFAVRAKYMTLDPEKNKYVKYIRNIRTIQDYLCKRADKHLVPKINNTNV 407
maize_a         VYITNEDKHMERFAVRAKYMTLDPAKNRYIKYIRNIRAIQEYLCNRADKHLVPKINNTNV 477
maize_b         VYITNEEKHMERFAVRAKYMTLDPAKNRYIKYIRNIRAIQEYLCNRADKHLVPKINNTNV 334
sorghum         VYITNEEKHMERFAVRAKYMTLDPAKNRYIKYIRNIRAIQEYLCNRADKHLVPKINNTNV 435
OsLPA1.1        VYIANEEKHMERFAVRAKYMTLDPAKNRYIKYIRNIRAIQDYLCNRADKHLVPKINNTNV 387
                .**..* ** **********.*** **.*.*** ***.** ***.****.****.*****

Vitis           DRSVAAIHATVFSCLRRXEAGENFYDSTTNTVXLIDEEYRNQCAANSLSSKGMFQLIQRK 497
rice(09g39870)  DRSVASIHATVFSCLRRRAAGDQLYDPATNTVAVVNEEYKNQCVANSMSSKGMFKLIQRL 428
Populus_a       DKSVAAIHATVFSCLRRREAGEQLYDPTTNTIALVDEEYRNQCAANSLSSKGMFQLIQRK 453
Populus_b       DKSVAAIHATVFSCLRRWDAGEQLYDPTTNTVALVDEEYRNQCAANSLSSKGMFQLIQRK 463
AtLPA1_a        DKSVATIHATVFGCLRRRETGEKLYDTTTNTVSVIDDEHRNQCAANSLTSKGMFQVIQRQ 443
AtLPA1_b        DKSVAAIHATVFSCLRRREAGEHLYDATTNTVSVIDDEYRNQCTANSLSSKGMFQLIQRK 467
maize_a         DQSVAAIHATVFSCLRRRAAGEQLYDLNTNTVAVVDEEYRNQRAANTLGSKGMFQLIQRK 537
maize_b         DQSVAAIHATAFSCLRRRAAGEQLYDLNTNTAAVVDEEYRNQRAANTLGSKGMFQLIQRQ 394
sorghum         DQSVAAIHATVFSCLRRRAAGEQLYDLNTNTVAVVDEEYRNQRAANTLGSKGMFQLIQRK 495
OsLPA1.1        DQSVAAIHATVFSCLRRREAGEQLYDLNTNTVAVVNEEYRNQRAANSLGSKGMFQLIQRQ 447
                *.***.****.*.****  .*...**  ***  ....*..** .**.. *****..***

Vitis           GSSRHMMALINTDGSVSKAWSVNM-AWGNR--SPTLCQGSKEGVENLMYGPLQITKAEPV 554
rice(09g39870)  GSSRKLMAIVNVDGSVSKAWPVES-SSGDG--KGGSENGSKKYVGDPIYGPLNIGRAESV 485
Populus_a       GSSRHLMALLNTDGSVAKAWPVDS-VDGNG--KPGSGQGTDSGKGIPMYGPLQIGKAEPV 510
Populus_b       GSSRHLMALLNTDGSVAKAWPVDS-VDGNG--KLGTGHGTDSGIGTPMYGPLQIGKAEPV 520
AtLPA1_a        GSSRRFMALCNTDGTVAKTWPVAS-VGKIR--KPVVNTEMDD--GTEH----QLHKAEPV 494
AtLPA1_b        GSSRHLMALLNTDGTFARTWPVTGKVDESG--KPVFCNEMIEENGMEHPVYGYLQKAEPV 525
maize_a         GSSRNLMALLNTDGSVTKAWHVSA-SDGNG-NLNGITNNKKSP-ENPMLDPSQIGKAEAV 594
maize_b         GSSRNLMALLNTDGSVTKAWHVGA-TDGGGENLGGNTSSGKKPDENRVLDASQIGKAEAV 453
sorghum         GSSRNLMALLNTDGSVTKAWHVGT-SDGNG-DLNGITKSRKSA-ENPMLDASQIGKAEAV 552
OsLPA1.1        GSSRNLMAILNTDGSVTKAWHVDK-NNGNG-SLDG-TSSDKST-KNPMYDT--FGKAEPV 501
                ****..**. *.**.....* *                   .           . .**.*

Vitis           NLPFGHFGISAWPSDVGGSSHTLSVNXSRTDEAESRSGXCSSTCRSPRMSDGPA------ 608
rice(09g39870)  NLQFGAFGISAWPTDAGCTSQAGSVNESWDNANEGTGSHVPSSSGSPKKLDGHC------ 539
Populus_a       NLQFGHFGISAWPSD-GGTSHAGSVDESRADGTDTGSRYYSSCCSSPRMVDGAA------ 563
Populus_b       NLQFGNFGISAWPSD-GGTSHAGSVDESRADGTDTGSRYYSSCCSSPRKPDGAA------ 573
AtLPA1_a        NLQFGHFGISAWPSD-GATSHAGSVDDLRADIIETGSRHYSSCCSSPRTSDGPS------ 547
AtLPA1_b        NLQFGLFGISAWPSD-GATSRAGSVDDCKADMAETSSRYYSSCCSSPRMSEGTS------ 578
maize_a         NLQFGPFGISAWMSDTGGTSHTGSVEDLRPDSVETGGRNYSSCCSSPKMSDSTS------ 648
maize_b         NLQFGPFGISAWMSDTGGTSHAGSVEDLRADGVETGGRNYSSCCSSPKTSDSTSKEMLSP 513
sorghum         NLQFGPFGISAWMSDTGGTSHTGSVEDLRADNVETGGRNYSSCCSSPKMSDSTS------ 606
OsLPA1.1        NLQFGSFGISAWMSDTGGTSHTGSVDDLRADGIETGGRYYSSCCSSPKVSDCPS------ 555
                ** ** ****** .* * .*.. **.    .  .  .   .* . **.  .  .

Vitis           KQLKEE-VWVSGSD-------EEVNNSPYMNSDGDLSDVGDRQIHEEIEGSVNEESTKSD 660
rice(09g39870)  KEIKES-AAASGSDDDEEEEEEAADVPPNSGSEEDLSEEDIRAIHEEMEGSVDEDCNRSD 598
Populus_a       KELKEE-LSVHGSD---EEADDP----PEVDSDEDPSDDDAEKHNHEEIGSVDEESSKSD 615
Populus_b       KELKEE-HSVNGSD---EEVDDP----PEVDSDEDLSDDD-DKHDHEEIGSVDEEYTKSD 624
AtLPA1_a        KELMEE-QSVNGSD---EDDEEGDDDFHEPDSDEDLSDNN-DERNRDEIGSVDEESTKSD 602
AtLPA1_b        KELKED-QSVHGSD---EEVED-----DPPEPDTDFSDDD-NKRDHDEVGSVDEQSTKSD 628
maize_a         KELMED-YSVYGSD---EE----ADDPRDAETDDDLTDEE-RDMHEIDAGSVDEHSTKSD 699
maize_b         RQLMED-YSVYVSE---EEEEEEADDPCDAETDEDLSDEE-RDMHEIDAGSVDEHSTKSD 568
sorghum         KELMED-YSVYGSE---EEE--EADDPRDAETDEDLTDEE-RDMHEIDAGSVDEHSTKSD 659
OsLPA1.1        KELMEDDYSVFGSE---ED----ADDPPDAGTDEDLTDEE-RDMHEIEAGSVDEHSTKSD 607
                ... *.   .  *.                 .. * ..      ..   ***.*. ..**

Vitis           EEYDDLAVQHVQENGYKKN--GYLSGDDESSDELMSACERWQQPTGEMLSRKKMIPSGEP 718
rice(09g39870)  EEYDDLAMRDCMENGFLTD--DGVVHTVFDGNGQKHSTLRKRQVNLRTLSK---IDLDSP 653
Populus_a       EEYDDLAMQDVQENGYWSD-DDEESKDRLPPISWGHSSPKRG----DKYRQNLERFLSTR 670
Populus_b       EEYDDLAMQDVQENGYWSD-DDEEPKDRLPPISGGNVSPNKI----DKYRQNLERFLGTR 679
AtLPA1_a        EEYDDLAMEDKS---YWTDNEEEESRDTISMVSQNNHNEASKTNKDDKYSQNLDLFLKTT 659
AtLPA1_b        EEYDDLAMEDKS---YWTD-EEEESRDTIAIMSEKNNKQATK---EDKYIQNLDLFLRTA 681
maize_a         EEYEDLAMRDVMENGDWSD--DDQ------------------------------AVGKTK 727
maize_b         EEYEDLAMRDVMESGDWSD--DEQQ-----------------------------GVSKTK 597
sorghum         EEYEDLAMRDVMENGDWSD--DEQ------------------------------VVSKTK 687
OsLPA1.1        EEYDDLAMQDVMENGYWSD--DEQ------------------------------AASSTK 635
                ***.***...       ..

Vitis           NDIKKQEFIRLPQANENDNRNGIKSDSNENEIPLPNLEENGDEANVGDNGDNGDDGAHGF 778
rice(09g39870)  DTARSSSALPISASSKRNGTRRWKRSLSESFRSRPRSAPSLVELTPKHKGSAVPEVAP-- 711
Populus_a       SEQVAEPLRSYSSLLREKSERRMLSSGSLKIRKRSLSIPAIGKHESMVGDPILSGAPRR- 729
Populus_b       SEQLAEPLCSYSSLLVEQGERRMLSSGSLKIRKRSLSIPAIRKHGSVISDPILSGAPQR- 738
AtLPA1_a        NQPLTESLELTS---EYRNRMGVAASDKAKMRKRSLSIPPVGKHGSIIDDQILANQTDSV 716
AtLPA1_b        NQQLVEPLQLCASLLTCENGNTRLWLGKEKMRKRSLSISAIGKHGSGLGDAILLGAP--- 738
maize_a         NSPALES-IHGSG---AAEDDGIKS-------RYHHNLDLFLKMSKEVAGT-RMPCAS-- 773
maize_b         NSPTLECSIHGSG---AAEDDGING-------RYHHNLDLFLKMSKEVVASNRMPCASS- 646
sorghum         NSPALEG-IHGSG---AAKDDGIKG-------RYHHNLDLFLKMSKEVAGT-GMPCAS-- 733
OsLPA1.1        NSSNQEKNIHGAADGDVVDDEGSGND------RFHHNLAFFLKMSKKVAAT-ELPCA--- 685
                .    .     .                              .             .

Vitis           YDKCSMSSNCSLLL 792
rice(09g39870)  -DK----------- 713
Populus_a       --------------
Populus_b       --------------
AtLPA1_a        L------------- 717
AtLPA1_b        --------------
maize_a         --------------
maize_b         --------------
sorghum         --------------
OsLPA1.1        --------------
```

Fig. 6 OsLPA1.1 is highly conserved in plants. The sequence alignment of OsLPA1.1 (EAY88043.1) with proteins from sorghum (Sbi_0.40583), maize_A (AC194424.2_FG002), maize_B (AC189706.4_FG007), *Arabidopsis thaliana*, AtLPA1a (At3g45090; NP_566873), AtLPA1b (At5g60760; NP_200884), *Populus*_A (fgenesh4_pg.C_LG_IX001462), *Populus*_B (fgenesh4_pg.C_scaffold_66000265), *Vitis vinifera* (CAN72472), and rice (LOC_Os09g39870, NP001063996) is shown. Sorghum, maize, and *Populus* accession numbers are from Gramene. Other accession numbers are from NCBI. Identical (*asterisk*) and similar (*dot*) amino acid residues are indicated

functions and could explain why the homozygous KBNT *lpa*1-1 mutant line appears to be as productive as its wild-type under standard production conditions (Rutger et al. 2004). Preliminary examination of *OsLpa*1 gene expression indicates that three splice variants are expressed in seedlings and the expression of each of these transcripts is similar in the two *lpa*1 mutants and their respective wild-types. Analysis of the expression of *OsLpa*1.1 indicates that this transcript can be detected in vegetative and reproductive tissues with stronger expression in the reproductive tissues as might be expected.

Two pathways, the inositol lipid-dependent and the lipid-independent, have been proposed for the biosynthesis of phytic acid (Raboy 2001). Recent studies involving the

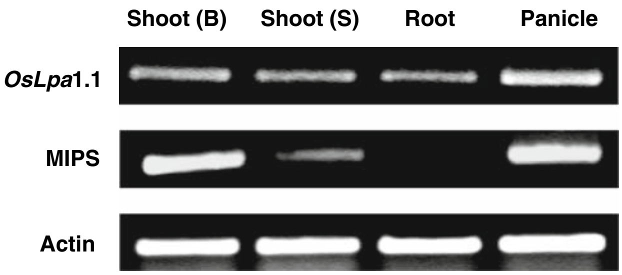


Fig. 7 Expression analysis of Os*Lpa*1.1. End-point RT-PCR analysis was performed using total RNA from various tissues [shoot (*B*) = leaf blade, shoot (*S*) = leaf sheath/stem, root and panicle] and primers for *OsLpa*1, rice MIPS and actin (control)

functional characterization of various plant inositol and inositol phosphate kinases have provided some evidence for both of these pathways (Shi et al. 2003, 2005, 2007; Stevenson-Paulik et al. 2002, 2005). However, some of the enzymes believed to be involved, particularly in the lipid-independent pathway, have yet to be identified. It is possible that *OsLpa*1.1 encodes a kinase involved in the early phosphorylation steps proposed in the lipid-independent pathway, but for which no genes have been cloned. Identification of this kinase activity would be definitive evidence of the lipid-independent phytic acid biosynthetic pathway in plants.

Another possibility is that the OsLPA1.1 protein indirectly impacts phytic acid biosynthesis and accumulation by catalyzing the formation of 2,3-bisphosphoglycerate (2,3-BPG). It has previously been shown that 2,3-BPG is a competitive inhibitor of inositol polyphosphate 5-phosphatases (Downes et al. 1982). These phosphatases breakdown the inositol polyphosphate intermediates leading to phytic acid. In this model, 2,3-BPG produced by OsLPA1.1 inhibits 5-phosphatases in the rice seed, thus allowing inositol polyphosphate intermediates to undergo further phosphorylation to form phytic acid. If this model is correct then rice *lpa*1 mutants should exhibit a reduction in 2,3-BPG and increased activity of 5-phosphatases in the developing rice seed.

Studies are currently underway to confirm the expression of OsLPA1.1 and to determine whether OsLPA1.1 is a kinase and its specific substrate(s). Functional characterization of OsLPA1.1 will contribute to our understanding of phytic acid biosynthesis in plants as well as the development of low phytate crops. The existence of alternative splice products as well as a homologue in the rice genome raises interesting questions regarding whether these predicted proteins are expressed and have any role in inositol phosphate metabolism.

Acknowledgments This was supported by National Research Initiative Competitive Grant 2005-35301-15708 from the USDA Cooperative State Research, Education, and Extension Service (T.H.T). We gratefully acknowledge technical assistance from P.M. Colowit and D. Farzaneh. We are also thankful to J.N. Rutger and V. Raboy for critical reading of the manuscript and helpful suggestions for improvement.

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