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ORIGINAL PAPER

Mutations of the multi-drug resistance-associated protein ABC transporter gene 5 result in reduction of phytic acid in rice seeds

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Abstract Phytic acid (PA, *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate) is important to the nutritional quality of cereal and legume seeds. PA and its salts with micronutrient cations, such as iron and zinc, cannot be digested by humans and non-ruminant animals, and hence may affect food/feed nutritional value and cause P pollution of groundwater from animal waste. We previously developed a set of low phytic acid (LPA) rice mutant lines with the aim of increasing the nutritional quality of rice. Two of these lines, *Os-lpa*-XS110-2 (homozygous non-lethal) *Os-lpa*-XS110-3 (homozygous lethal), contain two mutant alleles of a LPA gene (hereafter *XS-lpa*2-1 and *XS-lpa*2-2, respectively). In this study, we mapped the *XS-lpa*2-1 gene to a region on chromosome 3 between microsatellite markers RM14360 and RM1332, where the rice orthologue (*OsMRP5*) of the maize *lpa*1 gene is located. Sequence analysis of the *OsMRP5* gene revealed a single base pair change (C/G–T/A transition) in the sixth exon of *XS-lpa*2-1 and a 5-bp deletion in the first exon of *XS-lpa*2-2. *OsMRP5* is expressed in both vegetative tissues and developing seeds, and the two mutations do not change the level of RNA transcription. A T-DNA insertion line, 4A-02500, in which *OsMRP5* was disrupted, also showed the same high inorganic phosphorus phenotype as *Os-lpa*-XS110-3 and appeared to be homozygous lethal. PA is significantly reduced in *Os-lpa*-XS110-2 (~20%) and in 4A-02500 (~90%) seeds compared with their wild type lines, and no PA was detected in *Os-lpa*-XS110-3 using HPLC analysis. This evidence indicates that the *OsMRP5* gene plays an important role in PA metabolism in rice seeds.

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Introduction

Phytic acid (PA), known as *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate, is an important constituent in plant seeds and often exists in the form of a mixed salt (phytate or phytin) of mineral cations, including Zn^{2+} and Fe^{3+} (Raboy 1997; Lott et al. 2000). Phosphorus in PA form and divalent cation minerals in phytates are almost indigestible for monogastric animals, hence its abundance in grain food/feed is known to cause nutritional and environmental problems (Raboy 2001). Therefore, the development of low phytic acid (LPA) crops,

in which the PA content is significantly reduced in seeds, has become an important field in crop breeding, and more than a dozen LPA mutant lines have been generated during the past decade in major food crops (see review, Raboy 2007). The first LPA barley variety, 'Herald' has been released for commercial use (Bregitzer et al. 2007).

Accumulation of PA in seeds may be affected not only by genes directly involved in its biosynthesis, but also genes involved in its transportation and compartmentalization. In barley, it has been proven that at least six non-allelic mutations could affect PA accumulation, although none of them has been cloned (see review, Raboy 2007). In maize, two *LPA* genes, *Lpa* 2 gene and *Lpa* 3 have been identified as encoding inositol phosphate kinase (*IPK*) belonging to the Ins (1,3,4)P3 5/6-kinase gene family (Shi et al. 2003) and a *myo*-inositol kinase (*MIK*) (Shi et al. 2005), respectively. Another *LPA* gene, *Lpa* 1, encodes a multidrug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter (Shi et al. 2007). In rice, several homozygous lethal or non-lethal LPA mutations have been identified (Larson et al. 2000; Liu et al. 2007). The first rice *LPA* gene was recently identified and contains a region of homology to the 2-phosphoglycerate kinase (2-PGK) reported in hyperthermophilic and thermophilic bacteria (Kim et al. 2008; Zhao et al. 2008a). This rice *LPA* gene is different from all three maize *LPA* genes cloned to date. It is not yet clear how it regulates PA metabolism in rice. Several other genes also were recently identified to be potentially involved in phytic acid biosynthesis in rice (Josefsen et al. 2007; Suzuki et al. 2007), but it is not yet known whether mutation of these genes can result in PA reduction in rice seeds like the *MIPS*1 gene (Kuwano et al. 2006).

We previously developed a set of LPA mutants in rice, which involve at least four genes (Liu et al. 2007) and proposed a strategy for gene identification in rice by integrating mutation induction, gene mapping, mutation scanning and allelic mutation verification (Zhao et al. 2008a). In this study, two LPA lines, *Os-lpa*-XS110-2 (a homozygous non-lethal mutant) and *Os-lpa*-XS110-3 (a homozygous lethal mutant), were identified to carry two allelic mutations of the rice orthologue, *OsMRP5*, of the maize *Lpa1* gene (*ZmMRP4*). The expression of *OsMRP5* in rice and the effect of *OsMRP5* mutations on seed phosphates and *myo*-inositol content were investigated.

Materials and methods

Low phytic acid mutants and T-DNA insertion line

Two LPA rice mutant lines, i.e. *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3, were used in this study. Both were developed through gamma irradiation followed by NaN_3 treatment of Xiushui 110 (XS110), a commercial *japonica* rice variety (Liu et al. 2007). *Os-lpa*-XS110-2 has a ~23% reduction in seed PA (Frank et al. 2007), however, seed viability is comparable to wild type (WT) (Zhao et al. 2008b). *Os-lpa*-XS110-3 had the highest increase of inorganic P (Pi) level according to qualitative test among all LPA mutants we developed and is a homozygous lethal mutant line (Liu et al. 2007). However, the LPA mutations of *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3 are allelic (Liu et al. 2007), and the mutant alleles are designated as *XS-lpa*2-1 and *XS-lpa*2-2, respectively. The T-DNA activation tagging line, 4A-02500, and its parental variety 'Dongjin' were also used. The 4A-02500 line was identified using RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE). It contains a T-DNA fragment of vector pGA 2715 (Jenong et al. 2006) inserted into the genic region of the gene locus LOC_Os03g04920 (TIGR V5).

Since LPA seeds of both *Os-lpa*-XS110-3 and 4A-02500 cannot germinate naturally, seedlings were either produced from immature embryos or the embryo halves of mature seeds via in vitro culture on half strength MS media (Murashige and Skoog 1962). Seedlings were transplanted to paddy field and grown to maturity.

Development of mapping population

In 2005, *Os-lpa*-XS110-2 was crossed to a conventional *indica* rice variety Y0526. The inorganic P (Pi) levels of eight F_2 seeds from each F_1 plant were colorimetrically assayed as described in the next section. F_2 seeds from F_1 plants that produced seeds segregating for both high and normal Pi were used for gene mapping. The non-embryo halves of F_2 seeds were used for Pi level assays. The embryo halves of those seeds with a high Pi level were surface sterilized and cultured on half strength MS media (Murashige and Skoog 1962). The F_2 plants derived from those embryos were used for mapping. Seeds of those F_2 plants were harvested and the Pi levels of eight seeds of each F_2 plant were tested.

Determination of seed phosphorus and *myo*-inositol

The seed Pi level was determined by colorimetric assay using freshly prepared Chen's reagent (Chen et al. 1956). For qualitative analysis, de-hulled seeds were cut into embryo halves and non-embryo halves, and the non-embryo halves were transferred to 96-well plates for Pi level determination according to Larson et al. (2000) with slight modifications (Liu et al. 2007). Development of a blue color implies an increased level of Pi typical for LPA mutants, while colorless samples typified WT levels of parent varieties (Supplementary Fig. 1S). For quantitative analysis, brown rice grains were ground into rice flour and

passed 0.212 mm mesh screen. The Pi level of 1.00 g of brown rice flour was determined in triplicate according to Chen et al. (1956). Because no plants were obtained by embryo culture of homozygous LPA T_2 seeds of the T-DNA line 4A-02500, T_3 seeds harvested from hemizygous T_2 plants were used for quantitative Pi determination. The endosperm halves were qualitatively analysed for Pi level and their corresponding embryo halves were classified into either WT (endosperm with a normal Pi level) or mutant (endosperm with a high Pi level) and used for quantitative analysis of Pi level and for total P and inositol phosphates (see the next section).

Whole or embryo halves of brown rice grains were freeze-dried and stored in a freezer until analysis. The total P level of 1.00 g of brown rice flour was measured in duplicate by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, Tokyo, Japan) according to Bohn et al. (2007). Analysis of inositol phosphates was performed by high performance liquid chromatography (HPLC) using 0.50 g brown rice flour as described previously by Frank et al. (2007). Quantification of phytic acid was based on external calibration using standard solutions of phytic acid dodecasodium salt in the mobile phase. Technical solutions with standards of phytic acid and lower inositol phytates ($InsP_3$, $InsP_4$, and $InsP_5$) were analyzed.

Quantification of *myo*-inositol was performed by gas chromatography according to Frank et al. (2007) using freeze-dried brown rice flour as described above.

DNA and RNA isolation

Genomic DNA was extracted from plant leaves according to a modified CTAB method as previously described (Liu et al. 2007). Mapping of the XS-*lpa2-1* mutation was performed using DNA samples extracted from individual F_2 plants and a DNA pool prepared by directly extracting DNA from equally mixed leaf tissues of about 50 homozygous LPA F_2 plants of *Os-lpa*-SX110-2/Y0526. All DNA samples were quantified using the Unican UV300 (Thermo Electron Corporation, Cambridge, UK) and adjusted to a final concentration of about 25 ng/µl for PCR.

Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. For vegetative plant tissues, leaf blade, leaf sheath and roots of 21-day-old seedlings were used for RNA extraction. For developing seeds, rice grains were harvested from panicles 7, 14, 21, 28, and 35 days after anthesis, and were subjected to RNA isolation after removal of the hulls. Genomic DNA contamination was eliminated by DNase treatment using RQ1 RNase-Free DNase (Promega, USA).

Molecular mapping

A total of 348 SSR markers, which covered all 12 rice chromosomes, were used for analysis of the pooled DNA sample to identify markers potentially linked to the XS-*lpa*2-1 mutation. The PCR and detection of SSR markers were as described previously (Liu et al. 2007). The primer sequences were originally described by Temnykh et al. (2000, 2001) and acquired from the Gramene database (http://www.gramene.org/). After two SSR markers, RM22 and RM523, were identified to be potentially linked to the mutation, new SSR markers were identified according to the rice genome maps publicly available from Gramene and used for detailed analysis. The SSR markers, their genomic position and primer sequences are provided in the Supplemental Table 1S.

DNA sequencing and assembly

The full length of the TIGR locus LOC_Os03g04920 was sequenced for XS110 and the mutants *Os-lpa*-SX110-2 and *Os-lpa*-SX110-3. Seven pairs of primer were designed using the Primer Premier 5 software based on the genome sequence of the *japonica* cultivar 'Nipponbare' (http://www.gramene.org) and used for the amplification of the entire locus, with 100–200 bp overlaps between adjacent fragments (Fig. 1). PCRs were performed in 50 µl volumes with 50 ng genomic DNA, 1′ Taq Plus PCR buffer (20 mM Tri–HCl pH 8.8, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA), 300 nM each primer, 200 nM each dNTP, and 2.5 U Taq Plus enzyme. Standard PCR conditions with an annealing temperature suitable for each primer pair were used for amplification (Information on primer sequences, annealing temperature, and size of PCR product are provided in Supplemental Table 2S). The target bands were cut from agarose gels, purified using DNA Gel Extraction Kit (AP-GX-250, Axygen, Union City, CA, USA), and directly sequenced in the sequencing facility of Shanghai Invitrogen Biotech Co. Ltd. (Shanghai, China). Sequences were assembled using ContigExpress and aligned using ClustalX 1.83 and BioEdit7.0 program.

Analysis of activation tagging line

Based on the alignment of flanking sequence tags and the rice genome, the T-DNA activation line 4A-02500 was identified as carrying a T-DNA insertion in the first exon of LOC_Os03g04920 (Fig. 1). Two pairs of primer, T1F/T1R and T2F/T2R, were designed for the detection of the presence of the T-DNA insertion (Fig. 1). T1F (5′-CGT CATG CCTCAAGACCG-3′) and T1R (5′-CACGAAGGAGAC CACCCA-3′) flank the T-DNA insertion and amplifies a

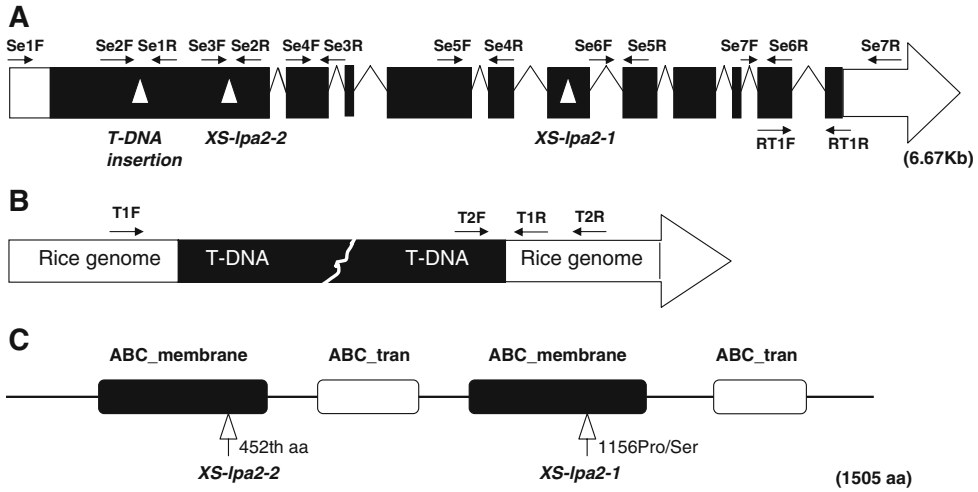

Fig. 1 Schematic diagram of the *OsMRP5* gene and its encoded protein with the sites of mutations. **a** Predicted gene structure of the *OsMRP5* gene, exons and introns are to scale and depicted as *boxes* and *solid lines* and *filled boxes* are predicted to be coding sequences (TIGR Rice Database, Release V5). The *white triangles* are sites of LPA mutations and T-DNA insertion. The position of PCR primers used for gene sequencing (Se1F/R through Se7F/R) and RT-PCR (RT1F/R) is also presented. **b** A diagram of the rice genome/T-DNA insertion region with the position of PCR primers (T1F/R for amplification of rice genomic DNA and T2F/R for detection of T-DNA), **c** The predicted structure of OsMRP5 protein with 1,505 amino acid residues. The two copies of each ABC membrane and ABC transporter (ABC_Tran) domain are to scale and depicted as *boxes*. The position of two LPA mutations is also indicated

308 bp fragment of rice genomic DNA. T2F (5′-TTAGC ACCCCAAGTTAGTCA-3′) is part of the left border of the T-DNA sequence and T2R (5′-CACCAACCAAGCAGA GGA-3′) is part of the rice genomic DNA adjacent to the left border (Fig. 1). These primers amplify a 355 bp fragment in the presence T-DNA. Standard PCR reactions and conditions were used as described above, with annealing temperatures of 61.5 and 55.3°C for T1F/T1R and T2F/T2R, respectively. Fragments were separated on 1.0% agarose gel in 0.5× TBE buffer and visualized by ethidium bromide staining.

Reverse transcription PCR (RT-PCR)

First-strand cDNA was transcribed using Oligo (dT) as the primer and M-MLV (RNase H^-) reverse transcriptase (TaKaRa) according to the manufacturer's instructions. Expression of the rice *MRP5* gene was monitored using the primers RT1F (5′-CGTATCCCGACGGTTATT-3′) and RT1R (5′-ATCCTACCTC CCTGTTCCT-3′), which amplify a 204 bp fragment of the cDNA (Fig. 1). Expression was normalized using the rice actin gene (forward primer 5′-TCAGCAACTGGGATG ATATGGAG-3′ and reverse primer 5′-GCCGTTGTGGTGAATGAGTAAC-3′, 385 bp product). PCR reactions and amplification were performed as described above using annealing temperatures of 61.5 and 55.3°C for the MRP5 and actin genes, respectively. Fragments were separated on 1. 0% agarose gel in 0.5× TBE buffer and visualized by ethidium bromide staining.

Results

Delimiting the XS-*lpa*2-1 locus and candidate gene identification

Two SSR markers, RM22 (genome position, 1.50 Mb) and RM523 (1.30 Mb) were identified to be linked to the XS-*lpa*2-1 mutation through bulked segregant analysis of LPA F_2 plants. Analysis of individual LPA F_2 plants showed that RM22 was more tightly linked to the LPA mutation. Therefore, new SSR markers, RM6013, 5761, 7072, 5628, 3807, 3392, 4352, 2790, 4492, 6038, in the direction from 1.63 to 4.80 Mb of chromosome 3, were used to analyze 128 LPA F_2 plants (the genotype was verified through analyzing the Pi level of F_3 seeds). Among them, RM5761 (2.11 Mb) completely co-segregated with the LPA mutation (Fig. 2). Additional SSR markers flanking RM5761 (between 1.93 and 2.86 Mb) were used for mapping and the XS-*lpa*2-1 was delimited within the 400 kb interval between RM14360 (2.03 Mb, 2 recombinants) and RM1332 (2.43 Mb, 1 recombinant) (Fig. 2).

A BLAST search of rice orthologues of genes involved in PA metabolism in other plant species was performed. Four genes, *myo*-inositol phosphate synthase gene (*MIPS*), *IPK*, *MIK*, and the multi-drug resistance-associated protein 5 gene (*MRP5*) were located on chromosome 3 (Fig. 2). The rice orthologue of the maize *Lpa*1 gene (*OsMRP5*) was the only one within the delimited region. Therefore, the locus LOC_Os03g04920 (*OsMRP5*) at the genome position between 2346960 and 2353626 bp on chromosome 3 was

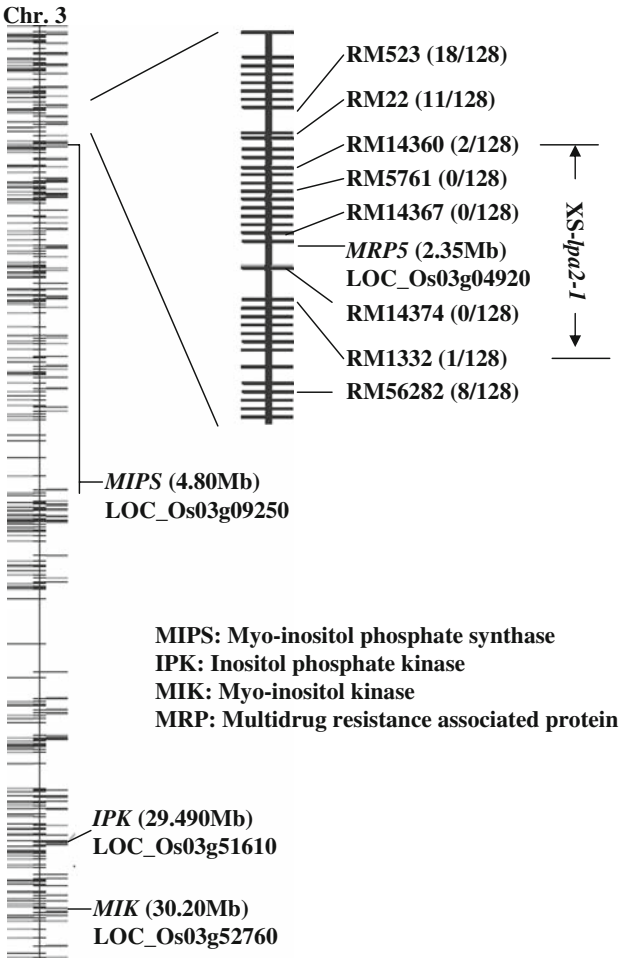

Fig. 2 Molecular mapping f the XS-*lpa2-1* mutation on rice chromosome 3. The position of other genes and loci known to be potentially involved in phytic acid biosynthesis is indicated according to the Nipponbare genome sequence information (TIGR Rice Database, Release V5). The number of recombinants between a marker and the mutation locus is indicated in *parenthesis* right after the SSR marker. A total of 128 homozygous LPA F_2 progenies from the *Os-lpa*-XS110-2/Y0526 cross were used for mapping

considered to be a strong candidate gene for the XS-*lpa*2-1 gene (Fig. 2).

Mutations in *OsMRP5* gene

While homozygous high Pi (mutant) seeds can be obtained from heterozygous *Os-lpa*-XS110-3 plants, they cannot naturally germinate, thus no homozygous mutant plants can be obtained. Attempts to produce homozygous mutant plants through in vitro culture of high Pi seeds resulted in limited success, since most of the embryos either did not germinate or were severely contaminated at a later stage after germination. However, a few plants were rescued from homozygous mutant seeds and were used to produce additional plants. Further attempts to recover homozygous mutant plants through in vitro culture of immature embryos (14–21 days after anthesis) were successful. About 500 immature embryos were obtained from the above rescued mutant plants and were cultured on MS media. About 70% germinated and developed into healthy plants, thus providing sufficient homozygous mutant seeds for further characterization including sequencing of the entire LOC_Os03g04920 locus.

Sequencing results revealed that there is a single base pair change (C/G–T/A transition) in XS-*lpa*2-1 and a 5-bp (GGTAG) deletion in XS-*lpa*2-2 (Fig. 1). According to TIGR (V5) annotation, the *OsMRP5* gene is composed of 11 exons and 10 introns (Fig. 1a). It encodes a multi-drug resistance-associated protein (MRP) ABC transporter that has 1,505 amino acid residues. Further analysis showed that it contains two copies of the modular structure consisting of an integral transmembrane domain (ABC_tran) and a cytosolic ATP-binding domain (ABC_membrane) (Fig. 1c). The XS-*lpa*2-1 point mutation is in the sixth exon and results in an amino acid change from Pro to Ser at the position 1156, which is located within the second ABC_membrane (Fig. 1c). The XS-*lpa*2-2 deletion is in the first exon and leads to a frame shift starting at aa 452 and resulting in a premature stop codon at aa 474, thus significantly truncating the encoded protein (Fig. 1c). Therefore, the XS-*lpa*2-2 represents a loss-of-function allele. These results strongly suggested that *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3 mutants are the result of mutations in *OsMRP5*.

OsMRP5 gene knockout and mutant phenotype

Confirmation of the involvement of OsMRP5 in rice seed phytic acid metabolism was obtained by analysis of a T-DNA activation tagging line, 4A-02500. This line contains a T-DNA insertion in the first exon of *OsMRP5* (Fig. 1a). High Pi level assay of 10 T_1 seeds of 4A-02500 showed that three seeds had a Pi level as high as *Os-lpa*-XS110-3, and the others had a normal Pi level (Supplementary Fig. 1S). The embryo halves of all ten seeds were cultured in vitro to recover seedlings. Two of the three mutant seed embryos and six of the seven wild type seed embryos germinated and were grown to maturity.

The T_2 seeds produced by the eight surviving T_1 plants were subjected to high Pi level assay. Two plants derived from seeds of a high Pi level produced T_2 seeds all exhibiting a high Pi level (4A-02500-1, -2), four plants derived from seeds of normal Pi content produced seeds that segregated for normal and high Pi levels (4A-02500-3, -4, -5 and -8), the remaining two plants derived from normal Pi T_1 seeds produced seeds all of normal Pi level (4A-02500-6, -7) (Supplementary Fig. 1S). The 1:2:1 segregation ratio of T_2 plants showed that the high Pi level mutation in 4A-02500 is controlled by a single gene mutation.

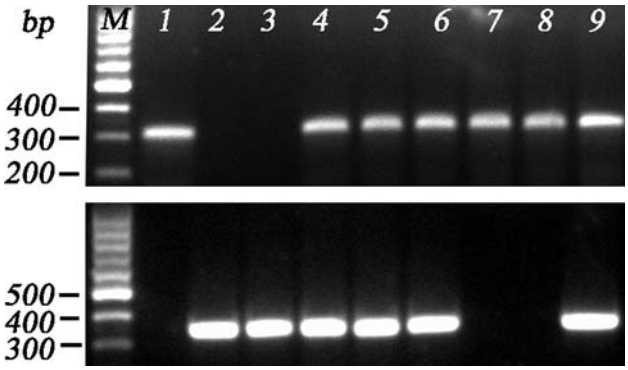

Fig. 3 PCR analysis of T_1 plants of the T-DNA activation tagging line 4A-02500. *Upper* amplification using T1F/T1R for detection of rice genome DNA, *bottom* amplification using primer T2F/T2R for detection of T-DNA fragment. *M* DNA size marker, *lane 1* Dongjin (non-transgenic parental variety), *lane 2–9* T_1 plants of 4A-02500

The surviving T_2 plants were further subjected to PCR analysis of the presence of T-DNA fragment. No fragment was amplified from 4A-02500-1 to 4A-02500-2 plants using T1F/T1R primers, but one fragment (~355 bp) was amplified using T2F/T2R primers (Fig. 3), which demonstrated that both copies of their *MRP5* were disrupted by the T-DNA insertion. On the opposite, one fragment (305 bp) was amplified from 4A-02500-6 to 4A-0250-7 using T1F/T1R but none using T2F/T2R (Fig. 3), indicating none of the two copies of *MRP5* was disrupted. From the remaining four plants, both T1F/T1R and T2F/T2R produced one fragment (4A-02500-3, -4, -5 and -8) (Fig. 3), indicating only one copy of *MRP5* was disrupted.

These results indicate that the high Pi mutant phenotype co-segregate with T-DNA insertion in the 4A-02500, which is consistent with the disruption of the *OsMRP5* gene resulting in a high Pi phenotype and our earlier findings with the *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3.

Expression of *OsMRP5*

The spatial and temporal expression of the *OsMRP5* gene was surveyed by RT-PCR on total RNA from various seedling tissues and seeds at different developmental stages. As shown in Fig. 4, the *OsMRP5* gene appeared to be expressed in both vegetative tissues and developing seeds; no significant spatial and temporal changes in expression were observed after normalizing against the *actin* gene (Fig. 4). The two LPA mutations, XS-*lpa*2-1 and XS-*lpa*2-2, did not seem to affect expression at the mRNA level (Fig. 4) although no results were available for developing seeds of *Os-lpa*-XS110-3 due to lack of sufficient material. Analysis of T_3 seedlings derived from embryo halves of mutant seeds of 4A-02500 with a high Pi level showed no expression of the *OsMRP5* gene (Supplementary Fig. 2S).

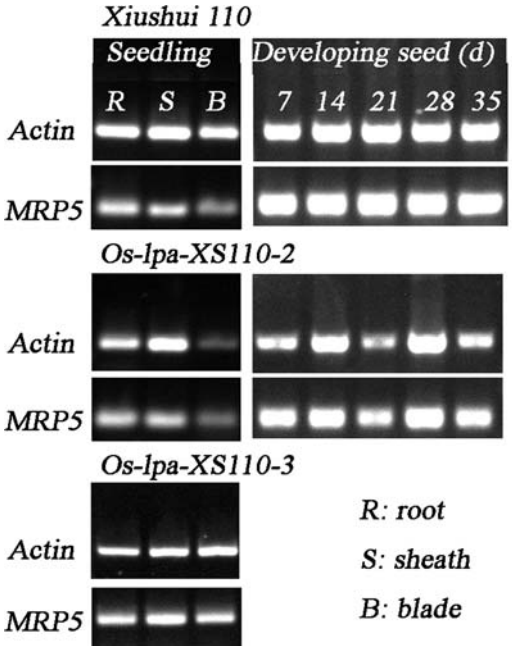

Fig. 4 Expression analysis of *OsMRP5* gene of wild type parental variety and LPA mutant plants. RT-PCR was performed using total RNA isolated from different tissues of 21-day-old seedlings and developing seeds. The *actin* gene was used as control

Phosphates and *myo*-inositol content

Initial qualitative analysis using the colorimetric assay showed that the Pi levels of homozygous mutant seeds of both *Os-lpa*-XS110-3 and 4A-02500 were much higher than other LPA lines, e.g. *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 (Supplementary Fig. 1S). This was confirmed by quantitative analysis which indicated that the Pi content of mutant seeds of *Os-lpa*-XS110-3 and 4A-02500 was about 10 times higher than their respective wild type lines (Table 1). HPLC analysis showed that no inositol polyphosphate except PA was detected in rice seeds of both wild type XS110 and mutant seeds (Supplementary Fig. 3S). The PA–P content was below the limit of quantification (<0.20 mg g^{-1}) in homozygous mutant seeds of *Os-lpa*-XS110-3 compared to 2.62 mg g^{-1} in the wild type XS110 seeds (Table 1). The PA-P content of mutant seeds of 4A-02500 was only about 10% of the wild type (Table 1). No significant differences in total P content were observed between mutant and wild type seeds of both lines (Table 1).

Analysis of *myo*-inositol content showed that mutant seeds of both *Os-lpa*-XS110-3 and 4A-02500 were significantly higher than their respective wild type progenitors, with an increase of more than 400% in *Os-lpa*-XS110-3 compared to XS110 (Table 1).

Table 1 Contents of *myo*-inositol, total phosphorus (P), inorganic P (Pi), and phytic acid P (PA–P) in brown rice grains of low phytic acid (LPA) and wild type (WT) rice lines

Material	*myo*-inositol (μg g^{-1})	Total P (mg g^{-1})	Pi (mg g^{-1})	PA–P (mg g^{-1})
I. Xiuhsui 110 (WT) and *Os-lpa*-XS110-3(LPA)[a]				
WT	47.7 ± 0.6	4.81 ± 0.24	0.30 ± 0.01	2.62 ± 0.18
LPA	242.0 ± 1.7*	4.50 ± 0.04	3.21 ± 0.06*	BLQ
II. Dongjin T-DNA insertion line 2A-02500[b]				
WT grains	78.0 ± 3.6	5.77 ± 0.40	0.35 ± 0.00	3.82 ± 0.17
LPA grains	147.5 ± 6.4*	5.46 ± 0.76	4.09 ± 0.06*	0.48 ± 0.00*

Data are shown in mean ± standard deviation

BLQ below limit of quantification (<0.20 mg g^{-1})

* Mean of the LPA is significantly different from its WT at $P = 0.01$ level

[a] Analysis was based on whole brown rice harvested from plants grown in Lingshui, Hainan, 2008 (Spring)

[b] Analysis was based on embryo halves of brown grains harvested from hemizygous T_2 plants grown in Jiaxing, Zhejiang, 2008 (Autumn)

Discussion

Dozens of LPA mutants have been developed in various crop species and a number of mutant genes have been either mapped or cloned (see review, Raboy 2007). A number of LPA mutants have been developed in rice, but only one gene corresponding to the mutants KBNT *lpa*1-1 and *Os-lpa*-XQZ-1 has been cloned (Kim et al. 2008; Zhao et al. 2008a). Using molecular mapping and comparative genomics analysis, we identified *OsMRP5*, the rice orthologue of the maize *Lpa1* gene *ZmMRP4*, as a candidate gene underlying two allelic LPA mutations, XS-*lpa*2-1 and XS-*lpa*2-2. Subsequent sequencing of the Os*MRP5* alleles of *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3 and analysis of a line containing a T-DNA insertion in *OsMRP5* indicated that the LPA phenotype is due to mutations in this gene.

Effect of mutations on gene function and phytic acid content

Although the nucleotide substitution of the *XS-lpa*2-1 mutation is predicted to result in an amino acid change from Pro to Ser, it would appear that the effect on the function of encoded protein would be limited since the amino acid change does not significantly change the tertiary structure of the protein (simulation using CPH models 2.0 Server from http://www.expasy.org/tools/, data not shown). On the other hand, the 5-bp deletion of the *XS-lpa*2-2 mutation causes a frame shift that introduces a premature stop code after the deletion site in the first ABC-tran domain (Fig. 1), therefore it is expected that the mutation would have a dramatic impact on its function. A T-DNA fragment was inserted into the first exon of the *OsMRP5* gene in the activation line 4A-02500 (Fig. 1). which is likely to have knocked out the *OsMRP5* gene. The predicted effects of these mutations on gene function match well with the observed LPA phenotypes of the mutant lines, with PA–P content reductions of ~23%, >90% and ~90% in mutant seeds of *Os-lpa*-XS110-2, *Os-lpa*-XS110-3, and 4A-02500, respectively (Frank et al. 2007; Table 1). Similar cases where allelic LPA mutations caused different effects on PA content have been observed in maize. The maize *lpa*241 mutant (Pilu et al. 2005), mutant line *lpa* 1-1(Raboy et al. 2000), and the knockout mutant *lpa1-mum1* (Shi et al. 2007), which all carry mutations in *ZmMRP4*, have PA reductions of ~90, ~60, and ~90%, respectively. The *lpa*1-1 mutation results in a change of a conserved amino acid (alanine to valine) in the second ATP-binding domain and the *lpa1-mum1* disrupts *ZmMRP4*; both mutations cause a ~90% PA reduction (Shi et al. 2007), which is comparable to that observed in mutant seeds of *Os-lpa*-XS110-3 and 4A-02500 in this study (Table 1). Since the embryo halves were used in determination of contents of PA and total P of 4A-02500, and PA and other forms of P are more concentrated in embryo and aleurone than in endosperm (O'Dell et al. 1972), therefore it is reasonable that high contents of total P, Pi and PA–P were observed in 4A-02500 compared to *Os-lpa*-XS110-3 (Table 1), although it could also be attributed to genotypic and environmental effects.

Effect of mutations on seed composition and seed viability

Different LPA mutations can have different effects on total P content and accumulation of lower inositol phosphates. Raboy (2007) summarized two types of LPA mutation: LPA (without change of total P, most LPA mutants identified to date belong to this type) and low phytic acid/low total P (barley *lpa*1); Another type of LPA mutant having an increased total P has also been reported in barley—the Type A *lpa* mutants of Pallas-P01 (Hatzack et al. 2000). LPA mutations can also result in accumulation of lower

inositol polyphosphates, e.g. seeds of the Type A mutants of barley (Hatzack et al. 2000), *lpa* 3 of maize (Shi et al. 2005), and *Gm-lpa*-ZC-2 of soybean (Yuan et al. 2007) all have increased levels of lower inositol polyphosphates. The total P contents remain unchanged and no intermediate inositol phosphates were detected in either 4A-02500, *Os-lpa*-XS110-3 (Table 1), or in *Os-lpa*-XS110-2 (Frank et al. 2007). These observations are consistent with that of *MRP4* mutants in maize (Shi et al. 2007).

Low phytic acid mutations can also result in content changes of other seed constituents, e.g. *myo*-inositol, sucrose and raffinose (Frank et al. 2007). Significantly higher *myo*-inositol contents were observed in LPA seeds of *Os-lpa*-XS110-3 (89.2%) and 4A-02500 (409%) compared to wild types (Table 1), which is consistent with the maize mutant line *lpa 1-1* (60%; Shi et al. 2007). However, our previous study showed that *Os-lpa*-XS110-2 had significantly decreased contents (40–48%) of *myo*-inositol compared to the wild type XS110 over four production environments (Frank et al. 2007). The differences in *myo*-inositol content change might be explained by the different effects of mutant alleles as described above.

Pleiotropic effects of *MRP5* mutation

While seeds containing homozygous mutant alleles of XS-*lpa*2-2 or T-DNA insertions cannot germinate naturally, the effect of XS-*lpa*2-1 on seed viability was minor (Zhao et al. 2008b). Similarly in maize, no significant effect on seed viability was observed for the *lpa*1-1 mutant (Raboy et al. 2000), while significant negative pleiotropic effects were detected for the *lpa*241 mutation, including reduced seed germination and seedling growth (Pilu et al. 2005). It would also be of interest and importance to analyze the content change of phosphates in vegetative tissues of mutant lines.

Role of OsMRP5 in phytic acid metabolism

Phytic acid can be synthesized through two distinct pathways in plants, namely, the lipid-dependent pathway and lipid-independent pathway. While much progress has been made in the biochemical steps involved in PA synthesis and breakdown, essentially no progress had been made concerning roles for compartmentalization and associated transport functions (see review, Raboy 2007). The maize ABC transporter gene Zm*MRP4* is the first gene to be identified to be potentially involved in process of PA compartmentalization and/or associated transport, but this function has not been confirmed.

There are two possible ways suggested for the *ZmMRP4* gene involving in PA biosynthesis (Shi et al. 2007). First, it might be involved in limiting the phosphate supply to *myo*-insitol and InsP kinase and therefore depriving these kinases of their substrates. This is supported by the observation of significant increase (~60%) of *myo*-inositol content in the *lpa1-1* mutant over its parent (Shi et al. 2007). Another possibility is that the MRP ABC transporter affects PA transport and compartmentalization. Presumably, the ABC transporter regulates synthesis or storage of PA by a negative feedback mechanism, that is, accumulation of PA in the cytosol may reduce its biosynthesis (Shi et al. 2007). Electron microscopy analysis suggested the PA is synthesized in cytosol, transported into the endoplasmic reticulum lumen, and moved in endoplasmic reticulum-derived vesicles to protein storage vacuoles (PSVs) (Greenwood and Bewley 1984; Otegui et al. 2002). *OsMRP5* has the same exon-intron structure as *ZmMRP4*, sharing 83% nucleotide sequence identity and 90% amino acid identity. Significant increases in *myo*-inositol and reductions in PA were observed in the mutant seeds of *Os-lpa*-XS110-3 and 4A-02500. Therefore, OsMRP5 may function as the ZmMRP4, directly or indirectly involved in transporting PA from the cytosol to PSVs.

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References

Bohn L, Josefsen L, Meyer AS, Rasmussen SK (2007) Quantitative analysis of phytate globoids isolated from wheat bran and characterization of their sequential dephosphorylation by wheat phytase. J Agric Food Chem 55:7547–7552

Bregitzer P, Raboy V, Obert DE, Windes JM, Whitmore JC (2007) Registration of 'Herald" Barley. Crop Sci 47:441–442

Chen PS, Toribara TY, Warner H (1956) Microdetermination of phosphorous. Anal Chem 28:1756–1758

Frank T, Meuleye Seumo B, Miller A, Shu QY, Engel KH (2007) Metabolite profiling of two low phytic acid (lpa) rice mutants. J Agric Food Chem 55(26):11011–11019

Greenwood JS, Bewley JD (1984) Subcellular distribution of phytin in the endosperm of developing castor bean: a possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. Planta 160:113–120

Hatzack F, Johansen KS, Rasmussen SK (2000) Nutritionally relevant parameters in low-phytate barley (*Hordeum vulgare* L.) grain mutants. J Agric Food Chem 48:6074–6080

Jenong DH, An SY, Park SH, Kang HG, Kim JY, Sim JY, Kim YO, Kim MK, Kim SR, Kim JW, Shin MS, Jung MY, An GH (2006) Generation of a flanking sequence-tag database for activation-tagging lines in japonica rice. Plant J 45:123–132

Josefsen L, Bohn L, Sorensen MB, Rasmussen SK (2007) Characterization of a multifunctional inositol phosphate kinase from rice and barley belonging to the ATP-grasp superfamily. Gene 397:114–125

Kim SI, Andaya CB, Goyal SS, Tai TH (2008) The rice OsLpa1 gene encodes a novel protein involved in phytic acid metabolism. Theor Appl Genet 117:769–779

Kuwano M, Ohyama A, Tanaka Y, Mimura T, Takaiwa F, Yoshida KT (2006) Molecular breeding for transgenic rice with low-phytic-acid phenotype through manipulating myo-inositol 3-phosphate synthase gene. Mol Breed 18:263–272

Larson SR, Rutger JN, Young KA, Raboy V (2000) Isolation and genetic mapping of a non-lethal rice (*Oryza sativa* L.) low phytic acid 1 mutation. Crop Sci 40:1397–1405

Liu QL, Xu XH, Ren XL, Fu HW, Wu DX, Shu QY (2007) Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor Appl Genet 114:803–814

Lott JNA, Ockenden I, Raboy V, Batten GD (2000) Phytic acid and phosphorus in crops seeds and fruits: a global estimate. Seed Sci Res 10:11–33

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

O'Dell BL, de Boland AR, Koirtyohann SR (1972) Distribution of phytate and nutritionally important elements among the morphological components of cereal grains. J Agric Food Chem 20:718–721

Otegui MS, Capp R, Staehelin LA (2002) Developing seeds of *Arabidopsis* store different minerals in two types of vacuoles and in the endoplasmic reticulum. Plant Cell 14:1311–1327

Pilu R, Landoni M, Cassani E, Doria E, Nielsen E (2005) The maize lpa241 mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci 45:2096–2105

Raboy V (1997) Accumulation and storage of phosphate and minerals. In: Larkins BA, Vasil IK (eds) Cellular and molecular biology of plant seed development. Kluwer, Dordrecht, pp 441–477

Raboy V (2001) Seeds for a better future: 'low phytate' grains help to overcome malnutrition and reduce pollution. Trends Plant Sci 6:458–462

Raboy V (2007) Forward genetic studies of seed phytic acid. Isr J Plant Sci 55:171–181

Raboy V, Gerbasi PF, Young KA, Stoneberg SD, Pickett SG, Bauman AT, Murthy PP, Sheridan WF, Ertl DS (2000) Origin and seed phenotype of maize low phytic acid 1–1 and low phytic acid 2–1. Plant Physiol 124:355–368

Shi JR, Wang HY, Wu YS, Hazebroek J, Meeley RB, Ertl DS (2003) The maize low-phytic acid mutant lpa2 is caused by mutation in an inositol phosphate kinase gene. Plant Physiol 131:507–515

Shi JR, Wang HY, Hazebroek J, Ertl DS, Harp T (2005) The maize low-phytic acid 3 encodes a myo-insitol kinase that plays a role in phytic acid biosynthesis in developing seeds. Plant J 42:708–719

Shi JR, Wang HY, Schellin K, Li BL, Faller M, Stoop JM, Meeley RB, Ertl DS, Ranch JP, Glassman K (2007) Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nat Biotechnol 25:930–937

Suzuki M, Tanaka K, Kuwano M, Yoshida KT (2007) Expression pattern of inositol phosphate-related enzymes in rice (*Oryza sativa* L.): implications for the phytic acid biosynthetic pathway. Gene 405:55–64

Temnykh S, Park WD, Ayers N, Cartinhour S, Hauck N, Lipovich L, Cho YG, Shii T, McCouch SR (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100:697–712

Temnykh S, DeClerck G, Lukasshova A, Lipovich L, Cartinhou S, McCouch SR (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Res 11:1441–1452

Yuan FJ, Zhao HJ, Ren XL, Zhu SL, Fu XJ, Shu QY (2007) Generation and characterization of two novel low phytate mutations in soybean (*Glycine max* L. Merr.). Theor Appl Genet 115:945–957

Zhao HJ, Liu QL, Ren XL, Wu DX, Shu QY (2008a) Gene identification and allele-specific marker development for two allelic low phytic acid mutations in rice (*Oryza sativa* L.). Mol Breed 22:603–612

Zhao HJ, Liu QL, HW FU, Xu HX, Wu DX, Shu QY (2008b) Effect of non-lethal low phytic acid mutations on grain yield and seed viability in rice. Field Crops Res 108:206–211