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ORIGINAL PAPER

Isolation and characterization of a low phytic acid rice mutant reveals a mutation in the rice orthologue of maize MIK

S. I. Kim · C. B. Andaya · J. W. Newman · S. S. Goyal · T. H. Tai

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Abstract Using a forward genetics approach, we isolated two independent low phytic acid (*lpa*) rice mutants, N15-186 and N15-375. Both mutants are caused by single gene, recessive non-lethal mutations, which result in approximately 75% (N15-186) and 43% (N15-375) reductions in seed phytic acid (inositol hexa*kis*phosphate). High-performance liquid chromatography and GC–MS analysis of seed extracts from N15-186 indicated that, in addition to phytic acid, inositol monophosphate was significantly reduced whereas inorganic phosphorus and *myo*-inositol were greatly increased when compared with wild-type. The changes observed in N15-186 resemble those previously described for the maize *lpa*3 mutant. Analysis of N15-375 revealed changes similar to those observed in previously characterized rice *lpa*1 mutants (i.e. significant reduction in phytic acid and corresponding increase in inorganic phosphorus with little or no change in inositol phosphate intermediates or *myo*-inositol). Further genetic analysis of the N15-186 mutant indicated that the mutation, designated *lpa* N15-186, was located in a region on chromosome 3 between the microsatellite markers RM15875 and RM15907. The rice orthologue of maize *lpa*3, which encodes a *myo*-inositol kinase, is in this interval. Sequence analysis of the N15-186 allele of this orthologue (Os03g52760) revealed a single base pair change (C/G to T/A) in the first exon of the gene, which results in a non-sense mutation. Our results indicate that *lpa* N15-186 is a mutant allele of the rice *myo*-inositol kinase (*OsMIK*) gene. Identification and characterization of *lpa* mutants, such as N15-186, will facilitate studies on the regulation of phytic acid biosynthesis and accumulation and help address questions concerning the contribution of the inositol lipid-dependent and independent biosynthetic pathways to the production of seed phytic acid.

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S. I. Kim · T. H. Tai (✉)
USDA-ARS Crops Pathology and Genetics Research Unit,
Department of Plant Sciences, University of California at Davis,
One Shields Avenue, Davis, CA 95616, USA
e-mail: Thomas.Tai@ars.usda.gov

C. B. Andaya · S. S. Goyal
Department of Plant Sciences, University of California at Davis,
One Shields Avenue, Davis, CA 95616, USA

J. W. Newman
USDA-ARS Western Human Nutrition Research Center,
Department of Nutrition, University of California at Davis,
430 West Health Sciences Drive, Davis, CA 95616, USA

Introduction

Phytate is a mixed salt of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate) that accumulates in seeds, making up from one to several percent of the dry weight (Raboy 1997). During germination, the breakdown of phytate into inorganic P, *myo*-inositol, and metallic cations (primarily K^+ and Mg^{++}) by phytases (Laboure et al. 1993) provides essential nutrients for seedling growth. Phytate, which accounts for over two-thirds of the total P in seeds, cannot be digested by monogastric animals and can strongly bind to nutritionally important mineral cations (e.g., Ca^{2+}, Zn^{2+}, and Fe^{2+}). Given these attributes, phytic acid is considered an anti-nutrient with regard to human and non-ruminant livestock nutrition (Brinch-Pedersen

et al. 2002; Raboy 2002). This has led to significant interest in developing cereal grains and legumes with reduced levels of seed phytic acid (Raboy 2002; Stevenson-Paulik et al. 2005; Shi et al. 2007).

One approach to developing low phytate crops has been the identification of induced mutants exhibiting increased levels of seed inorganic P. Such mutants are usually associated with low phytic acid. Over the past decade, several induced low phytic acid (*lpa*) mutants have been isolated in various crop species (Raboy 2007). This is an attractive approach to developing low phytate crops given the consumer sentiment opposing genetic modification of crops through recombinant DNA technology. Unfortunately, mutations affecting seed phytic acid content are often associated with decreased agronomic performance (Meis et al. 2003; Pilu et al. 2003; Oltmans et al. 2005; Shi et al. 2007) even though some exceptions have been described in barley (Bregitzer and Raboy 2006) and soybean (Yuan et al. 2007). The negative effect on agronomic performance is not surprising given that most of these mutations are likely to be involved in the phytic acid biosynthesis pathway, and therefore could affect *myo*-inositol and other metabolites that are important for normal development as well. In addition to serving as a storage compound for carbon and phosphorus in plant seeds, the role of phytic acid in a number of important cellular processes has been characterized in yeast (York 2006) and it has also been implicated in signaling in plant guard cells through the regulation of cytoplasmic calcium (Lemtiri-Chlieh et al. 2003).

Biosynthesis of phytic acid in plants is believed to proceed via both an inositol lipid-dependent and a lipid-independent pathway. During the past few years, a number of genes involved in phytic acid biosynthesis have been cloned and characterized from plants (Stevenson-Paulik et al. 2002; Shi et al. 2003, 2005; Josefsen et al. 2007; Sun et al. 2007; Suzuki et al. 2007); however, genes predicted to contribute key steps in these proposed pathways remain unidentified (Stevenson-Paulik et al. 2005). In particular, the lipid-independent pathway involving the stepwise phosphorylation of *myo*-inositol to phytic acid (Stephens and Irvine 1990; Brearley and Hanke 1996; Irvine and Schell 2001) remains controversial although the recent identification of a *myo*-inositol kinase in maize (Shi et al. 2005) may be relevant to the proposed lipid-independent pathway in plants.

In rice, about 70% of the total seed P is found in the form of phytic acid. Like wheat, seed phytic acid accumulates primarily in the pericarp and aleurone layers of rice seeds, which contrasts with maize where 90% of phytate is found in the germ (O'Dell et al. 1972). Larson et al. (2000) identified the first low phytic acid mutant of rice (*lpa*1), which results from a non-lethal recessive mutation and exhibits a 45 to 50% reduction in phytic acid with a molar-equivalent increase in inorganic P. Several *lpa* mutants have recently been identified in rice ranging from 34 to 64% reduction in seed phytic acid (Liu et al. 2007).

We report here the isolation of two rice low phytic acid mutants, N15-186 and N15-375, which exhibit 75 and 43% reductions in phytic acid, respectively. In addition to changes in phytic acid and inorganic phosphorus contents associated with all *lpa* mutants, the N15-186 mutant also exhibited a significant accumulation of *myo*-inositol and reduction in inositol monophosphate compared to wild-type. The N15-375 mutant did not show changes in these metabolites and thus resembles a rice *lpa*1 mutant. Genetic analysis of N15-186 indicated that the mutant is caused by a single gene, recessive mutation (*lpa* N15-186) that is located on chromosome 3 in the same region as the rice orthologue of the maize *lpa*3, which encodes a *myo*-inositol kinase (Shi et al. 2005). Sequence analysis of the N15-186 allele of this rice gene (Os03g52760) revealed a single base pair change from wild-type (C/G to T/A transition) in the first exon of the gene, which results in a nonsense mutation. Our results indicate that *lpa* N15-186 is a mutant allele of the rice *myo*-inositol kinase (*OsMIK*) gene. The identification and characterization of this gene will facilitate studies on the biosynthesis of phytic acid in plants.

Materials and methods

Plant materials and mutant screening

Mutant screening was conducted using M_2 seeds from single panicles of individual M_1 rice plants derived from seeds of the *Japonica* cultivar Nipponbare that were treated with 1 mM sodium azide (Az) and 15 mM methyl nitrosourea (MNU) as described (Till et al. 2007). A total of 540 lines (designated N15-# where "N" represents Nipponbare, "15" represents the Az-MNU mutagenesis, and # is the number of the line) were screened. Eight seeds from each line were assayed for a high inorganic phosphate (HIP) phenotype as described (Andaya and Tai 2005) with modifications as follows. Dehulled seeds were cut in half with a scalpel resulting in an embryo half and a non-embryo half. The non-embryo halves were transferred to a 96 deep well block and ground for 3 × 5 min in a Minibeadbeater-96™ (Biospec Products, Bartlesville, OK) with one stainless steel dowel pin (Small Parts, Inc., Miami Lakes, FL) per well. After removal of the dowels, the powdered samples were mixed with 100 µL of 0.4 N HCl using a vortex and incubated at 4°C overnight. The blocks were centrifuged for 1 min at 4,000 rpm and 10 µL of supernatant from each well was transferred to a clean microtiter plate and 90 µL of ultrapure water and 100 µL of Chen's reagent (Chen et al. 1956) were added. After mixing, reactions were incubated at

room temperature for 1 h and visually scored for the presence (blue) or absence (colorless) of HIP. The embryo half of seeds from lines segregating for HIP were surface sterilized with 50% household bleach for 20 to 30 min and rinsed several times with sterile water before plating on 0.5X MS media consisting of 2.2 g/L MS basal salts (Sigma-Aldrich, St Louis, MO), 10 g/L sucrose, and 2.5 g/L Phytagel® (Sigma-Aldrich), pH 5.8, and placed in a growth chamber at 25°C with a 12 h photoperiod. After about two weeks, seedlings were transferred to soil and grown in a greenhouse.

Determination of phytic acid, inorganic P and inositol phosphates

In order to ascertain if the HIP phenotype observed in N15-186 was indicative of a reduction in phytic acid, the total P, phytic acid-P and inorganic P (P_i) contents of mature seeds were determined as described (Kim et al. 2008). For each sample, single grains were weighed and 10 volumes of 0.4 N HCl were added. Extraction was performed as described above and seed extracts were centrifuged and supernatants were transferred into fresh tubes for both phytic acid and P_i determinations. Phytic acid-P and the P_i of each sample was expressed as P content on a dry weight basis. 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 ($P = 0.05$).

High-performance liquid chromatography (HPLC) was performed using a Dionex DX-500 ion chromatography system as described previously with minor modifications (Mitsuhashi et al. 2005). Supernatants from seed extracts were passed through 0.2 μ 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 the inositol phosphate standards were purchased from Sigma-Aldrich. The retention times for 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.

Determination of seed *myo*-inositol

myo-Inositol contents of dry, mature seeds were quantified using gas chromatography-mass spectrometry (GC–MS) according to Brunton et al. (2007) with minor modification. Dehulled seeds (brown rice) were ground using the mini-beadbeater and dowel pins as described above and 100 mg samples were placed into 7-mL scintillation vials. One mL of 70% (v/v) ethanol was added and the vials were shaken on an orbital shaker for 20 min at room temperature. The samples were centrifuged for 5 min at 1500*g* at 4°C and the supernatants were filtered through a 0.22 µm nylon membrane. Alditol hexa-acetate derivatives were prepared using a modified version of the method of Blakeney et al. (1983). Sample extracts were centrifuged at 12,000*g* for 10 min at 4°C and 500 µL aliquots of the supernatants were evaporated under a stream of N_2 gas at a temperature of 60°C. The residues were re-suspended in 200 µL of ultrapure water and 20 µL allose (1 mg/mL aqueous solution) was added as a surrogate standard. The pH of all the samples was raised by adding 20 µL of 15 M ammonium hydroxide, after which 1 mL of a sodium borohydride solution (0.5 M in dimethyl sulfoxide) was added to each sample. Samples were mixed by vortexing for 1 min, incubated at 40°C for 90 min vortexed again, and acidified with drop wise addition of 100 µL of glacial acetic acid and each sample was vortexed again. A 200 µL aliquot of 1-methylimidazole and 1 mL of acetic anhydride were then added, with vortexing after the addition of each reagent, and the samples were incubated at 40°C for 10 min to ensure that acetylation proceeded to completion. Finally, reactions were diluted with 2.5 mL of water and a 1 mL aliquot of dichloromethane was added and the samples were vortexed for 5 min prior to centrifugation at 1,000*g* for 10 min at 4°C. Approximately 80% of the upper aqueous phase was discarded and the majority of the lower solvent phase was recovered containing the acetylated alditols. Samples were diluted 10-fold (220 µL) and 20 µL of 44 nmol/mL of methyl tricosanoate (NuCheck Pre Inc., Elysian, MN) was added as an internal standard before transferring to 2 mL amber gas chromatograph vials fitted with 200 µL glass inserts.

myo-Inositol, as a hexa-acetate derivative, was quantified with a gas chromatograph (6890, Agilent, Palo Alto, CA) coupled with a mass spectral detector (5973N, Agilent). Measurements were performed in duplicate. One-microliter samples were introduced in the splitless mode onto a DB-225 column (30 m × 0.25 mm I.D., 0.15 µm film thickness; Agilent). The initial oven temperature of 70°C was held for 2 min then increased at 35°C min^{-1} to 225°C and held for 5 min. The inlet and transfer line temperatures were 225°C. Helium at a constant flow of 1 mL min^{-1} was the carrier gas. Electron impact (70 eV) mass spectra were collected after a 5 min solvent delay in selected ion monitoring/full scan mode. Full scan spectra were collected from charge/mass (m/z) ratio values of 50–550. The *myo*-inositol derivative was partially resolved from glucitol in the total ion chromatograms, therefore to enhance sensitivity and selectivity, quantification was executed using selected ion moni-

toring responses and two ions per compound. Standard curves for *myo*-inositol and other sugars (allose, glucose and galactose) were prepared from 200 µL aqueous standards of each of the sugars at concentrations of 20, 50, 100, and 200 µg/mL derivatized into alditol hexa-acetate derivatives as described above. Peaks eluted in the order of allitol hexaacetate (tR: 10.685 min) from allose, mannitol hexaacetate (tR: 9.794) from fructose, galactitol hexaacetate (tR: 10.217) from galactose, and glucitol hexaacetate (tR: 10.544) from glucose, and inositol hexaacetate (tR: 10.685). With the exception of inositol, alditol hexaacetates were quantified using m/z 115.1 with m/z 187.1 as the qualifier ion. Inositol hexaacetate was quantified using m/z 168.1 with m/z 210.1 as the qualifier ion. Methyl tricosanoate (tR: 8.373 min) was quantified using m/z 368.1 and 74.1 as the qualifier ion. The calibration curves bracketed all samples, were configured in internal standard mode, and the relative response factors were linear with correlation coefficients of between 0.995 and 0.999.

Genetic analysis of the N15-186 mutant

One plant, designated N15-186, was grown from the embryo half of a M_2 seed that tested positive for HIP, was used as a pollen donor in a cross with the *indica* cultivar Kasalath. The Kasalath/N15-186 cross was used to generate the K/N F_2 mapping population. Informative microsatellite markers were identified by surveying Nipponbare and Kasalath DNA for polymorphisms. K/N F_2 seeds were subjected to HIP phenotyping using the half-seed protocol and the F_2 plants grown from the corresponding half-seeds were genotyped with microsatellite markers. Where possible, F_3 seeds were used to confirm the phenotyping. DNA extraction and microsatellite marker genotyping were performed as previously described (Andaya and Tai 2005). Chi-square tests were performed to determine the goodness of fit between the Mendelian ratio of the F_2 mapping population and the segregation data for the low phytic acid (HIP) trait and the molecular markers.

Microsatellite marker analysis was performed as described previously (Andaya and Tai 2005). All microsatellite markers were selected from the Gramene database (http://www.gramene.org/) and surveyed for polymorphism prior to use. Markers used for initial linkage analysis were as follow: RM6, 7, 21, 25, 41, 55, 85, 104, 169, 215, 216, 225, 235, 248, 256, 274, 280, 298, 454, 482, 484, 519, and 536 (http://www.gramene.org). For additional mapping of the *lpa* locus, the following chromosome 3 markers were used: RM186, 5813, 15845, 15851, 15854, 15857, 15875, 15882, 15884, and 15907. The PCR reactions consisted of 50–100 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), 0.9 µM dNTPs, 0.46 µM fluorescein-12 or tetramethylrhodamine-6 labeled dUTP (Molecular Probes, Eugene, OR) and 0.6 U of Taq polymerase in a final volume of 8 µL. Amplifications were performed using MJ Research PTC-200 thermal cyclers (Waltham, MA) and 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. The PCR products were analyzed on an ABI 377 DNA sequencer using standard protocols and fragment analysis was performed using GENESCAN V3.1.2 (Applied Biosystem, Foster City, CA). Mapmaker/EXP V3.0 was used to perform linkage analysis between the *lpa* mutation and the molecular markers. Genetic distances were determined using the Kosambi function.

Sequence analysis of the *lpa* N15-186 candidate gene

Based on mapping, the candidate gene Os03g52760 (an orthologue of maize *lpa3*; Shi et al. 2005) was sequenced from N15-186. Primers were designed to amplify overlapping, 1 kb fragments using 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, 4 µL of 25 mM dNTPs, and 1 U of ExTaq polymerase (TaKaRa Bio USA, Madison, WI). PCR amplifications were performed using the same condition described above. Four independent PCR reactions for each line were combined and then purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) prior to sequencing. 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 for sequencing were designed manually or using the Primer 3 program (http://www.genome.wi.mit.edu/) and were purchased from Integrated DNA Technologies (Coralville, IA).

Gene structure of the *Os*MIK (Os03g52760) was obtained from the TIGR annotation database (http://www.tigr.org/). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) and additional homologies were identified in the Gramene website (http://www.gramene.org/). Multiple sequence alignments were generated using the ClustalW program (http://www.ebi.ac.uk/clustalw).

OsMIK gene expression analysis

For initial RT-PCR analysis total RNA samples were isolated from 7-day-old seedlings of N15-186 and Nipponbare using TRIzol® reagent (Invitrogen). For analysis of expression in various tissues, total RNA samples were prepared

from the shoots (leaf blade and leaf sheath) and roots of two week old seedlings and panicles tissue was harvested during grain filling. First-strand cDNA was synthesized from 5 μg of total RNA by random priming using SuperScript™ III reverse transcriptase according to the manufacturer's instructions (Invitrogen). The primer pairs were designed from the C-terminal and 3′ UTR region of the *OsMIK* sequence: *Os*MIK (forward 5′-GCTGTTAAACAAATAC TTGAGAAGG-3′ and reverse 5′-AGAGCACATAATTC AACAGAGAG-3′; 420 bp product) and actin (forward 5′-GAAGATCACTGCCTTGCTCC-3′ and reverse 5′-CGA TAACAGCTCCTCTTGGC-3′; 249 bp product). Five μL of single strand cDNA was used in 50 μL PCR reactions as described above and amplifications were performed using the same conditions as described above. RT-PCR products were separated and visualized using 1% agarose/1X TAE gels containing ethidium bromide. Expressed sequence tag data were obtained from the TIGR rice gene expression database (http://www.tigr.org/).

Accession number

Sequence data from this article can be found in the Genbank Database under the following accession number: Os MIK (EU366952).

Results

Identification of putative *lpa* mutants

M_2 seeds from 540 independent M_1 lines, obtained by chemical mutagenesis of Nipponbare seed, were screened to identify putative low phytic acid mutants. The M_2 seeds were cut in half ("embryo" half and "endosperm" half) and the endosperm half was subjected to the HIP assay. Two lines, N15-186 and N15-375, were identified as segregating for the HIP phenotype. Two out of eight M_2 half seeds from N15-186 exhibited the HIP phenotype (1:3; *lpa* mutant: wild-type) indicative of a single gene, recessive mutation. Two out of eight M_2 half seeds from N15-375 also exhibited the HIP phenotype (1:3; *lpa* mutant: wild-type), indicating that this mutant is due to a single gene, recessive mutation as well.

The remaining embryo half of the seeds were germinated to obtain M_2 plants for phenotypic and genetic analysis. One of two M_2 plants that grew from the HIP positive seeds for each line, was selected for seed increase and crossing. The M_3 seeds from the selected M_2 plants were scored for HIP and all seeds tested were HIP, confirming that both N15-186 and N15-375 lines were homozygous for the putative *lpa* mutation. Seeds from these plants appeared to have normal germination and seedling development based on casual observation.

Characterization of seed phosphorus, inositol polyphosphates, and *myo*-inositol

In order to confirm that these two mutants are *lpa* mutants, the seed total P, phytic acid-P (PA-P) and inorganic P (P_i) contents were examined (Table 1). Quantitative analysis of N15-186 and N15-375 seeds indicated that while the total P contents of mutant seeds were not significantly different compared to wild types, the P_i contents were significantly increased and the PA-P contents were significantly reduced in both mutants. N15-186 exhibited a much stronger *lpa* phenotype compared to N15-375 (75% reduction in phytic acid compared to wild-type for N15-186 versus 43% reduction in phytic acid relative to wild-type for N15-375).

Seed extracts of *lpa* mutants were subjected to HPLC analysis to determine if the other inositol phosphates were affected by the mutation. Comparison to wild-type Nipponbare and inositol phosphate standards revealed that N15-186 seeds are significantly reduced in PA and inositol monophosphate while exhibiting an increase in P_i (Fig. 1). No significant accumulation of other inositol phosphate intermediates ($InsP_2$ to $InsP_5$) was detected under the conditions used. The HPLC elution profile of N15-375 showed an increase in P_i and a decrease in PA, but no other significant changes were observed (Fig. 1).

Table 1 Comparison of seed *myo*-inositol, phytic acid and inorganic P contents of *lpa* mutants and wild-types

Line	Seed dry weight (mg)[a]	*myo*-Inositol (μg g^{-1} seed)[b]	Phytic acid P (mg g^{-1} seed)[a,c]	Inorganic P (mg g^{-1} seed)[a,c]	Total P (mg g^{-1} seed)[b,c]
Nipponbare (wild-type)	20.5 ± 1.6	53.2 ± 0.4	2.61 ± 0.31	0.10 ± 0.05	3.35 ± 0.27
N15-186	18.4 ± 2.6	608.4 ± 12.4*	0.65 ± 0.36*	1.64 ± 0.33*	3.39 ± 1.24
N15-375	18.2 ± 1.6	75.2 ± 1.2	1.48 ± 0.25*	0.53 ± 0.21*	3.03 ± 0. 54

* 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] Values are means and standard deviation of four independent assays

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

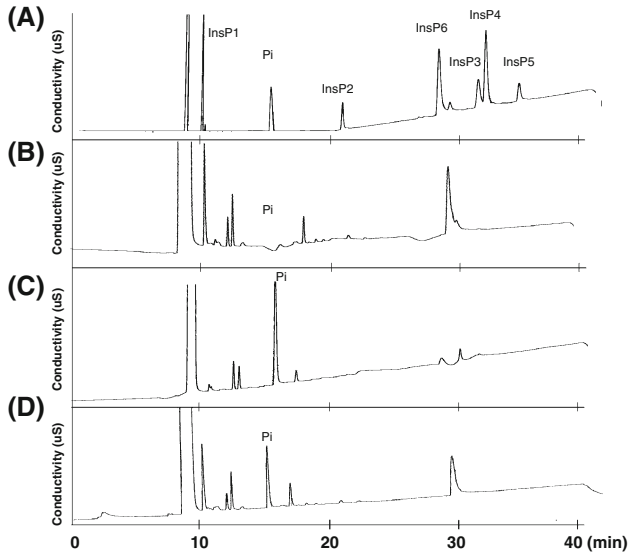

Fig. 1 HPLC chromatograms of inositol phosphates. **a** Inositol phosphate standards, retention times for the various inositol phosphates were: Ins(2)P_1, 9.5 min; P_i, 15.5 min, Ins(1,4)P_2, 21 min; Ins(1,3,4)P_3, 32 min; Ins(13,4,5)P_4, 33 min; Ins(1,3,4,5,6)P_5, 35 min; and Ins(1,2,3,4,5,6)P_6, 29 min. **b** Nipponbare wild-type, **c** N15-186 *lpa* mutant, **d** N15-375 *lpa* mutant

The GC–MS analysis revealed that the *myo*-inositol content, as well as those of other sugars (glucose, fructose and galactose), was increased in the N15-186 mutant compared to the wild-type (Fig. 2). The *myo*-inositol content of N15-186 seed was ninefold greater than wild-type, while the other sugars examined were 2–3 fold higher. In contrast, the *myo*-inositol content of N15-375 mutant seeds did not differ from wild-type.

Mapping and identification of *lpa* N15-186

The N15-186 mutant was selected for further genetic characterization based on a greater reduction in phytic acid content compared to N15-375. The M_2 plant used to generate seeds for analysis of phosphorus content was also used as a pollen donor for a cross with the cultivar Kasalath, which exhibits a wild-type HIP phenotype. Three F_1 progeny were obtained from the Kasalath/N15-186 (K/N) cross and confirmed to be true hybrids by analysis with microsatellite markers (C. Andaya, data not shown). Ninety-four K/N F_2 seeds from a single F_1 plant were scored for the HIP phenotype using a half-seed assay. Of the 94 seeds, a ratio of 21 HIP:73 wild-type was observed which was consistent with a single gene, recessive mutation ($\chi^2 = 0.355$; df = 1; *P* value = 0.552). The embryo half of each seed was germinated and DNA samples were extracted for genotyping. F_3 seeds were obtained from 77 of the F_2 plants. Phenotyping of these F_3 seeds indicated that out of the 77 F_2 plants producing seeds, 16 were *lpa*/*lpa*, 40 were *Lpa*/*lpa* and 21 were *Lpa*/*Lpa*. These results were also consistent with a single gene, recessive mutation ($\chi^2 = 0.766$; df = 2, *P* value = 0.682).

In order to map the *lpa* N15-186 locus, microsatellite marker data were generated from 81 of the 94 F_2 plants, which consisted of the 77 F_2 plants whose phenotypes were confirmed by progeny testing and four F_2 plants whose F_2 half seeds were scored as HIP (*lpa*/*lpa*), but did not produce F_3 seeds. A total of 23 polymorphic microsatellite markers were examined and two point linkage analyses were conducted using the segregation data for the markers and the *lpa* phenotype. This resulted in the identification of linkage

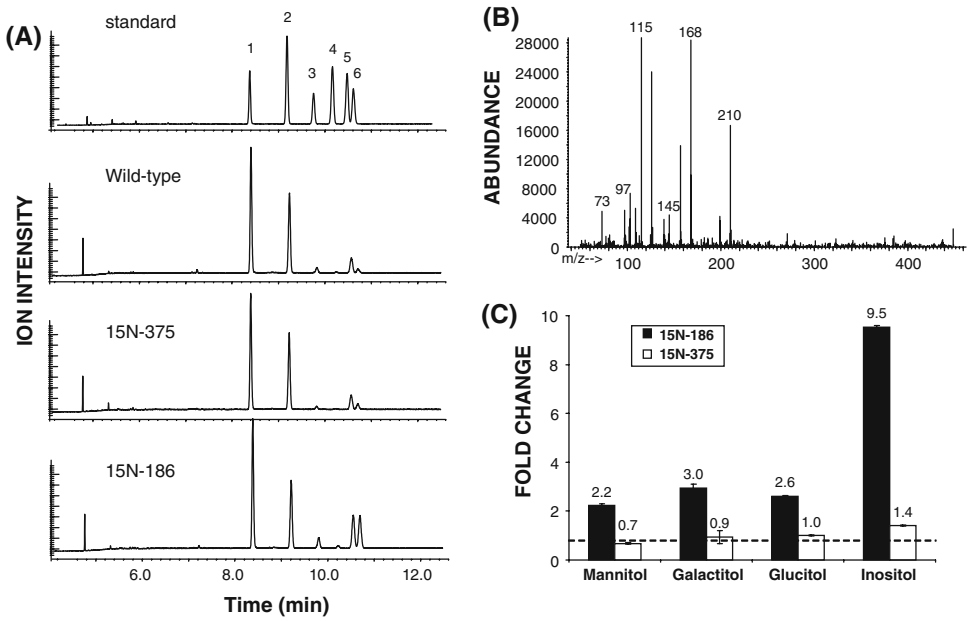

Fig. 2 GC–MS analysis of *myo*-inositol and other carbohydrates as alditol hexaacetate derivatives from seed extracts of wild-type, N-186 and N-375 mutants. **a** Representative chromatogram of standard and seed extracts Peaks indicate: 1: methyl tricosanoate (internal standard); 2: allitol hexaacetate (surrogate standard); 3: mannitol hexaacetate (fructose); 4: galactitol hexaacetate (galactose); 5: glucitol hexaacetate (glucose); 6: *myo*-inositol hexaacetate (*myo*-inositol). **b** Mass spectrum of *myo*-inositol hexaacetate. **c** Comparison of carbohydrate change in mutants relative to wild-type

between the *lpa* N15-186 locus and RM55 (27.9 cM), which is located on chromosome 3.

Using additional microsatellite markers near RM55, the *lpa* N15-186 locus was delimited to the interval between RM186 and RM5813 (Fig. 3). Additional F_2 plants (total 243 plants—original 77 and additional 166 plants) were analyzed to find recombinants between these markers. A total 45 F_2 recombinants were identified between RM186 and RM5813. The rice orthologues of maize *lpa*2 (ZmIPK) and *lpa*3 (MIK) reside within this interval. Further mapping indicated that the *lpa* N15-186 locus resides between RM15875 (one recombinant) and RM15907 (four recombinants), thus eliminating the maize *lpa*2 orthologue (Os03g51610) as a candidate (Fig. 3). The physical distance between RM15875 and RM15907 is about 500 kb.

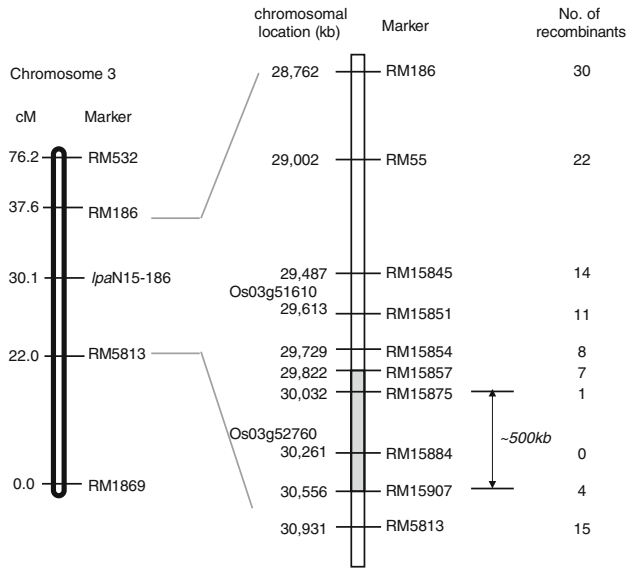

Fig. 3 Genetic and physical map of the *lpa* N15-186 locus on rice chromosome 3. The number of recombinants between a marker and the locus is indicated in the far right column. A total of 243 F_2 progeny from a Kasalath/N15-186 cross were examined to identify these recombinants

Sequence analysis of Os03g52760 (maize *lpa*3 orthologue) revealed a single base pair change (C/G to T/A) in the first exon at position 409 (relative to the predicted ATG) which results in a nonsense mutation (Fig. 4a). In addition, the markers RM15884, which is about 60 kb from Os03g52670, and RM15882, which is about 2 kb from Os03g52760, co-segregate with the *lpa* phenotype. These results indicate that *lpa* N15-186 is a mutant allele of Os03g52760, which we designated as *OsMIK* based on homology to the maize MIK gene.

A BLASTP search revealed that OsMIK is a highly conserved protein among higher plants (Fig. 5). Maize LPA3 (AAX14809) is 80% identical to OsMIK at the amino acid level. Sorghum has a theoretical protein (Sbi_0.14483), which exhibits 88% identity to OsMIK. *Populus* has two OsMIK orthologues showing 53% identity (gw1.IX.3754.1) and 51% identity (gw1.I.898.1), respectively. The *Vitis* genome has a protein (CAO21458) showing 50% amino acid identity and the *Arabidopsis* protein (NP_125260) encoded by the At5g5873 has 46% identity.

Gene structure and expression of *OsMIK*

Examination of the *OsMIK* gene structure indicates that the gene has a single open reading frame consisting of two exons (Fig. 4a) and is predicted to encode a protein of 397 amino acids. The transcript is predicted to be about 1.5 kb. RT-PCR analysis carried out using total RNA from N15-186 and Nipponbare seedlings indicated that the mutation does not appear to affect the expression of *OsMIK* at the transcriptional level (Fig. 4b). RT-PCR analysis of total RNA from various tissues including shoot, root, and panicle indicated that *OsMIK* is expressed in all tissues (Fig. 6). Higher expression was detected in the vegetative shoot tissue compared to the reproductive tissue with relatively lower expression in the roots. A search of publicly available rice EST data indicated that *OsMIK* is expressed in callus, leaf, root, shoot and panicle tissues (http://www.tigr.org/).

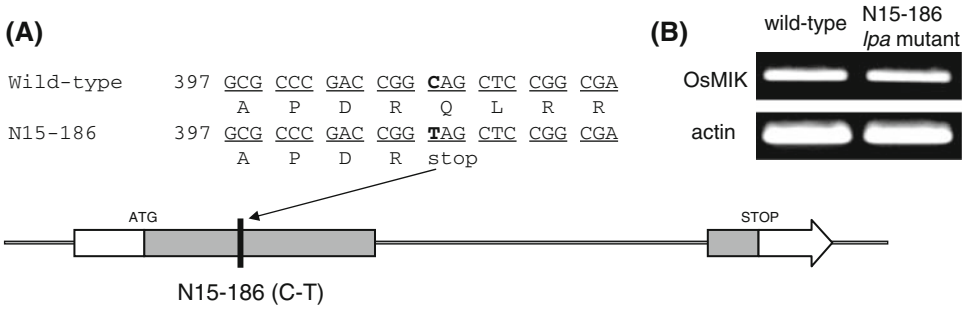

Fig. 4 Gene structure and analysis of OsMIK gene expression in the N15-186 mutant. **a** The structure of OsMIK gene and mutation Open boxes represent 5′ and 3′ UTR, filled boxes represent the coding regions, and the lines between boxes indicate introns. The N15-186 mutation (C → T change) was found in the first exon, **b** RT-PCR of OsMIK gene were performed using total RNA isolated from 7-day-old seedlings of wild-type and N15-186 mutant. Actin was used as a control

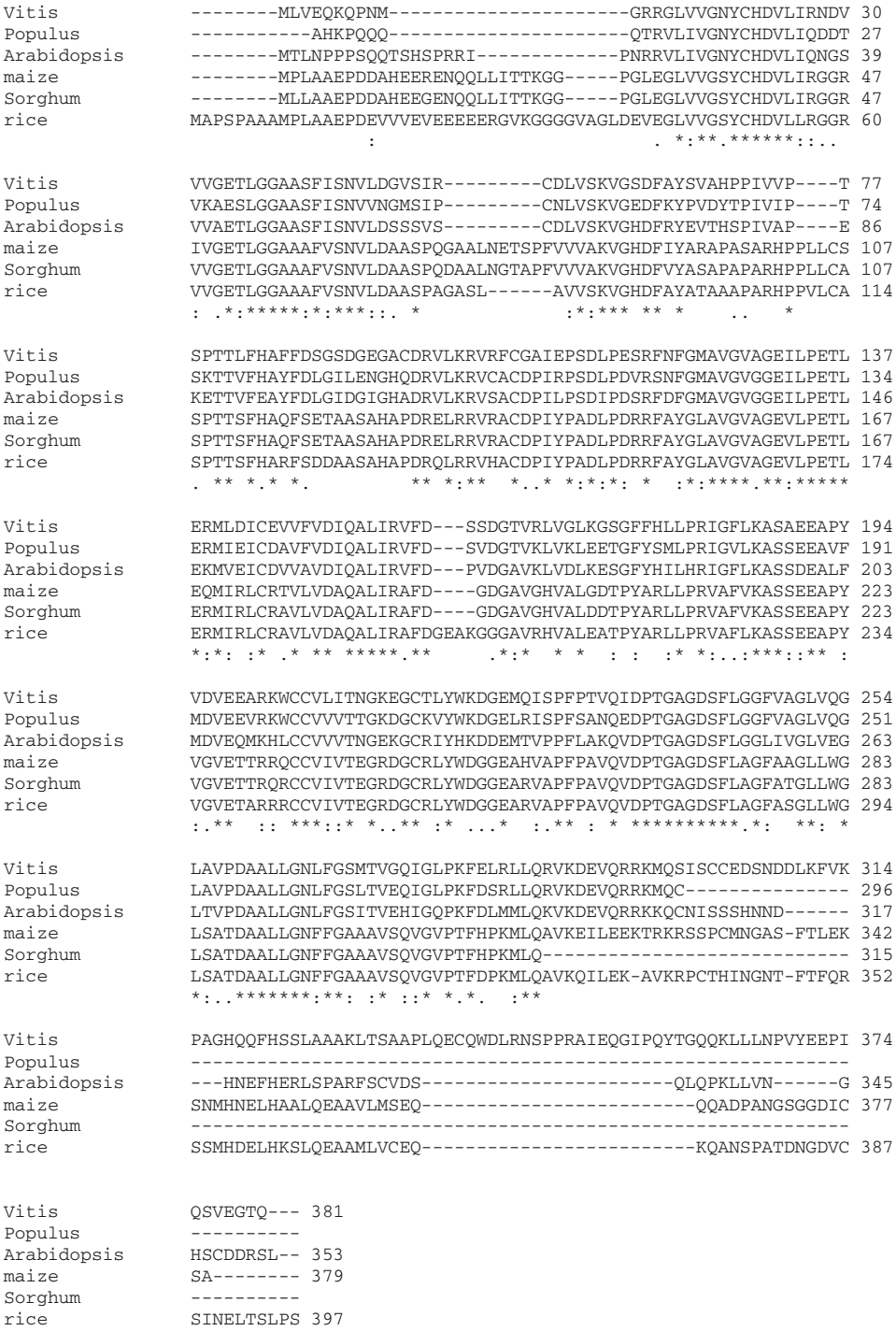

Fig. 5 Alignment of the deduced amino acid sequences from rice OsMIK (AAP03418), *Sorghum bicolor* (Sbi_0.14483) maize *Lpa3* (AAX14809), *Vitis* (CAO21458), *Arabidopsis thaliana* (NP_125260) and *Populus* (gw1.I.898.1). Sorghum, maize, and *Populus* accession numbers are from Gramene. Others are from NCBI. Alignments were displayed with the following symbols denoting the degree of conservation *asterisk* indicates that all the residues in the column are identical, *colon* indicates that conserved substitution has been observed, and *dot* indicates that semi-conserved substitution are observed

Discussion

A number of *lpa* mutants have been isolated in maize, barley, wheat, rice, soybean, with a reduction of PA in seeds ranging from 50 to >95% (Raboy et al. 2001). In maize, the *lpa*1, *lpa*2, and *lpa*3 mutants can be distinguished by differences in inositol and inositol phosphate content. Maize *lpa*1 mutants exhibit a reduction in PA which is accompanied by a molar equivalent increase in P_i, whereas *lpa2* mutants exhibit significant increases in inositol phosphate intermediates and *lpa*3 mutants accumulate P_i and *myo*-inositol but do not accumulate inositol phosphate intermediates (Raboy et al. 2000; Shi et al. 2005). The genes corresponding to each of these maize *lpa* mutants have been cloned (Shi et al. 2003, 2005, 2007). Larson et al. (2000) isolated the first non-lethal rice *lpa* mutant, *lpa*1, which exhibit about a 50% reduction in seed phytic acid and a molar equivalent increase in inorganic P. We recently reported the cloning of

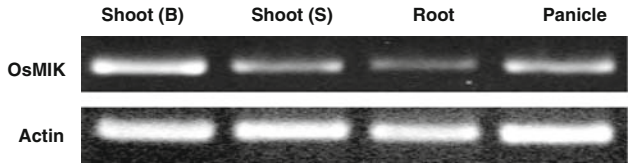

Fig. 6 Expression analysis of OsMIK in different tissues of wild-type plant. RT-PCR analysis was performed using total RNA from various tissues [shoot (B) = leaf blade, shoot (s) = leaf sheath/stem, root and panicle] of wild-type Nipponbare and primers for *Os*MIK and actin (control)

the rice *lpa*1 gene (*OsLpa*1), which is predicted to encode a protein with homology to 2-phosphoglycerate kinase from hyperthermophilic methanogens (Kim et al. 2008). Liu et al. (2007) reported the isolation of eight rice *lpa* mutants, one of which is allelic to rice *lpa*1 and another that was mapped to the same region on chromosome 3 harboring the rice orthologue of the maize *myo*-inositol kinase gene, which corresponds to maize *lpa*3.

We report here the isolation of two low phytic acid mutants, N15-186 and N15-375, resulting from recessive, single gene mutations. HPLC and GC–MS analysis revealed that the seeds of N15-186, unlike those of N15-375, have significantly higher levels of *myo*-inositol and reduced levels of inositol monophosphate in addition to the changes in PA and P_i that are typical of all *lpa* mutants. These results indicate the the N15-186 mutant has a similar phenotype to that of the maize *lpa*3 mutant (Shi et al. 2005). In addition to *myo*-inositol, other sugars such as glucose, fructose, and galactose accumulate in N15-186. The alteration of metabolically related sugar compounds has been observed in *lpa* mutants of barley (Karner et al. 2004) and rice (Frank et al. 2007). This is not surprising given the central role played by *myo*-inositol in numerous biosynthetic pathways in plants (Loewus and Murthy, 2000).

Since N15-375 appeared to be very similar to the rice *lpa*1 mutant, we selected the N15-186 mutant for further characterization. Genetic mapping of the N15-186 mutation, *lpa*-N15-186, indicated that the locus resides in the interval between microsatellite markers RM15875 and RM15907 on chromosome 3. This region is about 500 kb and contains the rice orthologue of the maize *lpa*3 gene, which encodes a *myo*-inositol kinase (MIK; Shi et al. 2005). MIK phosphorylates *myo*-inositol to form *myo*-inositol monophosphate and is believed to contribute to the lipid-independent biosynthesis of phytic acid in plants. The results of the metabolite analysis and genetic mapping strongly suggested that the N15-186 mutant was due to a mutation affecting the rice orthologue of *myo*-inositol kinase. To address this possibility, we sequenced the N15-186 allele of the rice MIK orthologue (Os03g52760) for comparison to the Nipponbare allele. A nonsense mutation was detected in the first exon of the gene from N15-186, indicating that the *lpa* N15-186 mutation is in Os03g52760, which we have designated as *OsMIK*. Based on RT-PCR analysis and EST data, *OsMIK* appears to be expressed in vegetative and reproductive tissues. No significant difference in expression of OsMIK was detected in our preliminary comparison of N15-186 to Nipponbare.

Phytic acid biosynthesis is thought to proceed via at least two distinct pathways in plants (Irvine and Schell 2001; Raboy 2001; Stevenson-Paulik et al. 2005). The inositol lipid-dependent pathway, originally defined in yeast and mammalian systems, involves the generation of $Ins(1,4,5)P_3$ from phosphatidylinositol-4,5-bisphosphate via phospholipase C followed by the sequential phosphorylation of $Ins(1,4,5)P_3$ to form phytic acid ($InsP_6$). The second pathway, proposed based on biochemical evidence in plants and *Dictyostelium discoideum*, is lipid-independent and is believed to involve the sequential phosphorylation of 1D-*myo*-inositol 3-phosphate [$Ins(3)P_1$] to form $InsP_6$ without the involvement of inositol lipids. $Ins(3)P_1$ is generated from glucose 6-phosphate by $Ins(3)P_1$ synthase (also known as 1L-*myo*-inositol 1-phosphate synthase or MIPS). It can also be produced by the phosphorylation of free *myo*-inositol by *myo*-inositol kinase (MIK) (Loewus at al. 1982; Stephens et al. 1990; Shi et al. 2005).

The importance of *myo*-inositol kinase in seed phytic acid metabolism has been established by the characterization of the maize *lpa*3 mutant, which is caused by a mutation in the maize *myo*-inositol kinase gene (Shi et al. 2005). Nevertheless, since $Ins(3)P_1$ is produced directly from glucose 6-phosphate by MIPS, it is not clear exactly why the activity of *myo*-inositol kinase is important for phytic acid biosynthesis. One possible explanation is that while the product of MIPS is $Ins(3)P_1$, MIK can produce multiple inositol monophosphates (Shi et al. 2005) which could provide more substrate diversity for the generation of inositol bisphosphate, the next step in the proposed lipid-independent pathway.

The expression of the MIPS gene in developing rice seeds coincides spatially and temporally with the accumulation of phytate, a mixed salt of phytic acid (Yoshida et al. 1999). MIK activity has been reported to be active in the aleurone layers of rice seed (Tanaka et al. 1976), which is the predominant site of phytic acid biosynthesis and accumulation. In general, *myo*-inositol serves as a precursor of several metabolic pathways including cell wall biogenesis through UDP-glucuronate by *myo*-inositol oxidation, auxin storage and transport though auxin-inositol conjugation, phosphatidylinositol signaling pathway through inositol phospholipd and synthesis of phytic acid through sequential phosphorylation. MIK could determine the fate of *myo*-inositol especially in seed. The identification of mutations in the maize and rice MIK that result in low seed phytic acid supports the importance of this enzyme in phytic acid bio-

synthesis in seeds. Also, significant reduction of phytic acid in both mutants suggested that the majority of phytic acid (50–75%) is synthesized via the lipid-independent pathway in plant seeds.

In the present study, we identified a new *lpa* mutation that appears to affect translation of the rice *myo*-inositol kinase (*OsMIK*) gene. Our results indicate that like the maize MIK, *OsMIK* is necessary for wild type levels of seed phytic acid, but not essential for viability. The identification of mutations in the maize and rice MIK that result in low seed phytic acid lends support to the existence of a lipid-independent biosynthetic pathway in plants. Analysis of *lpa* mutants, such as N15-186, will facilitate studies on the regulation of phytic acid biosynthesis and accumulation and help address questions concerning the contribution of inositol lipid-dependent and -independent biosynthetic pathways to the phytic acid content of seeds and other plant tissues.

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