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ORIGINAL PAPER

Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.)

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Abstract Phytic acid (PA, *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate), or its salt form, phytate, is commonly regarded as the major anti-nutritional component in cereal and legume grains. Breeding of low phytic acid (*lpa*) crops has recently been considered as a potential way to increase nutritional quality of crop products. In this study, eight independent *lpa* rice mutant lines from both *indica* and *japonica* subspecies were developed through physical and chemical mutagenesis. Among them, five are non-lethal while the other three are homozygous lethal. None of the lethal lines could produce homozygous *lpa* plants through seed germination and growth under field conditions, but two of them could be rescued through in vitro culture of mature embryos. The non-lethal *lpa* mutants had lower PA content ranging from 34 to 64% that of their corresponding parent and four of them had an unchanged total P level. All the *lpa* mutations were inherited in a single recessive gene model and at least four *lpa* mutations were identified mutually non-allelic, while the other two remain to be verified. One mutation was mapped on chromosome 2 between microsatellite locus RM3542 and RM482, falling in the same region as the previously mapped *lpa1-1* locus did; another *lpa* mutation was mapped on chromosome 3, tightly linked to RM3199 with a genetic distance of 1.198 cM. The latter mutation was very likely to have happened to the LOC_Os03g52760, a homolog of the maize *myo*-inositol kinase (EC 2.7.1.64) gene. The present work greatly expands the number of loci that could influence the biosynthesis of PA in rice, making rice an excellent model system for research in this area.

Keywords *Oryza sativa* L. · Low phytic acid (*lpa*) · Mutation · Gene mapping · *Myo*-inositol kinase

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Introduction

Phytic acid (PA), known as *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate, is a ubiquitous component and often exists in the form of a mixed salt (phytate or phytin) of mineral cations, including Zn^{2+} and Fe^{3+} in plant seeds (Raboy 1997; Lott et al. 2000). The role of PA reserves in plant seeds is still poorly understood—merely being viewed as a phosphorus (P) and mineral storage compound or as an important metabolite in P homeostasis (Raboy 1997; Lott et al. 2000). However, PA and its

salts are almost indigestible for monogastric animals; its abundance in grain food/feed is known to cause nutritional and environmental problems (Raboy 2001). In developing countries, high-phytate grain-based diets are often feared to exacerbate iron and zinc malnutrition, while in developed countries, where livestock is fed primarily with grain-based feed, the excreted phytate contributes to environmental P pollution by being washed into surface waters (Lott et al. 2000). These concerns have collectively provided a strong impetus to developing low phytic acid (*lpa*) crops, in which the PA–P content is significantly reduced in seeds.

Several *lpa* mutant lines have been generated by chemical and physical mutagenesis as well as genetic transformation in several crops, including maize (Raboy et al. 2000; Pilu et al. 2003; Shi et al. 2003, 2005), barley (Larson et al. 1998; Ramussen and Hatzak 1998), wheat (Guttieri et al. 2004), soybean (Wilcox et al. 2000) and rice (Larson et al. 2000). Genetic analysis has shown that the *lpa* phenotypes resulted from single locus mutations, and three types of *lpa* mutation, designated as *lpa1*, *lpa2* and *lpa3*, have been already identified and mapped to different positions of various chromosomes in maize (Raboy et al. 2000; Pilu et al. 2003; Shi et al. 2003, 2005), barley (Larson et al. 1998) and soybean (Walker et al. 2006). Human feeding studies showed that, in comparison with its wild type counterpart, *lpa* maize diets increase the absorption of several important minerals (Mendoza et al. 1998, 2001; Adams et al. 2001). Similarly, reduction of P content by 10–85% in wastes and increase of P bioavailability by 2–5 times were observed in several animal feeding studies using either *lpa* maize or barley (Spencer et al. 2000a, b; Veum et al. 2001; Yan et al. 2000, 2003; Li et al. 2000; Douglas et al. 2000; Loza et al. 2002).

Rice is the staple diet for nearly two billion people worldwide and the major food for over 50% of Asians, where mineral micronutrient malnutrition is a common occurrence. Therefore, *lpa* rice has become a potential means to alleviate mineral micronutrient malnutrition in these areas. In addition, since the by-product of rice grains, i.e. hull and bran including pericarp, seed coat, embryo and aleuronic cells, is an important constituent of daily animal feed in rice growing areas, *lpa* rice should be also beneficial to animal production and environmental protection. Up to now, only one *lpa* mutant line, KBNT *lpa1*-1 from Kaybonnet, has so far been isolated and characterized in rice (Larson et al. 2000; Rutger et al. 2004), of which the *lpa* mutation was fine mapped to a 47-kb region of chromosome 2L, though the causative gene has yet to be identified (Andaya and Tai 2005). Similar to most other *lpa* mutants (Rasmussen and Hatzack 1998; Raboy et al. 2000; Oltmans et al. 2005), KBNT *lpa1*-1 has an about 10% yield penalty as compared to other standard rice varieties (Rutger et al. 2004), which does not lend itself readily for breeding yield competitive *lpa* varieties. This issue might be solved through isolation of more *lpa* mutants and a wiser combination of different mutations (double mutant). For example, at least one *lpa* mutation (*lpa 1*-1) has proven not harmful to yield performance in barley (Bregitzer and Raboy 2006) and a combination of two mutant inositol phosphate kinase genes could lead to phytate-free seeds but without detectable yield reduction in *Arabidopsis* (Stevenson-Paulik et al. 2005). In the present study, several mutated *lpa* rice lines were generated through chemical and physical mutagenesis and some of them were genetically and chemically characterized at different levels, with the aim to develop new genetic resources for both breeding *lpa* varieties and facilitating research on PA synthesis in rice.

Materials and methods

Generation of *lpa* mutants

Six commercial rice varieties, i.e. one conventional *japonica* rice variety "Xiushui 110" (XS110), three *indica* cytoplasmic male sterile (CMS) maintainer lines "Zhenshan 97 B" (ZS97), "Xieqingzao B" (XQZ) and "Zhong 9 B" (Z9B), and two CMS restorer lines "Minghui 86" (MH86) and "R6547", were subjected to either gamma rays irradiation or additionally followed by NaN_3 treatment at routine doses (Table 1). Briefly, dried seeds (ca. 13.5% of moisture) were treated with 250–300 Gy γ rays from a ^{60}Co facility, at the Irradiation Centre of Zhejiang University, at a dose rate of 5 Gy per min, or from a ^{137}Cs facility, at the Irradiation Centre of Zhejiang Academy of Agricultural Sciences, at a dose rate of 0.8 Gy per min. The gamma-irradiated seeds of XS110 and R6547 were soaked in tap water for 8 h, and then treated in the solution of 1.0 mM NaN_3 for 2 h, followed by washing in running tap water for 2 h.

M_1 seeds were sown on seedling beds after indoor pre-germination; seedlings were transplanted to paddy fields with 3–5 plants per hill. M_2 seeds were harvested in bulk from M_1 plants and were sown again as was done for M_1 seeds. M_2 seedlings were transplanted as single plant per hill; seeds from M_2 plants, designated as $M_{2:3}$ seeds (the same for other materials, e.g. $M_{3:4}$ and $F_{2:3}$ seeds were those harvested from M_3 and F_2 plants, respectively) were harvested and stored on an individual plant basis.

Table 1 Low phytic acid mutant lines generated through physical and chemical mutagenesis in rice

Parental variety	Mutagenic treatment	No. of M_2 plants	Mutant line, generation and homozygous lethality[a]
Xieqingzao B (XQZ)	Dried seeds, 300 Gy ^{60}Co γ rays	4,500	*Os-lpa*-XQZ-1, M_3, non-lethal *Os-lpa*-XQZ-3, lethal
Xiushui 110 (XS110)	Dried seeds, 250 Gy ^{60}Co γ rays followed by soaking germinating seeds in 1.0×10^{-3} mol/L NaN_3 solution for 2 h	33,500	*Os-lpa*-XS110-3, lethal *Os-lpa*-XS110-2, M_3, non-lethal *Os-lpa*-XS110-1, M_2, non-lethal
Zhenshan 97 B (ZS97)	Dried seeds, 300 Gy ^{60}Co γ rays	1,060	None
R6547	The same as XS110	82,600	*Os-lpa*-R6547-3, lethal
Zhong 9 B (Z9B)	Dried seeds, 300 Gy ^{60}Co γ rays	5,150	*Os-lpa*-Z9B-1, M_2, non-lethal
Minghui 86 (MH86)	Dried seeds, 300 Gy ^{137}Cs γ rays	3,428	*Os-lpa*-MH86-1∼1/∼4, M_2, and *Os-lpa*-MH86-1∼5/∼7, M_3, non-lethal

[a] Mutant lines are named by using the first two letters of the crop Latin name (*Oryza sativa Os*), "*lpa*" short for recessive low phytic acid mutation, and followed by both an abbreviation of parent variety name (e.g. XQZ for Xieqingzao B) and the first order of numbering for non-allelic mutations with "−1" and "−2" for non-lethal lines and "−3" for lethal lines. When there are more than one allelic line from the same variety, the name is further extended by "∼1, 2, etc."

Generation generation at which an homozygous mutant plant was first identified

lethality lines in which no homozygous *lpa* plant could survive after seed propagation in field conditions

$M_{2:3}$ seeds were indirectly screened by using the colorimetric assay for high inorganic P (Pi) in 96-well plates, following the procedures of Larson et al. (2000) using freshly prepared Chen's reagent (Chen et al. 1956). Development of a blue color implies increased level of Pi typical for *lpa* mutants, while colorless samples typified wild-type levels of all parent varieties used in this experiment (Fig. 1). Seeds with an elevated level of Pi (stain in blue color) are hereafter referred to as high inorganic phosphorus (HIP) seeds. Eight $M_{2:3}$ seeds were tested for each M_2 plant, and the remaining seeds of corresponding M_2 plants with at least one HIP seed were grown into M_3 plants. Similarly, the Pi level was tested for seeds of $M_{3:4}$ and advanced generations. Three mutant lines, i.e. *Os-lpa*-XQZ-3, *Os-lpa*-XS110-3 and *Os-lpa*-R6547-3, however, did not yield in any homozygous plants through continuous self-pollinations. Seeds of these three lines were cross cut into two parts; the half without embryo was used for colorimetric assay for the Pi level, and the other half with embryo was rescued by culturing on MS basic medium (Murashige and Skoog 1962).

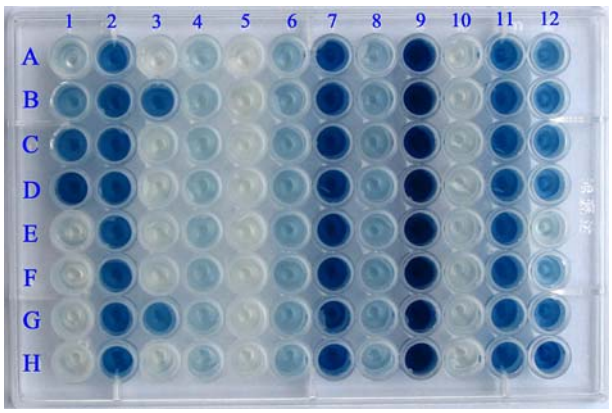

Fig. 1 Detection of high levels of inorganic P by molybdenum staining of hydrochloric extracts of rice grains. 1E–1H, XQZ; 2A–2H, *Os-lpa*-XQZ-1; 3A–3H, *Os-lpa*-XQZ-3; 4A–4H, *Os-lpa*-Z9B-1; 5A–5D, Z9B; 5E–5H, MH86; 6A–6H, *Os-lpa*-MH86-1; 7A–7H, *Os-lpa*-XS110-1; 8A–8H, *Os-lpa*-XS110-2; 9A–9H, *Os-lpa*-XS110-3; 10A–10 D, R6547; 10E–10H, XS110; 11A–11H, *Os-lpa*-R6547-3; 12A–12D KBNT *lpa1*-1; Both 1A–1D and 12E–12H are P standards at amounts of 0.155, 0.465, 0.93, and 1.395 μg, respectively. The P in each sample well equals the amount of inorganic P in 1 mg brown rice grain

Phosphorus analysis

Brown rice grains were used for analysis of all P compounds. Dried grains were ground into flour and passed through a 2-mesh sieve using a Cyclone Sample Mill (UDY Corporation, Fort Collins, Co., USA). Total P and Pi content was determined colorimetrically (Chen et al. 1956), following the procedures of Raboy et al. (1984) for total P and Wilcox et al. (2000) for Pi after minor modifications. Analysis of PA–P was performed through anion-exchange HPIC. Samples were pretreated following the procedures of Dorsch et al. (2003) after slight modification. Briefly, 5.0 g rice flour of each sample was extracted in 0.4 M HCl (20 ml) overnight; then centrifuged at 15,000*g* for 30 min, 10 ml aliquots of the supernatants were diluted with ddH_2O to 20 ml and added with 5 ml of 15 mM $FeCl_3$/0.2 M HCl solution in a 30 ml Corex centrifuge tube; the solutions were heated at 90°C for 30 min and centrifuged for 30 min at 8,000*g*, 30 min; the precipitate was converted to a soluble sodium inositol (Na Ins) phosphate solution and a $Fe(OH)_3$ precipitate by adding 1.0 ml

1.5 M NaOH; the solutions (1.0 ml) were transferred to 1.5 ml micro-centrifuge tubes and centrifuged at 12,000*g* for 15 min. An aliquot (300 µl) of supernatant was diluted to 1.0 ml, passing through a 0.2 µm filter for injection use. Ion chromatography assay was based on the procedure of Philliphy et al. (2003); aliquots of the sample solutions were separated on a Dionex IonPac AS7 anion-exchange column (Dionex, Sunnyvale, CA, USA), equipped with an IonPac AG7 guard column (Dionex), which had been equilibrated with 0.25 N HNO_3 at a flow rate of 1 ml/min. The effluent from the column was mixed with a colorimetric reagent [0.1% $Fe(NO_3)_3$ in 2% $HClO_4$] at a flow rate of 0.8 ml/min and passed through a plastic coil; the UV absorbance was monitored at 290 nm in a Waters Lambda Max model 480 LC spectrophotometer. An external standard of Na Ins P_6 (P-3168, Sigma, St Louis, MO, USA) was analyzed before and after every two samples.

All assays were performed in triplicate and both total P and other forms of P were expressed as their P (atomic weight = 31) content on a dry matter basis. Multiple comparison analysis was performed using the Statistical Analysis System (SAS 8.0 Institute, Inc., Cary, NC, USA). Data were expressed as a mean with standard deviation (SD) and compared by one-way analysis of variance (ANOVA).

Genetic analysis of *lpa* mutations

The inheritance modes of different *lpa* mutations were investigated using various populations, i.e. the segregating populations of the mutant progenies and the F_2 and F_3 populations of crosses between the mutants and their parents, or between mutants and other wild type varieties. For allelism test of various mutations, F_1 seeds were produced through artificial crossing between mutants and F_2 seeds through self-pollination of F_1 plants. The KBNT *lpa1*-1 mutant, developed by gamma irradiation by Larson et al. (2000), became available to us in 2004 and was used for some allelism tests. In the case of *Os-lpa*-XS110-1 and *Os-lpa*-SX110-2, doubled haploid (DH) plants were developed from F_1 plants through anther culture using a protocol routinely practiced in our breeding program (Shen et al. 2003). Seeds were defined as either HIP or wild type based on the color after staining (blue or colorless) in colorimetric assays as described above (Generation of *lpa* mutants).

Molecular mapping of *lpa* mutations

Initially, two F_2 populations, i.e. *Os-lpa*-XQZ-1/ XQZ and *Os-lpa*-XS110-1/ XS110, were developed. Since only a few polymorphic microsatellite (or simple sequence repeat, SSR) markers were observed between the mutant and its wild type parent, two other crosses, i.e. *Os-lpa*-XQZ-1/Huahui No. 7 and G0133/*Os-lpa*-XS110-1, were made to produce two new F_2 mapping populations. The phenotype of individual seeds was determined according to the color after staining in Pi assays as described above, and the genotype of F_2 plants was defined as homozygous *lpa* or wild type when all its $F_{2:3}$ seeds were high or low in Pi and as heterozygous when its $F_{2:3}$ seeds were of both types.

Genomic DNA was extracted from leaf tissues of F_2 plants and their parents following a modified CTAB method (Lu and Zheng 1992). DNA samples were quantified using the Unican UV300 (Thermo Electron Corporation, Cambridge, UK) and adjusted to a final concentration of about 25 ng/µl. DNA pool samples were also directly extracted from equally mixed leaf tissues of 20 homozygous HIP F_2 plants.

The primer sequences of these SSRs were originally described by Temnykh et al. (2000, 2001) and acquired through Rice Sequence Information Resources (http://www.dna-res.kazusa.or.jp/9/6/05/spl_table1/table1.pdf). All primers were synthesized in Shanghai Sangon Biological Engineer Technology & Services Co., Ltd (Shanghai, China). PCR amplification reactions were performed in 20 µl volumes containing approximately 50 ng genomic DNA, 1× PCR buffer, 400 nM each primer, 200 µM each dNTP, 2 mM $MgCl_2$ and 1 unit Taq enzyme. The SSR fragments were amplified according to Temnykh et al. (2000) and separated in 8% polyacrylamide electrophoresis gels, and silver-stained as described (Panaud et al. 1996) with slight modification. The bands were documented using the VersaDoc Imaging System Model 3000 (Bio-Rad Laboratories, Inc., USA).

A total of 348 SSR loci, which cover all 12 rice chromosomes, were used for screening polymorphic markers between *Os-lpa*-XQZ-1 and XQZ and between *Os-lpa*-XS110-1 and XS110, and the polymorphic SSR markers were later used for analysis of individual F_2 plants of the two crosses. The same set of SSR markers was used for identifying potentially linked markers in the F_2 population of G0133/*Os-lpa*-XS110-1, first by bulk analysis of 60 F_2 plants homozygous for the *lpa* mutation, which was pre-determined by seed Pi level. The SSR markers that appeared potentially linked to the *lpa* character were further analyzed for more individual F_2 plants. All SSR analysis was done in duplicate to minimize experimental error.

For obtaining SSR markers linked as closely to the target locus as possible, new SSR markers were identified according to the rice genomic maps publicly

available on website http://www.gramene.org and were tested for polymorphism between the parent lines. New polymorphic markers identified were further analyzed for individual plants of the populations.

The linkage and genetic distance analysis of *lpa* mutations with SSR markers was performed using the JoinMap3.0 program (van Oijen and Voorrips, 2001) (http://www.kyazma.nl/index.php/mc.JoinMap) and genetic map was drawn using MapDraw V2.1 (Liu and Meng 2003).

Fig. 2 Seedlings of *lpa* mutant line *Os-lpa*-XS110-3 and its wild type parent Xiushui 110 from mature embryos after a 25-day in vitro culture on regular MS medium

Results

Isolation of mutants

We isolated several bred-true *lpa* mutant lines from five of the six varieties after mutagenic treatments (Table 1). These mutants had substantially different staining in colorimetric assay from their wild type parents; different mutants, despite all staining blue as compared to their parents being colorless, were also different in color intensity, indicating that they had different Pi contents (Fig. 1). All the mutant lines in Table 1 were derived from different individual M_2 plants of various parent varieties, although the possibility could not be excluded that mutants might originate from the same M_1 plant due to bulk harvest of M_2 seeds.

Homozygous *lpa* plants were isolated as early as in the M_2 generation, since their $M_{2:3}$ seeds were of HIP type in the cases of *Os-lpa*-XS110-1, *Os-lpa*-Z9B-1, and *Os-lpa*-MH86-1∼1/∼4. While some other lines, such as, *Os-lpa*-XS110-2 and *Os-lpa*-MH86-1∼5/∼7, were identified one generation later in M_3 (Table 1). Besides, some *lpa* lines failed to appear homozygous through self-pollination, such as *Os-lpa*-XQZ-3, *Os-lpa*-XS110-3, and *Os-lpa*-R6547-3. The *Os-lpa*-XQZ-3 line has now been in its M_{10} generation but it still remains segregating for the *lpa* characteristic. Therefore, the mutations in these lines are probably causing homozygous lethality (Table 1).

For these lethal lines, some plants were rescued through in vitro culture of embryos, of which their endosperm appeared to be of the HIP phenotype. Even during in vitro culture, the performance of mutant lines was significantly inferior to their wild type parents, being lower in germination rate, more susceptible to contamination, and slower in growth than their sibling seeds of normal PA content (Fig. 2). Most of the recovered mutant seedlings did not survive in fields while most of the wild type seedlings grew well to maturity. Finally, we obtained two, ten and three plants for *Os-lpa*-XQZ-3, *Os-lpa*-XS110-3 and *Os-lpa*-R6547-3, respectively. Surprisingly, not all of the recovered plants produced HIP seeds as theoretically expected. One of the two rescued *Os-lpa*-XQZ-3 plants yielded only wild type seeds, the other produced seeds of both HIP and wild type. Among the ten rescued plants of *Os-lpa*-XS110-3, only three plants produced seeds all of HIP type, six plants produced seeds of both HIP and wild type, with HIP seeds being the majority; and the remainder only yielded wild type seeds. As for all three rescued *Os-lpa*-R6547-3 plants, they produced HIP seeds.

Change of Pi and PA–P level

All *lpa* lines had been grown at least for three locations/crop seasons, and their seeds consistently appeared to be significantly higher in Pi level than their counterpart wild type parent in colorimetric assays (Fig. 1 and data not shown). Quantitative analysis showed some variations in total P in *lpa* mutants as compared to their corresponding wild type parents, but not at a significant level except between Z9B and its mutant (Table 2). The Pi contents in all *lpa* mutants were significantly increased as compared to their wild type counterparts (Table 2), and the ratio of Pi/total P in *lpa* mutants increased from the least 15.5% (*Os-lpa*-Z9B-1) to as high as 46.8% (*Os-lpa*-XS110-1) while the maximum in wild type (Z9B) was 10.1% (Table 2). The PA–P content, on the other hand, was significantly reduced in all *lpa* mutant lines by 38.9, 63.6, 33.8, 44.0 and 44.5% in *Os-lpa*-XQZ-1 (seeds produced in 2004 in Hainan), *Os-lpa*-XS110-1, *Os-lpa*-XS110-2, *Os-lpa*-MH86-1 and *Os-lpa*-Z9B-1, respectively (Table 2). An environmental influence was observed on the content of all types of P in seeds of *Os-lpa*-XQZ-1 produced in different locations, however, the effect of the *lpa* mutation surpassed that of environment factor on Pi and PA–P level (Table 2).

Table 2 The concentrations of various forms of P in brown rice grains of *lpa* mutants and their parents

Material	Production [a]	Total P (TP)[b] mg g^{-1}	Inorganic P[b]		Phytic acid P[b]	
			mg g^{-1}	TP (%)	mg g^{-1}	TP (%)
Os-lpa-XQZ−1	JD	3.46 ± 0.24	1.55 ± 0.20**	44.8	1.22 ± 0.09*	35.3
XQZ	JD	3.31 ± 0.22	0.21 ± 0.03	6.3	2.28 ± 0.19	68.9
Os-lpa-XQZ-1	JX	3.68 ± 0.26	1.50 ± 0.16**	40.8	1.34 ± 0.09*	36.4
XQZ	JX	3.30 ± 0.28	0.25 ± 0.03	7.6	2.17 ± 0.14	65.8
Os-lpa-XQZ-1	HN #1	3.32 ± 0.29	1.24 ± 0.11**	37.3	1.29 ± 0.07*	38.9
XQZ	HN #1	3.33 ± 0.24	0.25 ± 0.03	7.5	2.11 ± 0.17	63.4
Os-lpa-XS110-1	HN #1	2.82 ± 0.21	1.32 ± 0.15A	46.8	0.71 ± 0.05C	25.2
Os-lpa-XS110-2	HN #1	2.99 ± 0.24	0.75 ± 0.09B	25.1	1.29 ± 0.10B	43.1
XS110	HN #1	3.07 ± 0.27	0.20 ± 0.03C	6.5	1.95 ± 0.13A	63.5
Os-lpa-MH86-1	HZ	N/T	1.15 ± 0.06**	N/A	1.44 ± 0.02*	N/A
MH86	HZ	N/T	0.26 ± 0.01	N/A	2.57 ± 0.04	N/A
Os-lpa-Z9B-1	HN #2	2.78 ± 0.05*	0.43 ± 0.04*	15.5	1.33 ± 0.08*	47.8
Z9B	HN #2	3.06 ± 0.06	0.31 ± 0.00	10.1	2.39 ± 0.04	78.1

N/*T* not tested, *N*/*A* not applicable

[a] HN #1 and #2 stand for seeds produced in Lingshui, Hainan in April 2003 and 2005, respectively; JD and JX for seeds produced in Jiande and Jiaxing, Zhejiang in September 2003, HZ for Hangzhou, Zhejiang in September 2004, respectively

[b] The value of a mutant with asterisk ** or * indicates significant difference from that of its parent at $P = 0.01$ or 0.05 level, respectively, for the samples produced at the same location; for the values of XS110 and its two mutants, multiple comparisons were undertaken and the marks A, B, C indicate significant differences at $P \leq 0.05$

The *lpa* mutations are recessive

All F_1 seeds from crosses between mutants and wild type varieties showed normal Pi level in colorimetric assays, which implied the recessive nature of the *lpa* mutations (photos not shown). No significant differences in Pi level between wild type parents and F_1 seeds in colorimetric assay were observed, indicating that the *lpa* mutations are completely recessive to wild type alleles.

The *lpa* mutations are inherited as a single locus

The segregation of *lpa* and wild type plants in advanced generations of the mutants and in the progenies of the crosses further showed that all the mutations so far investigated were inherited in a single recessive gene mode (Table 3). The exception was that less homozygous HIP F_2 plants were observed as expected in a single gene mode, which might result from the inferior growth of homozygous

Table 3 Segregation of rice seeds/plants for the *lpa* (LP) and wild-type (WT) phenotypes in various segregating populations

Population	No. of seeds				Number of plants with seeds				
	Generation[a]	LP	WT	$\chi^2_{(1:3)}$[b]	Generation[a]	LP	LP/WT	WT	$\chi^2_{(1:2:1)}$or $\chi_{(2:1)}$[b]
Os-lpa-XQZ-1	M5 (s)	46	146	0.06	–	–	–	–	–
Os-lpa-XQZ-1/XQZ	F_2	43	149	0.36	F_2	49	117	50	1.51
Os-lpa-XQZ-1/XQZ	F_3	514	1358	3.48	–	–	–	–	–
Os-lpa-XQZ-3	M_5 (s)	120	392	0.59	M_3 (s)	0	32	15	0.00
Os-lpa-MH86-1	M_5 (s)	54	138	0.77	–	–	–	–	–
Os-lpa-R6547-3	M_4 (s)	42	174	3.27	$M_{4:5}$ (s)	0	9	3	0.09
XS110/ *Os-lpa*-XS110-1	F_2	74	246	0.50	F_2	92	178	128	10.94*
G0133/Os-lpa-XS110-1	F_2	265	887	2.34	F_2	5	61	70	63.57*
Os-lpa-XS110-2/XS110	F_2	159	513	0.57	–	–	–	–	–
Os-lpa-XS110-3	M_3(s)	193	623	0.72	$M_{3:4}$ (s)	0	51	23	0.08

Values with an asterisk are statistically significant at 0.05 level in χ^2 test

[a] The letter "s" in parenthesis indicates that the materials investigated were either heterozygous sib-lines of the five non-lethal mutants or segregating lines of the three lethal lines; seeds from the single plant of earlier generations were used for plant analysis, i.e. F_2 plants were derived from the seeds harvested from a single F_1 individuals, while M_3 plants were from seeds of a single heterozygous M_2 plant

[b] The significant values for theoretical segregation ratio 1:3 in seed analysis and 1:2 in plant analysis of lethal lines is $\chi^2_{c\ 0.05,1} = 3.84$; for ratio 1:2:1 in plant analysis is $\chi^2_{\ 0.05.2} = 5.99$

lpa seeds to normal seeds in plant establishment (Table 3).

The allelism of *lpa* mutations

The allelism of *lpa* mutations was first analyzed through functional complementation using F_1 seeds; the results were summarized in Table 4. A wild type Pi level of F_1 seeds suggests the mutations complement one another, and hence the *lpa* mutations are non-allelic. In contrast, a mutant Pi level of hybrid seeds indicates the two mutations occur in the same locus and hence are allelic. Since no homozygous lines of *Os-lpa*-XQZ-3 and *Os-lpa*-XS110-3 were available for allelism tests, several plants of a segregating line were selected, marked and crossed with other *lpa* lines; the genotype of plants used for crossing was determined by Pi test of their self-pollinated seeds. F_1 seeds from crossing with wild type plants were excluded from further analysis; only the F_1 seeds from crosses with plants heterozygous or homozygous (from embryo rescue) at the mutation locus were used for *lpa* allelism tests, and in the heterozygous case, half of the F_1 seeds should appear mutant type if two mutations are allelic while all seeds are of wild type if they are non-allelic.

Based on the above presumption, the *lpa* mutations of all seven mutant lines (*Os-lpa*-MH86-1∼1/∼7) from MH86 proved to be mutually allelic (data not shown), and these seven mutants were treated as a single homozygous *lpa* line in subsequent allelism tests with other mutations. The KBNT *lpa1*-1 line (Rutger et al. 2004) appeared to have similar Pi content as *Os-lpa*-XQZ-1 (Fig. 1). The F_1 seeds of these two *lpa* lines had also similar Pi levels as KBNT *lpa1*-1, which indicated that the mutations of these two lines are allelic. The F_1 seeds from crosses between KBNT *lpa1*-1 and *Os-lpa*-XQZ-1 with other mutant lines all showed wild type Pi level (Table 4), which also supported their allelic relationship. Since the seeds from crosses among *Os-lpa*-XQZ-1, *Os-lpa*-XS110-1, *Os-lpa*-XS110-3 and *Os-lpa*-MH86-1 all had low Pi levels similar to wild type materials, therefore, the mutations involved in these lines should be mutually non-allelic (Table 4). The F_1 seeds of crosses *Os-lpa*-XS110-2/*Os-lpa*-XS110-3 had the same Pi level as *Os-lpa*-XS110-3 (Table 4), indicating the two mutations occurred in a same locus, despite their significant differences in Pi level. The phenotype of F_1 hybrids of *Os-lpa-XS110-2*/*Os-lpa*-XS110-1 was quite ambiguous because they were stained in blue, but lighter than *Os-lpa-XS110-2* in colorimetric assay (Table 4).

If the mutations are non-allelic and not closely linked, wild type seeds should appear in the F_2 population derived from crosses between two *lpa* mutant lines. Otherwise, all F_2 seeds should have a mutant type of Pi level. This was confirmed by the results of Pi tests, where all wild type seeds were detected from crosses between non-allelic lines (data not shown). For the cross of *Os-lpa-XS110-2*/*Os-lpa*-XS110-1, seeds of 35 DH lines were additionally tested to avoid the difficulty in clearly phenotyping individual F_2 seeds. Three types of lines, i.e. wild type, *Os-lpa*-XS110-1 and *Os-lpa*-XS110-2 type, were observed in these DH lines, which demonstrated that the *lpa* mutations in these two lines are non-allelic.

Although the mutation in *Os-lpa*-XQZ-1 and *Os-lpa*-XQZ-3 must be non-allelic since all their 69 F_1 seeds appeared in wild type, the relationship of the *lpa* mutations in *Os-lpa*-XQZ-3 and other mutants could not be concluded because the F_1 seeds were limited (Table 4). The allelic relationship between *Os-lpa*-MH86-1 and *Os-lpa*-Z9B-1 is yet not clear because no crosses were made due to their differences in maturity.

Table 4 Allelism test of *lpa* mutations in rice

Mutant lines	*Os-lpa*-XQZ-1	*Os-lpa*-XQZ-3 (H)	*Os-lpa*-XS110-1	*Os-lpa*-XS110-2	*Os-lpa*-XS110-3	*Os-lpa*-MH86-1	*Os-lpa*-Z9B-1
KBNT *lpa1*-1	15 (HIP)	NT	16 (WT)	16 (WT)	NT	NT	8 (WT)
Os-lpa-XQZ-1	NT	69 (WT)	53 (WT)	56 (WT)	NT	110 (WT)	8 (WT)
Os-lpa-XQZ-3 (H)	NT	NT	4 (WT)	8 (WT)	NT	NT	3 (WT)
Os-lpa-XS110-1	NT	NT	NT	24 (WT/HIP)	NT	48 (WT)	13 (WT)
Os-lpa-XS110-2	NT	NT	NT	NT	3 (HIP_3)	16 (WT)	8 (WT)
Os-lpa-XS110-3 (H)	64 (WT)	8(WT)	12(WT)	3(WT); 5(HIP_3)	NT	NT	NT
Os-lpa-XS110-3	NT	NT	NT	NT	NT	9 (WT)	6 (WT)

NT not tested, *HIP* or *WT* in parenthesis indicates that the seeds appeared in a mutant (high Pi, blue in color) or a wild type (low Pi level, without color), *HIP_3* indicates the Pi level similar to *Os-lpa*-XS110-3, *WT/HIP* showed an ambiguous level between those of WT and HIP type material *Os-lpa*-XZQ-3 (H) and *Os-lpa*-XS110 (H) were heterozygous plants used in crossing

The figures in the table represent the number of hybrid seeds tested

Molecular mapping of *lpa* mutations

Initial tests of the SSR markers, reportedly linked to the *lpa1*-1 mutation (Larson et al. 2000), showed that *Os-lpa*-XQZ-1 and XQZ are polymorphic at the RM48 locus. Analysis of 40 homozygous mutant type F_2 plants from the cross of *Os-lpa*-XQZ-1/XQZ showed that the RM48 is closely linked with the *lpa* mutation in *Os-lpa*-XQZ-1. Similar analysis was carried out for the F_2 population of *Os-lpa*-XQZ-1/Huahui No.7, using the four mutually linked SSR markers RM208, RM48, RM207, and RM482. Only RM208 appeared to be polymorphic between the two parents and to be closely linked with the mutation. Since the *lpa* mutation in *Os-lpa*-XQZ-1 was later found to be allelic to the *lpa1* locus in KBNT *lpa1*-1, which was recently fine mapped to a region of 47 kb on chromosome 2 (Andaya and Tai 2005), no fine mapping was further carried out in the present study.

Although eight of the 348 SSR markers (RM3, -4, -232, -253, -270, -492, -498, -583) were found to be polymorphic between *Os-lpa*-XS110-1 and XS110, none of them was linked with the *lpa* mutation in their F_2 population. Therefore, another F_2 population was developed through crossing *Os-lpa*-XS110-1 with an *indica* rice variety G0133. Since G0133 is a wide-compatible line, so their F_1 plants had normal seed setting and grain-filling. Through bulk DNA analysis of homozygous mutant and homozygous wild type F_2 plants of G0133/*Os-lpa*-XS110-1, the *lpa* mutation was found correlated with SSR marker RM143 on chromosome 3. New SSR markers RM468, -514, -565, -570, and -571, all neighboring the RM143 locus, were further analyzed; only RM468 and RM571 appeared polymorphic between *Os-lpa*-XS110-1 and G0133. One hundred and nine individual F_2 plants, of which 61 were homozygous for the mutant type and 48 homozygous for the wild type, were analyzed for these three polymorphic SSR loci. The *lpa* mutation was above RM468, far from RM571 and RM143 (Fig. 3).

At the time when the above results were obtained, Shi et al. (2005) reported that a new gene, *lpa3*, was found to cause a new type of *lpa* mutation in maize. Through Blast and search of Genbank, we also found a homolog to the *lpa 3* gene in the rice genome at the locus LOC-Os03g52760. This putative gene is at the genome position between 30194828-30197720 bp of chromosome 3, which is only about two million nucleotides away from RM468. Subsequently, four new SRR markers, RM2593, RM3199, RM3525 and RM5172, with genome position of 30327757-30327957, 30372573-30372764, 30344279-30344457, and 30379401-30379698, respectively, were synthesized and analyzed for *Os-lpa*-XS110-1 and G0133. However, only RM3199 was successfully amplified from both *Os-lpa*-XS110-1 and G0133 and showed polymorphism between them. The above 109 plants plus another 166 individual plants were analyzed for this marker locus. The results showed that RM3199 is more tightly linked to the *lpa* mutation than RM468, with a genetic distance of 1.198 cM (Fig. 3).

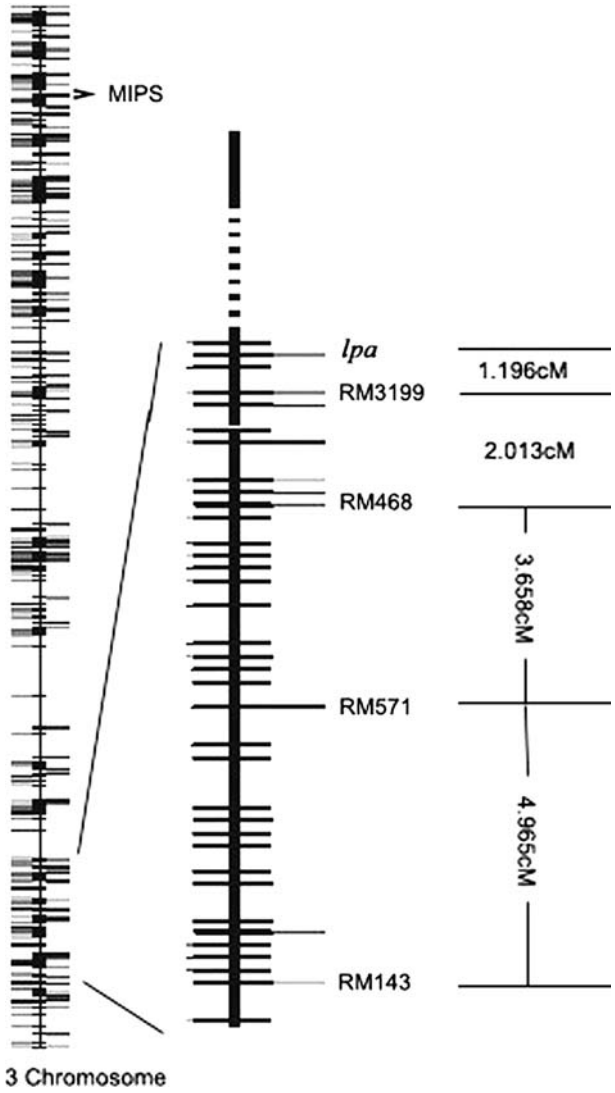

Fig. 3 Mapping of the *lpa* gene on chromosome 3 using microsatellite markers (SSR). The F_2 population of GO133/*Os-lpa*-XS110-1 was used and the genetic distances between the *lpa* gene and SSR markers were expressed in cM. MIPS, the *myo*-inositol 3-phosphate synthase gene

Discussion

In this study, we generated a total of eight independent *lpa* mutant lines from five rice varieties through artificial

mutagenesis and at least four non-allelic loci were identified, of which three are reported for the first time, responsible for phytic acid synthesis. We further mapped two mutations on chromosome 2 and 3, one in the same region as the *lpa1*-1 gene and the other around the locus homologous to the *lpa 3* gene in maize. These genetic resources, together with the relevant chemical and genetic findings, will be tremendously useful and of some significance not only for breeding *lpa* rice, but also for identifying various genes and their roles in PA synthesis.

Induced mutation has become an important source of genetic variation in plant breeding programs. According to the FAO/IAEA Mutant Variety Database (http://www.-mvd.iaea.org/MVD/default.htm), more than 2,500 mutant varieties including about 500 rice varieties were developed worldwide during the past 40 years. As part of our regular work on rice genetic improvement through mutation induction, we initiated *lpa* mutant screening using the two already available mutant populations in stock, that is, XQZ and ZS97. Two mutant lines were identified from the former one but none from the latter, because the population size of the latter was rather small (Table 1). We then developed and screened four populations for this study, and succeeded in isolating *lpa* mutant lines, though the mutation frequency varied from variety to variety (Table 1). The variety-dependency of mutation frequency has been known as a common feature in mutation induction for a long time (van Harten 1998). Our study also proved that isolation of novel mutations using conventional mutagenic treatments could be achieved in a relatively short period and in a cost-effective way.

The only rice *lpa* mutant line previously reported, KBNT *lpa1*-1, was also developed through gamma irradiation (Larson et al. 2000). The PA portion of seed P in KBNT *lpa1*-1 is reduced from 71 to 39% and the Pi portion is increased from 5 to 32%, with little effect on total seed P (Rutger et al. 2004). Our results were consistent with those previous observations in that there were no significant differences of total P level between mutants and their parents, except for a significant reduction in *Os-lpa*-Z9B-1 (Table 2). The significant increase of Pi level in all mutants is a logical result because the selection was based on the color of Pi staining. Variation of Pi increase in seeds produced at different locations was observed in some mutant lines, like *Os-lpa*-MH86-1, which changes the blue color intensity in colorimetric assays as was the case for Pi level in KBNT *lpa1*-1 (Rutger et al. 2004). However, the change of PA–P and Pi level was insignificant in seeds of *Os-lpa*-XQZ-1 produced in different locations (Table 2), therefore, the environment effect was rather line-dependent. The degree of PA content reduction was quite different among the mutants generated in the present study, and further study is needed to investigate the changes of various forms of P in the rice mutants. It will not only help us with the correct classification of the *lpa* mutations involved, but also with possible identification of new *lpa* types, since we have indeed generated more than three non-allelic mutations and thus it is reasonable to postulate that there might be additional P profiles.

Three types of low phytic acid mutation have already been identified and characterized, *lpa 1* and *lpa 2* (Raboy 2001; Shi et al. 2003) and *lpa 3* (Shi et al. 2005) in maize. The *lpa 1* type mutants usually have a decreased PA–P content, accompanied by mole equivalent increase of Pi; *lpa 1* was postulated to be a mutation related to the *myo*-inositol phosphate synthase (MIPS) gene (Shukla et al. 2004; Pilu et al. 2005), but it is now known to be a mutation in one of the multidrug resistance protein (MRP) genes, ZmMRP3, a gene encoding an ABC transporter (Shi et al. 2006); *lpa 2* type of mutation also results in the reduction of PA–P content, but is complemented by an increase of both Pi and lower inositol phosphates (LIns–P), and may involve an inositol phosphate kinase gene and therefore the Pi level in *lpa1* mutants is usually higher than in *lpa2* mutants (Raboy et al. 2000); and *lpa 3* involving the *myo*-inositol kinase (MIK) gene, is characterized by increased *myo*-inositol levels and a lack of significant amounts of *myo*-inositol phosphate intermediates in seeds (Shi et al. 2005). However, the three types of *lpa* mutations were only named according to the order in which they were studied and isolated. Therefore, the *lpa 1* mutation in maize might differ in genetic basis from the *lpa 1* mutations in other crops. For example, the *lpa 1*-1 mutant in maize (Raboy et al. 2000) and the KBNT *lpa 1*-1 (Larson et al. 2000) and *Os-lpa*-XQZ-1 in rice have total P levels similar to those in their corresponding wild type parents; however, the *lpa 1*-1 mutant in barley has a lower total P level than its parent (Dorsch et al. 2003). The different effect of *lpa* mutation on P content and profile might also provide some clues of their genetic basis. For example, the gene mutated in *Os-lpa*-Z9B-1 may function similarly to the one in *lpa 1*-1 in barley, because *Os-lpa*-Z9B-1 also had a lower total P level than its wild type parent (Table 3). This is at least not contradicted with the finding that the *lpa* mutation in *Os-lpa*-Z9B-1 is non-allelic to the *lpa 1* mutation in KBNT *lpa 1*-1 and *Os-lpa*-XQZ-1 (Table 4).

An homozygous lethal *lpa* mutation was also reported previously in rice where seeds homozygous at the *lpa* locus failed to germinate and grow into plants

(Larson et al. 2000). Reduction of seed viability was also reported in maize (Raboy et al. 2000; Pilu et al. 2005) and soybean (Meis et al. 2003). Although no homozygous lethality was observed in other *lpa* mutations, such as *Os-lpa*-XS110-1, the less-than-expected number of homozygous *lpa* F_2 plants in segregating populations also indicated that homozygous *lpa* seeds are inferior to heterozygous or wild type seeds in competition (Table 3). We isolated three mutants of lethal type from three different varieties, *Os-lpa*-XQZ-3, *Os-lpa*-XS110-3, and *Os-lpa*-R6547-3. The success of in vitro rescue through tissue culture in our study confirmed the assumption that homozygous lethality is caused by the loss of seed viability. The seeds of these lines demonstrated the deepest blue in colorimetric assay, indicating the greatest reduction of PA–P than in other mutant lines (Fig. 1). It is therefore possible that a minimum PA amount is needed for seed germination, but not for seed development as observed in maize (Raboy et al. 2000). The success of in vitro rescue provided a possibility for using these mutants for thorough analysis of all genes that are involved in genetic control of PA synthesis in rice. Also needing clarification is why heterozygous plants, even wild type plants, were produced through in vitro culture of homozygous *lpa* seeds. Although genetic (out-crossing) or mechanical (mixture) contamination could not be excluded, it is highly unlikely that most of the "unexpected plants" resulted from such contaminations, since these rescued plants were under strict monitoring and all labeled and harvested individually. One possible explanation is that the *lpa* mutations constitute a "genetic stress" to the rice genome and caused RNA-mediated reversion to normal type, a phenomenon recently found in plants (Lolle et al. 2005) and animals (Rassoulzadegan et al. 2006), known as RNA-mediated non-mendelian inheritance. Detailed studies are being undertaken to investigate all these possibilities.

F_1 materials are widely used in allelism tests of various mutations involved in the same biological process, e.g. the biosynthesis of PA in the present study. Clear-cut conclusions could be made based on experimental results in most circumstances, for example, wild type F_1 seeds (colorless in Pi assay) of two high Pi *lpa* mutants (dark blue in Pi assay) undoubtedly indicates non-allelic mutations. However, ambiguity could also arise when the mutant had a relatively low level of Pi and hence was not substantially different from wild type on an individual seed basis. More problematic were artificially produced F_1 seeds, because the glumes are usually half-cut during emasculation and the Pi levels of half-naked kernels are more sensitive than intact rice seeds to environmental factors during grain-filling and post-harvest storage. In such circumstances, analysis of F_2 seeds and/or seeds of individual lines are helpful. In the present study, a double haploid population derived from *Os-lpa*-XS110-2/*Os-lpa*-XS110-1 was successfully used for the analysis of the two mutations.

The result of allelism test between *Os-lpa*-XQZ-1 and KBNT *lpa-1*-1 matched the mapping finding which indicated the *lpa* mutation has a similar location in both. Although more studies are needed to identify the nature of the mutation in these two lines, it is very possible that the mutations might be the result of different changes in DNA in the same gene. The result that *lpa* mutations in *Os-lpa*-XS110-2 and *Os-lpa*-XS110-3 are allelic was rather unexpected, because they differ significantly in both Pi level and seed viability. However, it was also reported in maize that the *lpa* mutations, *lpa 1-1* and *lpa 241*, which were independently generated by Raboy et al. (2000) and Pilu et al. (2003) and had different PA reductions and phenotypic effects, were later shown to be allelic (Pilu et al. 2005). The discovery of such allelic *lpa* mutations, with significant different phenotypic effects in some cases, indicates that multiple alleles of a given gene could be generated through experimental mutagenesis.

An allelic mutation to *lpa 1*-1 was identified in our mutant line *Os-lpa*-XQZ-1; this mutation could be therefore named as *lpa 1-2*. Larson et al. (2000) and Andaya and Tai (2005) discussed in detail the possible biological and genetic basis of the *lpa 1* mutation in rice, but no conclusion could be made yet. One possibility is that the *lpa 1* mutation affects trans-acting regulatory factors that controls expression or activity of the MIPS gene in rice. In maize, the *lpa 1* mutation is closely linked with the 1S-MIPS gene, and the expression of 1S-MIPS was reduced in mutants, the sequence of all exons and introns is identical between mutants and their corresponding wild type lines (Shukla et al. 2004; Pilu et al. 2005). Although the *lpa 1* mutation in maize is now known to be a mutation in ZmMRP3 encoding an ABC transporter, the relationship between mutations in this transporter and either inositol synthesis or phytic acid synthesis is not clear yet (Shi et al. 2006). Although the rice homolog of ZmMRP13, OsMRP13, is putatively encoded by the locus on chromosome 3 (LOC_Os03g04920) (Shi et al. 2006), it is not located near the rice *lpa 1* locus that is located on chromosome 2. Therefore, more studies are needed to uncover the molecular genetic basis and molecular biological mechanisms leading to the *lpa* phenotype in all these crops, and rice should become a useful model crop in such endeavors.

The *lpa* mutation in *Os-lpa*-XS110-1 was mapped on chromosome 3 in this study. This site is very close to

the locus LOC_Os-03g52760, which encodes the protein OSJNBb0016H12.26, the putative MIK gene in rice, therefore we postulate that *Os-lpa*-XS110-1 is an *lpa 3* mutant, though more genetic and biochemical studies are needed. Interestingly, the MIPS gene (LOC_Os03g09250, genome location 4804749-4808864) is on the short arm of rice chromosome 3, while the putative MIK gene is located on the long arm (genome location 301994828—30197720) of the same chromosome. Although the maize MIPS and MIK genes are both mapped on the short arm of maize chromosome 1, the relative position of MIPS and MIK on rice and maize chromosomes is similar (Raboy et al. 2000; Shi et al. 2005).

The pathway and the genes related to PA synthesis have not been well studied in plants, particularly in rice. The materials developed in this study are probably the most extensive collection of *lpa* mutant lines developed to date in any plant species, in that they carried at least four non-allelic mutations and multiple alleles of a few loci. Therefore, this collection will provide a unique means for investigating genes and their functions in PA synthesis and inositol phosphate metabolism. In addition, the intermediate metabolites of PA, inositol 1,4,5-trisphosphate (IP_3) and 1,4,5,6-tetra*kis*phosphate (IP_4), are crucial second messengers which regulate calcium homeostasis, and if the mutation of some of the mutant lines are the results of the disruption of genes controlling IP_3 and IP_4 production, these types of mutants will also be useful for investigating the functions of these chemicals (see review, Xia and Yang 2005).

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