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ORIGINAL PAPER

Generation and characterization of two novel low phytate mutations in soybean (*Glycine max* L. Merr.)

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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 soybean meal. Organic phosphorus (P) in PA is indigestible in humans and non-ruminant animals, which affects nutrition and causes P pollution of ground water from animal wastes. Two novel soybean [(*Glycine max* L. (Merr.)] *low phytic acid* (*lpa*) mutations were isolated and characterized. *Gm-lpa*-TW-1 had a phytic acid P (PA-P) reduction of 66.6% and a sixfold increase in inorganic P (Pi), and *Gm-lpa*-ZC-2 had a PA-P reduction of 46.3% and a 1.4-fold increase in Pi, compared with their respective non-mutant progenitor lines. The reduction of PA-P and increase of Pi in *Gm-lpa*-TW-1 were molar equivalent; the decrease of PA-P in *Gm-lpa*-ZC-2, however, was accompanied by the increase of both Pi and lower inositol phosphates. In both mutant lines, the total P content remained similar to their wild type parents. The two *lpa* mutations were both inherited in a single recessive gene model but were non-allelic. Sequence data and progeny analysis indicate that *Gm-lpa*-TW-1 *lpa* mutation resulted from a 2 bp deletion in the soybean D-*myo*-inositol 3-phosphate synthase (MIPS1 EC 5.5.1.4) gene 1 (*MIPS1*). The *lpa* mutation in *Gm-lpa*-ZC-2 was mapped on LG B2, closely linked with microsatellite loci Satt416 and Satt168, at genetic distances of ~4.63 and ~9.25 cM, respectively. Thus this mutation probably represents a novel soybean *lpa* locus. The seed emergence rate of *Gm-lpa*-ZC-2 was similar to its progenitor line and was not affected by seed source and its *lpa* mutation. However, *Gm-lpa*-TW-1 had a significantly reduced field emergence when seeds were produced in a subtropic environment. Field tests of the mutants and their progenies further demonstrated that the *lpa* mutation in *Gm-lpa*-ZC-2 does not negatively affect plant yield traits. These results will advance understanding of the genetic, biochemical and molecular control of PA synthesis in soybean. The novel *lpa* mutation in *Gm-lpa*-ZC-2, together with linked simple sequence repeat (SSR) markers, will be of value for breeding productive *lpa* soybeans, with meal high in digestible Pi eventually to improve animal nutrition and lessen environmental pollution.

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Introduction

Soybean meal is a very important protein source for humans and is commonly used in animal feed world wide. Like other agriculturally important crops, 60–80% of total seed P is in the form of phytic acid (*myo*-inositol hexa*kis*phosphate, PA or IP6) (Raboy et al. 1984). On average, soybean seed contains about 4.3 g kg^{-1} phytic acid P (PA-P) and 0.6 g kg^{-1} inorganic P (Pi) (Wilcox et al. 2000). PA can chelate with important mineral micronutrients, e.g., Zn, Fe, Ca rendering them virtually indigestible by humans and non-ruminant livestock. It is commonly

regarded as a major anti-nutrient in legumes (Erdman et al. 1979). Widespread use of soybean meal results in PA-P being excreted in livestock waste, which is a threat of pollution to stream and groundwater. The addition of Pi or microbial phytase (E.C.3.1.3.8) to feed is commonly practiced to increase available P to animals. It would be desirable to reduce the level of PA-P and simultaneously increase the available Pi in soybean seed, via genetic means. These available P for use in humans and livestock would improve P availability without the need for supplemental P for diets (for review, Raboy 2002).

Several genetic methods have been exploited to solve the phytate issue in food and feed. In soybean, transgenic plants expressing a recombinant fungal phytase (Denbow et al. 1998) or over-expressing the soybean phytase gene (*GmPhy*) (Chiera et al. 2005) were produced; reduction of PA-P in soybean seeds was recently achieved through (partially) silencing the *myo*-inositol 1-phosphate synthase (MIPS) gene using RNAi technology (Nunes et al. 2006). Soybean with reduced PA has also been produced through chemical mutagenesis (Wilcox et al. 2000; Hitz et al. 2002). However, breeding of high yielding, *low phytic acid* (*lpa*) soybean varieties has been hindered by the inherent defects of the *lpa* mutations. For example, the LR33 mutant, with a 50% reduction in phytate has an increase of seed sucrose levels and a decrease in seed raffinosaccharides, both highly desirable traits in soybeans (Hitz et al. 2002; Chappell et al. 2006). However, its seeds have a very low field emergence rate, particularly when seeds were produced in tropical and subtropical environments, making it of little commercial value in breeding (Meis et al. 2003). Mutant line, M153, shows a reduction in seed phytate $\geq$75% (Wilcox et al. 2000) and has low seedling emergence (Oltmans et al. 2005). Also undesirable saturates, palmitate, and stearate, in seed oil are greater than wild type (WT) soybeans (Hulke et al. 2004).

There are at least four non-allelic mutations for *lpa* seeds in barley (Bregitzer and Raboy 2006) and rice (Liu et al. 2007). In maize, three types of *lpa* mutation have been characterized at the gene level (Raboy et al. 2000; Shi et al. 2003, 2005, 2006). In barley, it has been shown that the *lpa 1* mutation has no negative effects on agronomic performance. Therefore, generating more *lpa* genetic resources in soybean would be useful for developing acceptable *lpa* varieties. Soybean mutants with altered PA content are also needed for investigations on PA biosynthesis and metabolism. Knowledge of biosynthetic pathway(s) and genes responsible for each step was limited in soybean, where only the MIPS genes, have been characterized (Hegeman et al. 2001; Hitz et al. 2002; Chappell et al. 2006).

In this study, we report two new soybean *lpa* mutants, the genetics of these mutations and their effects on seeding emergence to determine their use in breeding and studies on PA syntheses.

Materials and methods

Mutant population development

About 2,000 dried seeds, each of the two commercial cultivars, Taiwan 75 and Zhechun no. 3, were treated with 150 Gy gamma rays at the Irradiation Center of Zhejiang University. The M_1 seeds were directly sown in fields. M_2 seeds were harvested from about 1,000 surviving M_1 plants. About 25,000 of Zhechun no. 3 and 8,000 of Taiwan 75 M_2 plants were grown in rows spaced 40 cm apart by row and 20 cm by plant. All seeds of M_2 plants ($M_{2:3}$ seeds) were harvested and bulked on an individual M_2 plant basis. A subsample of each plant was used for screening of putative *lpa* mutants, via screening seeds that are high inorganic P (HIP) (see below).

Spring and autumn crops were conducted on the Experimental Farm of Zhejiang Academy of Agricultural Sciences in Hangzhou, Zhejiang Province, while those of winter-spring crops were performed in the Winter Breeding Station of Zhejiang University in Lingshui, Hainan Province. All M_1 populations were grown as a spring crop in 2002, and M_2 populations as an autumn crop in the same year.

Detection of seed of high inorganic P

Six $M_{2:3}$ seeds from each M_2 plants were individually analyzed for HIP phenotype associated with homozygosity for an *lpa* mutation. In total, about 25,000 and 8,000 $M_{2:3}$ seed samples of Zhechun no. 3 and Taiwan 75, respectively, were screened according to the protocol of Wilcox et al. (2000) with slight modifications. Namely, each seed was cross-cut into halves; the half without embryo was extracted overnight in a solution of 12.5%(v/w) TCA: 25 mM $MgCl_2$ at room temperature; an extract aliquot of 8 µl from each seed was used for assay of Pi level as described by Wilcox et al. (2000). WT soybean seeds typically contain Pi of $\leq$0.7 mg g^{-1}. Therefore, seeds with a Pi level >1.0 mg g^{-1} were recorded as HIP seeds. The $M_{2:3}$ seeds of M_2 plants, in which HIP seeds were identified, were grown into M_3 plants in 2002/3 winter/spring seasons in Hainan, and their $M_{3:4}$ seeds were again individually tested for the HIP mutant phenotype.

Analysis of seed phosphorus fractions

Mature seeds were dried for 72 h at 70°C, were ground into flour in a Cyclone Mill (UDY Corporation, Fort Collins, CO, USA), and passed through a 60-mesh screen. Flour samples were stored in desiccators for analysis of various P fractions. A colorimetric assay (Chen et al. 1956) was used for quantitative determination of total P, Pi and total inositol phosphates (Ins-P), using 1.000*g*, 0.400*g*, and 0.600*g*

samples, respectively. Pi was analyzed by extracting Pi by magnetically stirring the flour overnight in 20 ml of 12.5% (w/v) TCA: 25 mM $MgCL_2$.The supernatant was centrifuged (10,000*g*, 10 min) and used for the assay (Wilcox et al. 2000). Total P analysis on soybean flour was conducted according to Raboy et al. (1984) with slight modification. The ferric-precipitation method was used according to Raboy et al. (1984) for determination of total Ins-P. Six hundred micrograms of soybean flour was placed into a 50 ml centrifuge tube with 30 ml solution of 0.4 M HCL: 0.7 M Na_2SO_4, and the mixture was shaken for 18–24 h at 4°C. The tubes were centrifuged at 15,000 rpm at 4°C for 15 min and 10 ml of supernatant was removed to a second tube. Ten milliliters of ddH_2O and 5 ml mixture of 15 mM $FeCL_3$ + 0.2 M HCL + 5% Na_2SO_4 were added. Tubes were then heated for 30 min in a boiling water bath. After cooling on ice, the tubes were centrifuged at 15,000 rpm at 4°C for 15 min and the ferric precipitate was washed twice with 10 ml 0.2 M HCL. The ferric pellets were then wet-ashed according to Raboy et al. (1984) and used for assay of total Ins-P according to Chen et al. (1956).

Phytic acid P was determined using anion-exchange chromatography (Zhang and Zhu 2005). Briefly, 20 ml 0.4 M HCL was added into a 50 ml tube with 400 mg defatted soybean flour and mixed by shaking overnight. The tubes were centrifuged at 15,000 rpm at 4°C for 30 min and 10 ml supernatant was removed to a second tube. Ten milliliters of ddH_2O and 5 ml mixture of 0.2 M HCL: 15 mM $FeCL_3$ were added, and the tubes were heated for 30 min in a boiling water bath. The tubes were ice-cooled and centrifuged at 10,000 rpm at 4°C for 15 min. The ferric precipitates were washed twice in 0.2 M HCL. The pellets were converted to a soluble Na Ins phosphate solution and an $Fe(OH)_3$ precipitate by adding 1.0 ml 1.5 M NaOH. The tubes were centrifuged at 12,000 rpm for 10 min and the supernatants were transferred to 2 ml tubes. An aliquot of supernatant was diluted 5–6 times with ddH_2O, passing through a 0.2 μm filter. Aliquots were fractionated 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^{-1}. The effluent from the column was mixed with a colorimetric reagent [0.1% $Fe(NO)_3$ and 2% $HCLO_4$] at a flow rate of 1 ml min^{-1} 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 $InsP_6$ (P-3168, Sigma, St. Louis, MO, USA) was analyzed before and after every two samples.

Genetic analysis

The *lpa* mutants, *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2, were crossed with WT varieties Zhechun no. 3 and Wuxing no. 4, respectively. About 100–200 F_2 seeds were harvested from individual F_1 plants. For eliminating false F_1 plants from self-pollination, six F_2 seeds from each F_1 plant were tested and only the F_1 plants that produced segregating F_2 seeds for the HIP phenotype were used for subsequent analysis. Seeds from individual F_2 plants ($F_{2:3}$ seeds) were harvested on an individual plant basis and 11–23 seeds of each F_2 plant were analyzed for their Pi level through colorimetric assay (Wilcox et al. 2000). A cross was also made between *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 to determine the allelism of the *lpa* mutations.

Molecular mapping of *lpa* mutation

The $F_{2:3}$ seeds of *Gm-lpa*-ZC-2 × Wuxing no. 4 were used for molecular mapping. The genotype of an F_2 plant was determined through tests of its $F_{2:3}$ seeds for Pi level (Wilcox et al. 2000). An F_2 plant producing $F_{2:3}$ seeds of only HIP type or WT were designated as either homozygous HIP (−/−) or homozygous WT (+/+), while plants with seeds segregating for HIP and WT were designated as heterozygous (HT, +/−). Genomic DNA was extracted from extended cotyledons of 10 $F_{2:3}$ seeds of each F_2 plants using the modified CTAB method (Keim et al. 1988). 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^{-1}. PCR was performed in a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany).

For microsatellite (or simple sequence repeat, SSR) analysis, primer sequence information was obtained from the SoyBase website (http://soybase.org/resources/ssr.php). PCR primers were synthesized in Shanghai Sangon Biological Engineer Technology & Services Co. Ltd., Shanghai, China. PCR reactions were carried out in a final volume of 20 μl containing ~50 ng genomic DNA, 1× PCR buffer, 400 nM each primer, 200 μM each dNTP, 2 mM $MgCL_2$ and 1 U Taq enzyme. PCR conditions followed Li et al. (2001), namely, 94°C for 5 min followed by 35 cycles at 94°C for 25 s; 47°C for 30 s, and 72°C for 30 s, with a final extension for 5 min at 72°C. The PCR products were separated through polyacrylamide gel electrophoresis (PAGE), consisting of 8% bis-acrylamide, 0.5% TBE buffer, 0.07% APS, and 0.035% TEMED. Two microliters of loading buffer (6×) were added to PCR products and a 5 μl sample was loaded on the gel. The gel was run at constant 120 V for about 3 h in 1× TBE buffer. The gel was silver stained according to Li et al. (2001) and was documented using the VersaDoc Imaging System Model 3000 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

About 800 SSR markers were first screened for their polymorphism between *Gm-lpa*-ZC-2 and Wuxing no. 4. The polymorphic SSR markers (~170) were used for the

bulked segregant analysis (BSA) of a DNA pool composed of 20 homozygous HIP F_2 plants. Individual F_2 plants were analyzed for the putatively linked SSR markers. The linkage and genetic distance analysis of the *lpa* mutation with SSR markers was performed using the JoinMap3.0 program (http://www.kyazma.nl/index.php/mc.JoinMap).

Analysis of the *MIPS1* gene

Initially, two pairs of PCR primers were designed to distinguish the single nucleotide polymorphism (SNP) G/T between LR33 and its parent variety reported by Hitz et al. (2002), based on the sequence of the cultivars "Wye" (accession number: AY038802). The forward PCR primer was 5′ ATCTGGGAAACAATGATGGTATG/T 3′ and common reverse primer was 5′ AATAGGAGCAGCTAAG AGGGAAT 3′.

Since the G/T SNP was not observed between Taiwan 75 and *Gm-lpa*-TW-1, and the full sequence of the *MIPS1* gene later became available (Chappell et al. 2006), we then sequenced the *MIPS1* gene of both Taiwan 75 and *Gm-lpa*-TW-1. PCR primers were designed according the sequence information of the cultivars Williams 82 (accession number: DQ323904) (Chappell et al. 2006) and synthesized in Shanghai Sangon Biological Engineer Technology & Services Co. Ltd. PCR reactions were carried out in a final volume of 20 µl containing ~50 ng genomic DNA, 1× PCR buffer, 400 nM of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$ and 1 U Taq plus+ enzyme (Shanghai Sangon Biological Engineer Technology & Services Co. Ltd.). Reaction conditions were 94°C for 5 min followed by 35 cycles of 94°C for 25 s, 52°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 5 min. The PCR products were separated in 2% agarose gel electrophoresis and amplified fragments were cut from the gel and purified using the Axy-Prep DNA Gel Extraction Kit (Vitagen, Hangzhou, China). DNA fragments were directly sequenced in Takara Biotechnology (Dalian) Co. Ltd., Dalian, China. The full sequences of Taiwan 75 and its mutant were blasted against reported sequence of *the MIPS1* genes in the Genbank, then aligned for identification of SNPs and indel mutations using Clustal W1.8 (Thompson et al. 1994).

PCR-based markers and progeny analysis of *MPIS1* alleles

A 2 bp deletion was detected in the third exon of *Gm-lpa*-TW-1 *MIPS1* gene compared to WT alleles. A series of PCR-based markers were developed to differentiate the mutant and WT alleles of *MIPS1* gene. The primer sequences, matching either the WT or mutant allele, and expected size of amplicons are given in Table 3.

An F_4 population of *Gm-lpa*-TW-1 × Zhongdou 27, composing 50 lines each homozygous for *lpa* and WT was used for assessing the relationship between the 2 bp deletion and the *lpa* phenotype. The F_4 lines were derived from individual F_3 plants and classified after Pi analysis of their $F_{3:4}$ seeds, using the same procedure as in genetic analysis.

The procedure of DNA extraction and quantification, and PCR reaction volume and composition was the same as in *MIPS1* Gene Analysis. However, PCR conditions were changed into 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min, with a final extension of 72°C for 10 min. The PCR products were separated in 1.5% agarose gel electrophoresis.

Agronomic performance analysis

A comparative test of agronomic performance was conducted for the two *lpa* mutants and their parents in Hangzhou 2005. In addition, seeds were produced in three crop seasons, i.e., spring (April–July) and autumn (August–November) in Hangzhou 2004, and winter/spring (December–March of the second year) in Lingshui 2004/5. Field emergence experiments were carried out in a randomized complete-block design in triplicates, in which seeds were sown in 50 holes (two seeds/hole), spacing at 10 cm by hole and 40 cm by row. The percentage of field emergence was determined between V2 and V4 stage according to Fehr and Caviness (1977) in Hangzhou in April 2005.

For the evaluation of potential effect on agronomic performance of the *lpa* mutation in *Gm-lpa*-ZC-2, the mutant line was crossed with two WT varieties, i.e., Wuxing nos. 1 and 4. The $F_{2:3}$ seeds were produced in 2005/2006 winter/spring crop season in Hainan. After tests for seed Pi level, three types of F_3 line were identified—homozygous HIP lines, homozygous WT lines, and HT lines. These F_3 lines were tested in the spring crop season in Hangzhou in 2006. All field and laboratory measurement were conducted according to regular protocols (Wang and Wang 1992). To test its potential for release as a new variety, *Gm-lpa*-ZC-2 was also evaluated in the Regional Trial of New Spring Soybean Varieties in Southern China in six locations of four provinces in 2006. All experimental data were analyzed with the general linear models (GLM) procedure of the SAS software package (release 9.0).

Results

Development and chemical characterization of *lpa* lines

Among some 25,000 M_2 plants of Zhechun no. 3, one plant, initially coded as 1216, produced $M_{2:3}$ seeds that were all high in Pi level. This line was grown in different environments and its seeds (M_3 and M_4) were tested again for Pi level. All seeds consistently displayed the HIP phenotype,

which indicated that the mutation conferring this HIP phenotype is non-lethal and can be maintained as a homozygote. This mutant line was formally named *Gm-lpa*-ZC-2 in 2006. In addition, one M_2 plant initially coded as 4297, from some 8,000 of Taiwan 75, was HT for the HIP phenotype. The remaining seeds of this plant were grown into M_3 plants, and one homozygous HIP plant was identified in M_4. This non-lethal homozygote mutant was named *Gm-lpa*-TW-1 in 2006.

In qualitative colorimetric assays, both *Gm-lpa*-TW-1 and *Gm-lpa*-ZC-2 appeared to have significantly higher Pi levels than their WT parents, and *Gm-lpa*-TW-1 apparently had a greater Pi content than *Gm-lpa*-ZC-2 (data not shown).

Quantitative analysis of various seed P fractions of the two *lpa* lines and their parent varieties was conducted using seeds produced in different locations/crop seasons (Table 1). While the change of total P was only marginal and non-significant in both mutant lines compared with their corresponding parents, both the increase of Pi level and decrease of PA-P content was substantial. However, the degree of PA-P decrease and Pi increase were different in these two lines (Table 1). *Gm-lpa*-TW-1, on average, had 66.6% PA-P less and increased 6.8× in Pi compared to its parent Taiwan 75, while *Gm-lpa*-ZC-2 had a PA-P decline of 46.3% and Pi increase of 209.0% (Table 1). The reduction of PA-P was more or less accompanied by a molar equivalent increase of Pi in *Gm-lpa*-TW-1, but it was much greater than the increase of Pi in *Gm-lpa*-ZC-2. The difference between PA-P reduction and increase of Pi was compensated by the significant and consistent increase of lower Ins-P in *Gm-lpa*-ZC-2 (Table 1).

Significant HPLC peaks in addition to the one corresponding to PA were detected in seeds of *Gm-lpa*-ZC-2, while only minor peaks and one major peak for PA existed in *Gm-lpa*-TW-1 and the two parent varieties (Fig. 1). The major peaks are highly likely peaks for IP3, IP4, IP5, and IP6 (PA), although only IP6 was confirmed by the standard control. The presence of significant mounts of non-IP6 Ins-P suggested that the mutation occurred at a late stage in the PA biosynthetic pathway in *Gm-lpa*-ZC-2. In contrast, little change in non-IP6 Ins-P occurred in *Gm-lpa*-TW-1, which implied that the disruption happened at an early step of PA synthesis.

The content of various P fractions varied in seeds of both mutant and parental lines produced in different locations/crop seasons. For example, the Pi level of *Gm-lpa*-ZC-2 was significantly higher in seeds produced in Lingshui, Hainan (winter/spring season, 2004/2005) than in Hangzhou (autumn season, 2004); its increase over its parent Zhechun no. 3 reached 247.0% in Lingshui while it was only 164.3% in Hangzhou (Table 1). The change of PA-P and Pi level was consistently greater in *Gm-lpa*-TW-1 than in *Gm-lpa*-ZC-2 when compared with their corresponding parent varieties (Table 1), which implied that the mutation in *Gm-lpa*-TW-1 must have a larger effect on PA synthesis than the one in *Gm-lpa*-ZC-2.

Genetic nature of *lpa* mutations

The F_1 seeds of both *Gm-lpa*-ZC-2/Wuxing no. 4, and *Gm-lpa*-TW-1/Zhechun no. 3 stained colorless in colorimetric assay of Pi level (data not shown), which indicated that the F_1 seeds had normal Pi levels similar to WT varieties. This suggested that the HIP phenotype is under the control of allele(s) recessive to WT. In both F_2 populations, WT seeds to HIP type seeds fit the 3:1 Mendelian model, which

Table 1 Phosphorus concentration of various fractions in soybean seeds of two *lpa* mutant lines (M) and their parents (P)

Materials	Total P (mg g^{-1})	Inorganic P (mg g^{-1})	*Myo*-inositol P (mg g^{-1})		
			PA-P	Lower Ins-P	Total Ins-P
Hangzhou, spring season, 2004					
TW 75-P	7.54 ± 0.18	0.51 ± 0.01**	4.66 ± 0.42**	0.39 ± 0.31**	5.05 ± 0.12**
TW75-M	8.13 ± 0.24	3.59 ± 0.05	2.12 ± 0.11	0.83 ± 0.11	2.95 ± 0.11
Hangzhou, autumn season, 2004					
ZC3-P	7.46 ± 0.07	0.56 ± 0.01**	3.73 ± 0.63**	1.15 ± 0.20**	4.88 ± 0.40
ZC3-M	7.69 ± 0.37	1.48 ± 0.01	1.87 ± 0.07	2.52 ± 0.10	4.39 ± 0.27
TW75-P	7.67 ± 0.15	0.58 ± 0.01**	4.07 ± 0.24**	1.08 ± 0.20**	5.15 ± 0.02**
TW75-M	7.94 ± 0.13	4.01 ± 0.16	1.24 ± 0.06	0.26 ± 0.02	1.50 ± 0.01
Lingshui, winter/spring season, 2004/2005					
ZC3-P	9.97 ± 0.27	0.66 ± 0.01**	4.65 ± 0.49**	1.20 ± 0.15**	5.85 ± 0.03
ZC3-M	10.45 ± 0.36	2.29 ± 0.04	2.63 ± 0.06	3.16 ± 0.25	5.79 ± 0.31

Data are presented in mean ± SD; ZC3-P and ZC3-M stand for Zhechun no. 3 and its *lpa* mutant GM-*lpa*-ZC-2, and TW75-P and TW75-M for Taiwan 75 and its *lpa* mutant GM-*lpa*-TW-1, respectively; PA-P and Lower Ins-P are phytic acid P and lower inositol phosphate P

**P level of the parental variety was significantly different from that of its mutant, grown at the same location ($P = 0.01$)

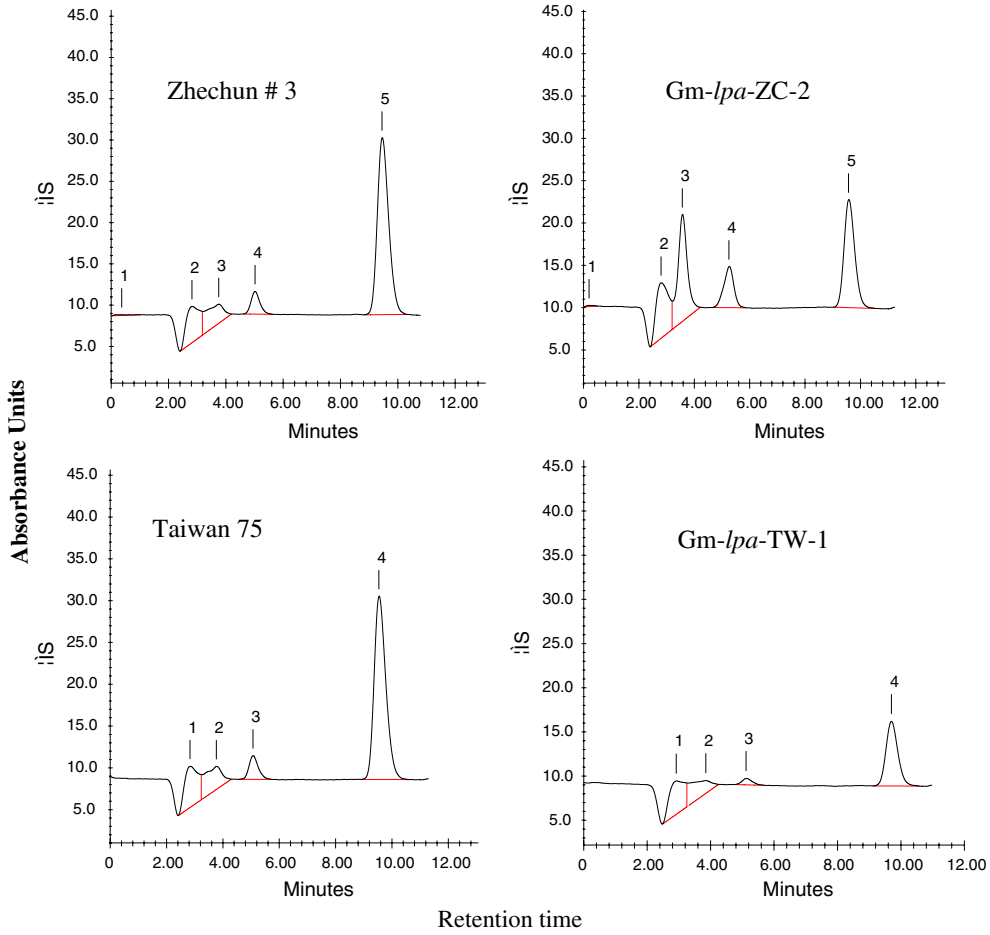

Fig. 1 Anion-exchange high performance liquid chromatography of acid-soluble *myo*-inositol (*Ins*) phosphates in soybean seeds of wild type varieties (Zhechun no. 3 and Taiwan 75) and their *lpa* mutant lines (*Gm-lpa*-ZC-2 and *Gm-lpa*-TW-1). Seeds were produced in the same field in Hangzhou during the 2005 autumn crop season. The peak 5 of Zhechun no. 3 and *Gm-lpa*-ZC-2, and peak 4 of Taiwan 75 and *Gm-lpa*-TW-1, are phytic acid (Ins P_6)

demonstrated the HIP characteristic was inherited as a recessive allele of a single gene (Table 2). Further assay of $F_{2:3}$ seeds of the cross-*Gm-lpa*-ZC-2/Wuxing no. 4 revealed, as expected, three types of F_2 plants, i.e. homozygous HIP plants of which all $F_{2:3}$ seeds were high in Pi level; homozygous WT plant of which all $F_{2:3}$ seeds had a low Pi level; and HT plants of which $F_{2:3}$ seeds were in segregation, either high or low in Pi level. The segregation ratio again fit well into a 1:2:1 ratio for HIP, HT and WT plants, reconfirming the single gene model (Table 2).

Twenty F_1 seeds of the cross between *Gm-lpa*-ZC-2 and *Gm-lpa*-TW-1 were also assayed for Pi level. All F_1 seeds had a low Pi level as WT varieties, which indicated that two mutations could be mutually complemented in functionality, hence they were not allelic to each other.

Molecular mapping of the *lpa* mutation in *Gm-lpa*-ZC-2

Among about 800 SSR genetic markers screened, 180 SSR loci were polymorphic between *Gm-lpa*-ZC-2 and Wuxing

Table 2 Segregation of soybean seeds for inorganic P level in progenies derived crosses between low phytic acid mutants and wild type varieties

Crosses	Materials	HIP	HIP/WT	WT	χ^2-value
Gm-lpa-TW-1 × *Gm-lpa*-ZC-2	F_1 seed	0	–	20	–
Gm-lpa-ZC-2 × Wuxing no. 4	F_2 seed	26	–	70	$\chi^2_{(1:3)} = 0.34$
Gm-lpa-TW-1 × Zhechun no. 3	F_2 seed	44	–	154	$\chi^2_{(1:3)} = 1.22$
Gm-lpa-ZC-2 × Wuxing no. 4	$F_{2:3}$ seed	81	162	79	$\chi^2_{(1:2:1)} = 0.04$

HIP represents seeds high in inorganic P and WT stands for seeds of wild type; $\chi_c{}^2{}_{0.05,\,1} = 3.84$ for the ratio 1:3 and $\chi^2{}_{0.05.\,2} = 5.99$ for the ratio 1:2:1, $P = 0.05$

no. 4. These polymorphic SSR markers were used in the BSA of the DNA pool of 20 homozygous HIP F_2 plants. Among them, two SSR markers were identified to be potentially linked with the HIP characteristic.

Two hundred and sixty-one F_2 plants (72 homozygous for WT, 63 homozygous for HIP and 126 HT) were individually analyzed for these two SSR markers. Finally, the two SSR markers, Satt168 and Satt416 on linkage group (LG) B2, were found to be closely linked with the *lpa* mutation in *Gm-lpa*-ZC-2, at a distance of ~9.25 and ~4.63 cM, respectively (Fig. 2).

Mutation in *MIPS1* gene

The first step in PA biosynthesis is the de novo synthesis of *myo*-inositol, which is catalyzed by the enzyme D-*myo*-inositol 3-phosphate synthase (MIPS EC5.5.1.4). It is already known that a nucleotide substitution in the *MIPS1* gene caused a 50% reduction in seed PA level in the mutant LR33 and concomitantly a decrease of raffinosaccharide level (Hitz et al. 2002). Because we observed that *Gm-lpa*-TW-1 had similar changes to LR33 not only in alteration of P fractions, i.e. the reduction of PA-P was accompanied by a molar-equivalent increase of Pi, but also in the reduction of raffinosaccharide level (data not shown), hence we postulated that the *lpa* mutation in *Gm-lpa*-TW-1 might have also happened to the *MIPS1* gene.

We tested whether the same G to T base change of the *MIPS1* gene of LR 33 (Hitz et al. 2002), at position 1,188 from the start codon of its cDNA, had occurred in *Gm-lpa*-TW-1. PCR primers capable of identifying this mutation were designed according to Hitz et al. (2002) and used in analysis of the *MIPS1* gene of Taiwan 75 and *Gm-lpa*-TW-1. The results, however, showed both Taiwan 75 and *Gm-lpa*-TW-1 to be similar to wild-type at this site, which demonstrated that the *lpa* mutation of *Gm-lpa*-TW-1 was not caused by this base change (data not shown). Therefore, we further sequenced the *MIPS1* gene of Taiwan 75 and

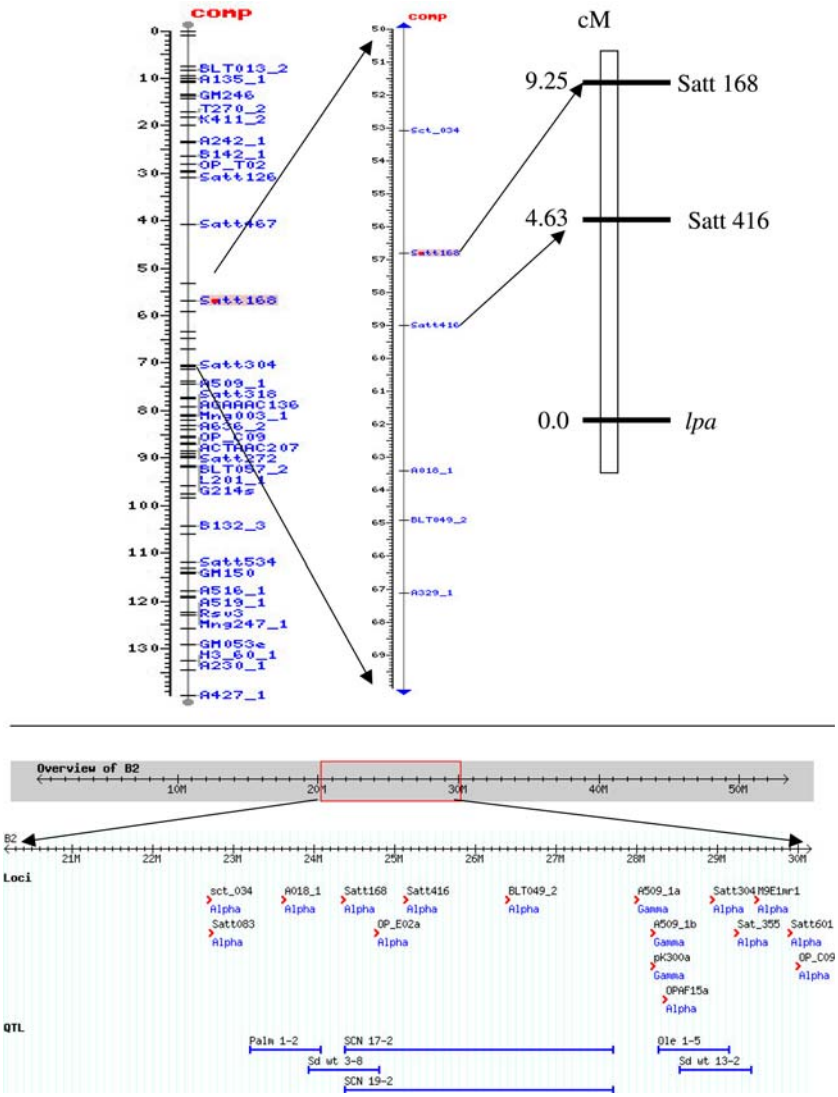

Fig. 2 Genetic mapping of the low phytic acid (*lpa*) mutation in GM-*lpa*-ZC-2. Part a (*upper*): *left*, the genetic map of linkage group (*LG*) B2 of soybean from http://www.ncbi.nlm.nih.gov/mapview; center, an enlarged part of LG B2; *right*, the mapping result of the *lpa* mutation. Part b (*lower*): the physical map of LG B2 and the region around Satt416 and Satt168 from http://soybeangenome.siu.edu/cgi-bin/gbrowse/soybeanv4., and some quantitative trait loci (*QTL*) mapped in this region

Fig. 3 The structure of the soybean *MIPS1* gene and allelic variations. Exons and introns are to scale and are depicted as *black boxes* and *solid lines*. Deletions and single nucleotide polymorphisms among three lines were elaborated by their position from the start code and base changes highlighted in *bold lower case letters*

Gm-lpa-TW-1. There was also no change at any other position except a 2 bp deletion in the third exon of *Gm-lpa*-TW-1 compared with Taiwan 75 (Fig. 3). The complete genomic sequences of Taiwan 75 and *Gm-lpa*-TW-1 have been deposited in the GenBank under the accession numbers EF155568 and EF155569, respectively. This 2 bp deletion can potentially lead to frame shift and internal stop codons in the conceptual translation of the coding region of mRNA, and possibly to a loss of function at the molecular or biochemical level of the gene.

Alignment of the *MIPS1* gene sequences of Taiwan 75 and *Gm-lpa*-TW-1 with that of Williams 82 (accession DQ323904) showed that the three lines have the same *MIPS1a* allele type in that the 39-bp deletion does not exist in the final intron (Chappell et al. 2006). However, Taiwan 75 had a single base deletion at position +1,503 in the sixth intron of the gene as compared with Williams 82 (Chappell et al. 2006) (Fig. 3). We further found that the last base of the sixth intron of Taiwan 75, which was an A (position +1,653 of the gene), was different from that of Williams 82, which was a G (Fig. 3). In addition, we also detected a base change in the eighth exon at position +1,812 of the gene, from an A in Williams to a C in Taiwan 75 (Fig. 3). However, this base change does not lead to any amino acid change because both "CCA" and "CCC" encode the amino acid proline.

Allele-specific markers for *MIPS1* gene

The *MIPS1* gene was amplified using PCR primer specific to either WT or mutant allele (Table 3). DNA fragments with expected size were amplified only in *Gm-lpa*-TW-1 for mutant allele specific markers (MIPS-M1 and 2), but were produced in both *Gm-lpa*-TW-1 and Taiwan 75 for the marker MIPS-WT specific for the WT allele (Fig. 4). The same was observed for Zhongdou 27 (a WT variety) and its F_4 lines derived from a cross with *Gm-lpa*-TW-1. Analysis of 100 homozygous F_4 lines, half normal and half low in PA-P, showed the 2 bp deletion existed in all 50 F_4 lines of mutant type (MT) but in none of WT, either using pooled samples (Fig. 4) or individual lines (data not shown).

Impact of *lpa* mutants on agronomic performance

The two mutant lines, together with their corresponding parent varieties, were tested in field conditions to check whether the *lpa* mutant lines had inferior field emergence compared to their parents, a phenomenon that has become a bottleneck for the use of previously reported *lpa* mutants in soybean (Meis et al. 2003; Oltmans et al. 2005). Furthermore, the agronomic characters were compared among three types of plant family derived from crosses of *Gm-lpa*-ZC-2 with two conventional varieties to further investigate any possible effect of the *lpa* mutation on agronomic performance. We also tested the yield potential of *Gm-lpa*-ZC-2 through participating in a national yield trial of new soybean varieties.

The field emergence rate seemed to be affected by both genotype and seed source. Both mutants and parents had good field emergence rates when seeds were produced in the autumn season in Hangzhou and winter/spring season in Lingshui (Fig. 5). For the seeds produced in the spring season in Hangzhou, both Taiwan 75 and *Gm-lpa*-TW-1 had a

Table 3 DNA makers and their primer sequence used for differentiating wild type and low phytate mutant alleles of soybean *MIPS1* gene

Marker	Forward primer (5′–3′)	Reverse primer (5′–3′)	Expected amplification	Expected size (bp)
MIPS1_WT	TGGGCTACAAAGGACAAG	GAAGCAGGCTCTTGAATG	Wild type allele	121
MIPS1_M1	GGGCTACAAAGGACA**at**	TTAACTACAACAAGTGGG	Mutant allele	219
MIPS1_M2	CTACGAAACCACCGAACT	TTGGCTTGTTGA**at**TGTC	Mutant allele	400

Nucleotides in capital lettees are shared between wild type and mutant alleles and those in bold lower case are different between mutant and wild type alleles

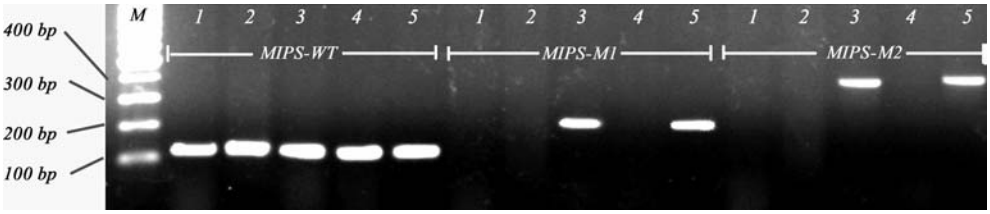

Fig. 4 Amplification of soybean *MIPS1* gene using three sets of PCR primers MIPS-WT, MIPS-M1 and MIPS-M2 (see Table 3). PCR products were separated on 1.5% agarose gel. Lane M, DNA ladders; Lanes 1, 2, and 3 represents Taiwan 75, Zhongdou 27 and *Gm-lpa*-TW-1, respectively. Lanes 4 and 5 are pooled F_4 lines ($n = 50$) of *Gm-lpa*-TW-1 × Zhongdou 27, homozygous for wild and low phytate phenotype, respectively

very low field emergence rate, but the parent did perform significantly better than the mutant (Fig. 5). On the other hand, seeds of *Gm-lpa*-ZC-2 and parent Zhechun no. 3 were low (75–78%) but better than *Gm-lpa*-TW-1. These results suggested that the *lpa* mutation in *Gm-lpa*-ZC-2 does not negatively affect seedling emergence, while the one in *Gm-lpa*-TW-1 could exert negative influence on this character when seeds are produced in unfavorable environments.

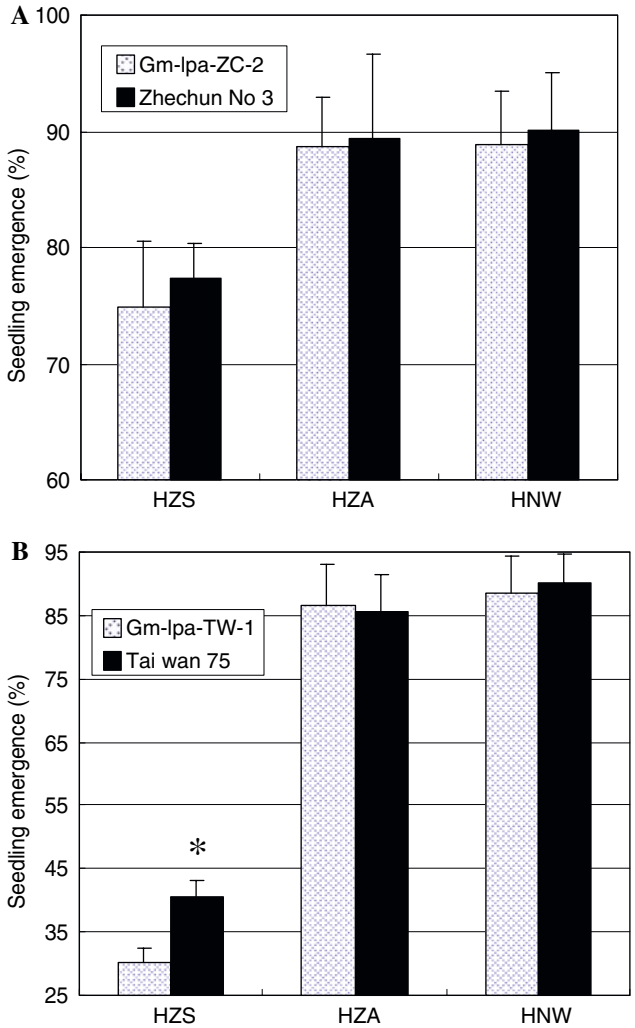

Fig. 5 Effect of seed source on seedling emergence of two low phytic acid mutant lines and their wild type parents From seeds produced in *HZS* Hangzhou spring season in 2004, *HZA* Hangzhou autumn season in 2004, *HNS* Hainan winter/spring season in 2004/2005. *Data* presented mean ± SE of three replications. An *asterisk* indicates a significant difference to the respective mutant line at the same location ($P < 0.05$)

Two F_3 populations were generated by crossing GM-*lpa*-ZC-2 with two conventional varieties Wuxing nos. 1 and 4 (Crosses I and II, respectively), for assessing the potential effect of the *lpa* mutation on agronomic performance. In each cross, F_3 lines were classified as either homozygous for MT (all $F_{2:3}$ seeds were in HIP phenotype), or homozygous for WT (all $F_{2:3}$ seeds were low Pi), or HT ($F_{2:3}$ seeds were segregating for HIP and WT). Comparative analysis showed there were significant differences for plant height, individual plant yield and seed size between WT and MT lines in both or one cross, while no differences were observed for other characters including plant density (Fig. 6).

The only consistent difference in the two crosses was the plant height, of which the MT lines were significantly shorter than WT lines (Fig. 6). The MT lines had a significant lower individual plant yield than the WT lines in Cross-I, while it was the opposite in Cross-II; the trend of seed size was similar to plant yield, where the MT lines had non-significant but larger seed size than WT ones in Cross-II, while the former type of lines had significantly smaller seed size than the latter type in Cross-II (Fig. 6). These results demonstrated the *lpa* mutation does not exert any negative effect on agronomic traits with the exception of plant height.

Comparative yield potential of *Gm-lpa*-ZC-2 with other commercial soybean varieties of similar growth type was tested in the Regional Trial of New Spring Soybean Varieties in Southern China in 2006. Based on data averaged over individual sites, yield of *Gm-lpa*-ZC-2 was not significantly different from the commercial control variety Xiangdou no. 10, which had a similar maturity date to that of *Gm-lpa*-ZC-2 (Fig. 7). Lines had yields lower than usual in Shanxia and Zhongqing due to a sustained and severe drought, but *Gm-lpa*-ZC-2 performed better than the control (Fig. 7).

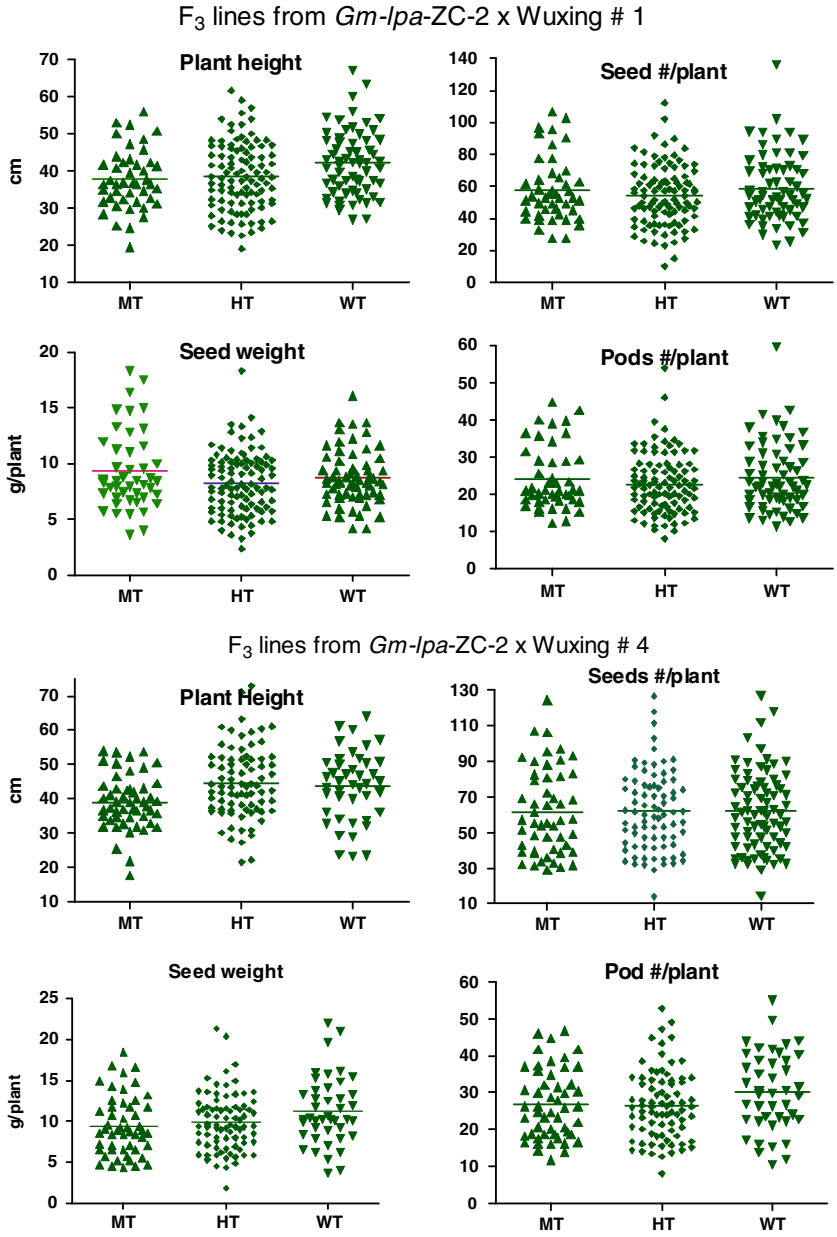

Fig. 6 Performance of four agronomic traits of three F_3 genotypes from two crosses between a low phytate mutant and two wild type varieties. *Above*: *Gm-lpa*-ZC-2 × Wuxing no. 1; *Below*: *Gm-lpa*-ZC-2 × Wuxing no. 4. *WT* homozygous wild type lines, *HT* heterozygous lines, *MT* homozygous mutant type lines

Discussion

In this study, we generated two *lpa* soybean mutant lines through artificial mutagenesis using gamma irradiation. Biochemical and genetic analysis demonstrated that *Gm-lpa*-ZC-2 is completely distinct from the three *lpa* mutant lines previously reported, M156 (and its derivatives) and M766 (Wilcox et al. 2000), and LR33 (Sebastian et al. 2000) in soybean. We further mapped the *lpa* mutation on LG B, tightly linked with two SSR markers. We also sequenced the *MPIS1* gene of *Gm-lpa*-TW-1 and its parent variety and discovered a 2 bp deletion in *Gm-lpa*-TW-1, which likely represents the *lpa* mutation in this line. We also demonstrated that the *lpa* mutation in *Gm-lpa*-ZC-2 does not negatively affect seedling emergence rate or seed yield. The genetic resources created in the present study will be useful not only for breeding *lpa* soybean, but also for the study of PA synthesis.

Mutation induction has become an important tool for generating novel genetic variation in plant breeding programs. According to the FAO/IAEA Mutant Variety Database (http://www-mvd.iaea.org/MVD/default.htm), more than 100 soybean varieties were developed worldwide by induced mutation during the past 40 years. Following chemical mutagenesis and screening of about 4,000 M_2 plants, Wilcox et al. (2000) identified two plants that produced M_3 seeds that were both high and low in Pi level. We selected one homozygous mutant among about 25,000 M_2 plants of

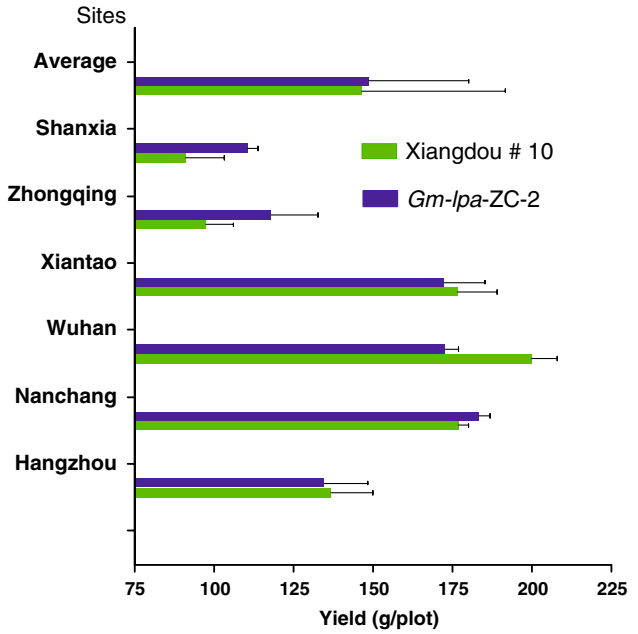

Fig. 7 Yield performance of *Gm-lpa*-ZC-2 and Xiangdou no. 10 at test locations in the Regional Trial of New Spring Soybean Varieties in Southern China in 2006. *Data* represent mean ± SE of three replications for individual site, and mean ± SE of six locations for the average

Zhechun no. 3 and one HT mutant among about 8,000 M_2 plants of Taiwan 75, following gamma rays treatment. Therefore, the *lpa* mutation frequency, although variable among populations, was more or less within the same magnitude, which was also within the range of mutation frequency of similar single gene controlled traits (van Harten 1998). Although genetic transformation can also be used to reduce phytate content in soybean (Nunes et al. 2006), chemical or physical mutagenesis is a more cost-effective, technically simpler, and more publicly accepted option.

All previously reported soybean *lpa* mutants (LR 33, M156, and M733) had a PA-P reduction of about 50–70% and always had molar equivalent increase of Pi (Wilcox et al. 2000; Hitz et al. 2002). In the present study, *Gm-lpa*-TW-1 had similar PA-P reduction (54.5–69.5%) and also had a molar equivalent increase of Pi (Table 1). However, the change of P fractions in *Gm-lpa*-ZC-2 was substantially different from that of *Gm-lpa*-TW-1. The reduction of PA-P was about 43.4–49.9% in *Gm-lpa*-ZC-2, which was accompanied by the increase of both Pi and lower Ins-P (Table 1, Fig. 1). The presence of significant amounts of lower Ins-P suggested that *Gm-lpa*-ZC-2 was very similar to the *lpa 2-1* mutant in barley (Dorsch et al. 2003) and the *lpa 2-1* in maize (Shi et al. 2003).

The similarity of P fraction change and the concomitant reduction of raffinosaccharides (Yuan et al. unpublished results) between *Gm-lpa*-TW-1 and LR 33 prompted us to sequence the *MIPS1* gene. Although we did not observe the base change in the exon as reported in LR33, we found a 2 bp deletion in the third exon of *Gm-lpa*-TW-1 *MIPS1* gene (Fig. 3). The inability of the DNA marker using PCR primers specific for WT allele of the *MIPS1* gene to distinguish WT varieties from mutant lines is understandable because it is already known that there are four highly conserved copies of the *MIPS* gene in soybean (Chappel et al. 2006). Analysis of F_4 lines of *Gm-lpa*-TW-1 × Zhongdou 27 revealed the precise relationship between the deletion and *lpa* phenotype, suggesting that the 2 bp deletion in the *MIPS1* gene caused the *lpa* phenotype of *Gm-lpa*-TW-1. This deletion caused a frame shift of the mRNA and internal stop codons, which consequently could lead to the dysfunction of the *MIPS1* gene in *Gm-lpa*-TW-1.

The sequencing results also showed that the *MIPS1* gene is highly conserved in soybean; only one single base deletion, one SNP in the sixth intron and one SNP in the eighth exon were identified between Taiwan 75 and Williams 82 (Fig. 3). This result was consistent with the observation made by Chappel et al. (2006) in that only one deletion was identified in addition to the 39 bp deletion in the last intron between alleles *MIPS1a* and *MIPS1b*. Chappel et al. (2006) also demonstrated that the *lpa* mutation in CX1834-1-3/6 was not related to the *MIPS1* gene, which is reasonable because it is already known that the *lpa* mutation of CX1834-1-3/6 was controlled by two independent genes (Oltmans et al. 2004).

Walker et al. (2006) recently mapped the two genes, *pha 1* and *pha 2*, that control the *lpa* trait in CX1834 on LG N near Satt237 and LG L close to Satt527. We mapped the mutation in *Gm-lpa*-ZC-2 on LG B2 closely linked with Satt168 and Satt 416 at genetic distances of 9.25 and 4.63 cM, respectively (Fig. 2). It should be noted that the genetic distance between Satt168 and Satt416 observed in our present study reached 4.6 cM, which was more than double the previously reported value ~2.2 cM (Fig. 2). Because the physical distance between Satt168 and Satt416 is only about 0.8 Mbp (Fig. 2), we are inclined to accept that our genetic distance data is somehow greater than the actual value, possibly due to the small size of our mapping population. If this is the case, the *lpa* mutation in *Gm-lpa*-ZC-2 should nicely overlap the region where one quantitative trait locus (QTLs) for palmitate content (*Palm 1–2*) is located (Fig. 2). Coincidently, Hulke et al. (2004) noted soybean lines homozygous for *lpa* derived from a cross between CX1843-1-3 and a low palmitate line, had a higher palmitate content than lines homozygous for the WT allele. Since high palmitate is nutritionally undesirable in soybean, e.g., a low level of palmitate is preferred for soybean oil when used as salad oil (Primomo et al. 2002), this might reduce the value of *lpa* soybean. Further studies are therefore needed to explore the relationship between the *lpa* mutation and palmitate content in soybean.

Although four non-allelic *lpa* mutations were reported in both barley (Bregitzer and Raboy 2006) and rice (Liu et al. 2007), only the three non-allelic *lpa* mutations in maize, *lpa 1*, *lpa 2*, *lpa 3*, were characterized at the gene level (Raboy et al. 2001; Shi et al. 2003, 2005, 2006). In soybean, the *lpa* mutation in LR33 is already known to be a nucleotide substitution in the *MIPS1* gene (Hitz et al. 2002), while the *lpa* trait of CX1834-1-3 was controlled by two independent genes, *pha1* and *pha 2* (Oltmans et al. 2004), which was recently mapped on LG N and L (Walker et al. 2006). It was demonstrated that the *lpa* trait of *Gm-lpa*-TW-1 is possibly a result from a lesion in the *MIPS1* gene. On the other hand, similarity of the P fraction profile of *Gm-lpa*-ZC-2 to that of *lpa 2* maize and *lpa 2* barley, suggested that it might result from a mutation in an orthologue of the Ins-P kinase gene as found in maize (Shi et al. 2003). These findings implied that the soybean *lpa* mutations, reported in the present study and in previous experiments, have not covered all possible types of *lpa* mutations, and hence there is still a potential to identify more *lpa* mutations, either through a conventional forward genetics approach like the present study, or by using reverse genetics tools, i.e. Targeting Induced Local Lesion IN Genomes (TILLING) (Chappell et al. 2006).

Lower seed yield and reduction of seed viability are common consequences of most *lpa* mutations. Meis et al. (2003) observed that the field emergence rate of homozygous *mips* lines, derived from the LR 33 line (Hitz et al. 2002), was significantly lower than WT lines. They also observed a significant seed source effect: reduction of field emergence percentage was better for seeds produced in the temperate area than for those from the tropical location (Meis et al. 2003). In the present study, a significant effect of crop season was also observed for the field emergence rate of both Taiwan 75 and its mutant line *Gm-lpa*-TW-1 (Fig. 5). However, the differences between the parent and its mutant were only significant when their seeds were produced in Hangzhou in the spring season, of which the climate is similar to a subtropical environment. If the *Gm-lpa*-TW-1 *lpa* mutation is confirmed to have occurred in the *MIPS1* gene, this suggests that different mutant alleles of the *MIPS1* gene could have different effects on field emergence. In our study on rice, we also observed significant effects of two alleles on seed viability (Liu et al. 2007). In another study, although on a completely different genetic basis, Oltmans et al. (2005) also observed an adverse effect of the *lpa* mutation in CX1834-1-3 on seed viability, when seeds were produced in a subtropical environment. In our study, the field emergence rate of *Gm-lpa*-ZC-2 was no less than Zhechun no. 3 for seeds produced in all three seasons (Fig. 5). In addition, the *lpa* mutation in *Gm-lpa*-ZC-2 showed no negative effect on yield components (Fig. 6); *Gm-lpa*-ZC-2 had no yield reduction compared to an adapted cultivar of similar maturity based on performance in regional yield trial (Fig. 7). Therefore, this *lpa* line would be beneficial to use in practical breeding programs to develop low phytate soybeans.

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