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ORIGINAL PAPER

Identification and characterization of the soybean IPK1 ortholog of a low phytic acid mutant reveals an exon-excluding splice-site mutation

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Abstract Phytic acid (*myo*-inositol 1, 2, 3, 4, 5, 6 hexa*kis*phosphate) is an important constituent of soybean meal. Since phytic acid and its mineral salts (phytates) are almost indigestible for monogastrics, their abundance in grain food/feed causes nutritional and environmental problems; interest in breeding low phytic acid has therefore increased considerably. Based on gene mapping and the characteristics of inositol polyphosphates profile in the seeds of a soybean mutant line *Gm-lpa*-ZC-2, the soybean ortholog of inositol 1,3,4,5,6 penta*kis*phosphate ($InsP_5$) 2-kinase (IPK1), which transforms $InsP_5$ into phytic acid, was first hypothesized as the candidate gene responsible for the low phytic acid alteration in *Gm-lpa*-ZC-2. One IPK1 ortholog (*Glyma14g07880*, *GmIPK1*) was then identified in the mapped region on chromosome 14. Sequencing revealed a G → A point mutation in the genomic DNA sequence and the exclusion of the entire fifth exon in the cDNA sequence of *GmIPK1* in *Gm-lpa*-ZC-2 compared with its wild-type progenitor Zhechun No. 3. The excluded exon encodes 37 amino acids that spread across two conserved IPK1 motifs. Furthermore, complete co-segregation of low phytic acid phenotype with the G → A mutation was observed in the F_2 population of ZC-*lpa* x Zhexiandou No. 4 (a wild-type cultivar). Put together, the G → A point mutation affected the pre-mRNA splicing and resulted in the exclusion of the fifth exon of *GmIPK1* which is expected to disrupt the GmIPK1 functionality, leading to low phytic acid level in *Gm-lpa*-ZC-2. *Gm-lpa*-ZC-2, would be a good germplasm source in low phytic acid soybean breeding.

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Introduction

Commonly known as phytic acid (PA), inositol 1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$) is the major storage form of phosphorus (P) in plant seeds and often exists as a mixed salt (phytate) of mineral cations, including Zn^{2+} and Fe^{3+} (Lott et al. 2000; Raboy 2001). Phosphorus in the PA or phytate form (PA-P) and divalent cation minerals in phytate are almost indigestible for monogastric animals, and undigested PA-P excreted in manure has become an important source of environmental phosphorus pollution (Abelson et al. 1999; Sharley et al. 2008). Therefore, the abundance of PA-P in grain food/feed has a number of nutritional, agricultural and environmental consequences and hence there is considerable interest in generating low phytic acid (LPA) crops (Raboy 2001, 2009).

Both forward and reverse genetics approaches have been used to study seed PA metabolism and to engineer LPA crops (see reviews Raboy 2007, 2009). In plants, there are two parallel PA biosynthetic pathways, known as

lipid-dependent and lipid-independent pathways (Raboy 2007), both starting with the production of inositol-3-phosphate [Ins(3)P_1] from glucose 6-P and ending with $InsP_6$ biosynthesis from Ins(1,3,4,5,6)P_5 ($InsP_5$). Mutation or silencing of genes involved in both pathways could result in PA reduction and simultaneous increase of inorganic P (Pi), e.g., silencing of the *myo*-inositol phosphate synthase (MIPS) gene in rice (Kuwano et al. 2009) and soybean (Nunes et al. 2006), mutation of the *myo*-inositol kinase gene in rice (Kim et al. 2008a) and maize (Shi et al. 2005), and of inositol polyphosphate kinase genes in maize (Shi et al. 2003) and *Arabidopsis* (Stevenson-Paulik et al. 2005). In addition, the multi-drug resistance-associated protein (MRP) ATP-binding cassette (ABC) transporter gene has also proven to be involved in PA metabolism, possibly by facilitating PA transportation between cellular compartments (Nagy et al. 2009), and its mutations or knockdown could cause seed PA reduction (Shi et al. 2007; Xu et al. 2009; Panzeri et al. 2011). Another LPA gene with function yet to be known in PA metabolism but with a domain homologous to the 2-phosphoglycerate kinase (2-PGK) gene of hyperthermophilic and thermophylic bacteria (Lehmacher et al. 1990) was identified in rice (*OsLpa1*, Kim et al. 2008b; Zhao et al. 2008) and *Arabidopsis* (*At5g60760*, Kim and Tai 2010).

Soybean has an effective deficiency of P and minerals, despite high levels of minerals and P in the meal (Raboy 2007). Several soybean LPA mutant lines have been developed by various groups (see review Raboy 2007). A few soybean LPA lines are already known to be mutants of two genes, i.e., *GmMIPS1* (Hitz et al. 2002; Yuan et al. 2007) and *GmMRP5* (Gillman et al. 2009; Maroof et al. 2009). Another soybean LPA mutant line, *Gm-lpa*-ZC-2, was generated through gamma rays mutagenesis and had a ~50 % PA reduction and elevated levels of inorganic P (Pi) in seeds (Yuan et al. 2007). Unlike other LPA mutants, *Gm-lpa*-ZC-2 appears to have excellent seed viability (both germination and field emergence) (Yuan et al. 2007). Further profiling indicated that, compared with its wild-type progenitor Zhechun No. 3, *Gm-lpa*-ZC-2 had increased levels of intermediate inositol polyphosphates ($InsP_3$, $InsP_4$ and $InsP_5$) (Frank et al. 2009a), similar levels of divalent mineral cations (Frank et al. 2009b) and elevated total isoflavone content in seeds (Yuan et al. 2009). Therefore, *Gm-lpa*-ZC-2 has the potential for improving quality or functional property of soybean grains. Although the gene responsible for the LPA phenotype of *Gm-lpa*-ZC-2 was mapped on chromosome 14 (Yuan et al. 2007), its molecular genetic basis is yet to be uncovered. In the present study, the mutant gene was further fine mapped and the soybean ortholog of the Ins(1,3,4,5,6)P_5-2 kinase gene *IPK1* (*GmIPK1*) was identified within the mapped region and subjected to detailed molecular characterization.

Materials and methods

Plant materials, inorganic P test and phytic acid content determination

The soybean LPA line *Gm-lpa*-ZC-2 (ZC-*lpa* hereafter) used in this study was developed through ^{60}Co gamma irradiation of Zhechun No. 3, a commercial soybean variety (Yuan et al. 2007). It is featured with ~50 % PA reductions and increased levels of Pi, *myo*-inositol phosphate and lower inositol polyphosphates in seeds (Yuan et al. 2007; Frank et al. 2009a). ZC-*lpa* was crossed with Zhexiandou No. 4, a non-LPA WT cultivar, and F_2 and F_3 populations were developed from self-pollinated F_1 and F_2 plants, respectively. $F_{2:3}$ seeds of each F_2 plant were harvested individually. All field experiments were carried out in the experimental farm of Zhejiang Academy of Agricultural Sciences in Hangzhou, Zhejiang Province during 2008–2011.

The Pi level of soybean seeds was colorimetrically tested according to the protocol of Wilcox et al. (2000) after slight modification (Yuan et al. 2007). Development of a blue color in the test indicates a high seed Pi level, typical for ZC- *lpa* mutant and its homozygous mutant progenies, while colorlessness indicated a WT, normal seed Pi level (Yuan et al. 2007). The Pi level of six $F_{2:3}$ seeds of each F_2 plant were colorimetrically tested.

PA content was determined using anion-exchange chromatography according to Yuan et al. (2007) with slight modification. Briefly, 20 ml 0.4 M HCl was added into a 50-ml tube with defatted soybean flour (~1.0000 g) or ground vegetative tissues (root, leaf and stem of 40-day-old plants, ~800 mg) and mixed by shaking overnight (soybean flour and tissue powder had been dried at 105 °C for 2 h and stored in glass tubes in desiccators before analysis). The tubes were centrifuged at 12,000*g* at 4 °C for 30 min. For seed samples, 10 ml of supernatant was transferred to a second tube, added with 20 ml of ddH_2O and 5 ml mixture of 0.2 M HCl:15 mM $FeCl_3$. For samples of vegetative tissue, 15 ml of supernatant was transferred. After heating for 30 min in a boiling water bath, the tubes were cooled to room temperature and centrifuged at 8,000*g* at 4 °C for 30 min. After washing twice in 0.2 M HCl, the ferric precipitates were converted to a soluble Na Ins phosphate solution and an $Fe(OH)_3$ precipitate by adding 10.0 ml of 1.5 M NaOH. After centrifugation at 12,000*g* for 10 min, 2.5 ml of supernatant was transferred to a volumetric flask and diluted to 50 ml (no dilution for vegetative tissues). The (diluted) supernatant solutions were further passed through a 0.22-μm filter and through an IC-RP column and an IC-H column (Bonna-Agela Technologies, China). The IC-RP column was used to get rid of hydrophobic compounds, and the IC-H column to remove residual alkaline earth metal ions, transition metal ions and carbonate ions.

Analysis of PA was performed on Anion-exchange Ion Chromatography ICS-2000 (Dionex, Sunnyvale, CA, USA). Aliquots were fractionated on a Dionex IonPac AS11-HC analytical column, equipped with an IonPac AS11-HC guard column and an EluGen cartridge KOH generator tank. The effluent was equilibrated with 50 mM KOH at a flow rate of 1 ml min^{-1}. PA was determined using a conductivity detector by measuring 25 µl solution keeping the suppressor current at 124 mA. An external standard of Na InsP6 (P-3168, Sigma, St. Louis, MO, USA) was analyzed before and after every two samples, and each material was analyzed in triplicate.

Microsatellite analysis

For fine mapping, microsatellite markers nearby Satt416 and Satt168 on chromosome 14 (equal to LG B2) were obtained from the SoyBase (http://soybase.org/resources/ssr.php). Additional microsatellites were identified in the targeted region using SSR search program SSRIT (http://www.gramene.org/microsat/) against the soybean genomic sequences downloaded from Phytozome (http://www.phytozome.net/search.php?show=blast). All microsatellite PCRs were carried out in a final volume of 20 µL containing approximately 50 ng of genomic DNA, 1× PCR buffer, 400 nM each primer, 200 µM each dNTP, 2 mM $MgCl_2$ and one unit 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 on polyacrylamide gel (8 % bis-acrylamide, 0.5 % TBE buffer, 0.07 % APS and 0.035 % TEMED) through electrophoresis at constant 120 v for about 3 h in 1× TBE buffer. The gel was silver stained according to Li et al. (2001) and documented using the VersaDoc Imaging System Model 3000 (Bio-Rad Laboratories, Inc., USA).

Identification and characterization of *IPK1* orthologs

The soybean genes homologous to *IPK1* were identified using basic local alignment search tool (BLAST) search implemented on databases of the National Center for Biotechnology Information (http://www.ncbi.clm.nih.gov/Database) and the Phytozome for soybean (http://www.phytozome.net/soybean). The *Glyma14g07880* locus, identified to be an *IPK1* ortholog in the present study, was amplified by one PCR using primer pair SQ1 (Fig. 1) and subsequently sequenced.

For gene expression analysis, total RNA was isolated from leaves, stems and roots of 14-day-old seedlings, flowering inflorescences and developing seeds (7–42 days after flowering, DAF) using an E.Z.N.A.^{TM} plant RNA kit (Omega Bio-tek, Inc., USA) according to the manufacturer's protocol. Genomic DNA contamination was eliminated by RQ1 RNase-Free DNase (Promega, USA). All RNA samples were quality assessed on 1.2 % agarose gels and adjusted to a final concentration of ~500 ng/µl after quantification. The first-strand cDNA was transcribed using random + oligo (dT) primer and reverse transcriptase (ReverTra Ace + RNase inhibitor, Toyobo Co., Ltd. Japan) according to the manufacturer's instruction. A single bulk cDNA synthesis reaction was performed for each sample to produce cDNA sufficient for a number of PCRs.

The full-length cDNA of *Glyma14g07880* was amplified by RT-PCR using primer pair RT1 (Fig. 1) and subsequently sequenced. Two pairs of primers (RT3 and RT6, Fig. 1) were used to analyze the effect of mutation on exon–intron splicing. PCRs were performed in 20 µl volume with 50 ng cDNA, 1× PCR buffer, 400 nM of each primer, 200 µM of each dNTP, 2 mM $MgCl_2$ and one unit Taq plus + enzyme (Shanghai Sangon Biological Engineer Technology and Services Co., Ltd.), using the following program: 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension of 72 °C for 10 min. Semi-quantitative RT-PCRs were performed using the primer pair qRT1, which amplifies a 452-bp fragment of the *Glyma14g07880* cDNA (Fig. 1), with soybean *Actin* gene as internal control (forward primer 5′-CAACCCAAAGGTCAACAG-3′ and reverse primer 5′-CAGCGAGATCCAAACGAA-3′). Similarly, semi-quantitative RT-PCRs were also performed for two homologs of *GmIPK1*, namely *Glyma04g03240* and *Glyma06g03310*, using primer pairs of Glyma04g03240 F/R and Glyma06g03310 F/R (Table 1).

Quantitative real-time PCRs were performed for *Glyma14g07880* in developing seeds with primer pair qRT1 using a SYBR^{R} Green Real time PCR Master Mix (TOYOBO Biotech Co., Ltd) in a Roche (USA) instrument according to the manufacturer's instruction. Each 20 µl reaction comprised 2 µl template, 10 µl SYBR Green Realtime PCR Master Mix-Plus, 1.2 µl (10 µM) of each primer, 2 µl Plus Solution and 3.6 µl ddH_2O. The quantification of gene expression was performed in triplicate using the relative $-2^{\Delta\Delta C_T}$ method by comparing the data with the internal control gene *Actin*.

Genomic DNA extraction, primer design, DNA sequencing and sequence alignment

For gene mapping and cloning, genomic DNA was extracted from plant leaves of ZC-*lpa*, Zhechun No. 3, Zhexiandou No. 4 and F_2 plants using a modified CTAB method (Keim et al.

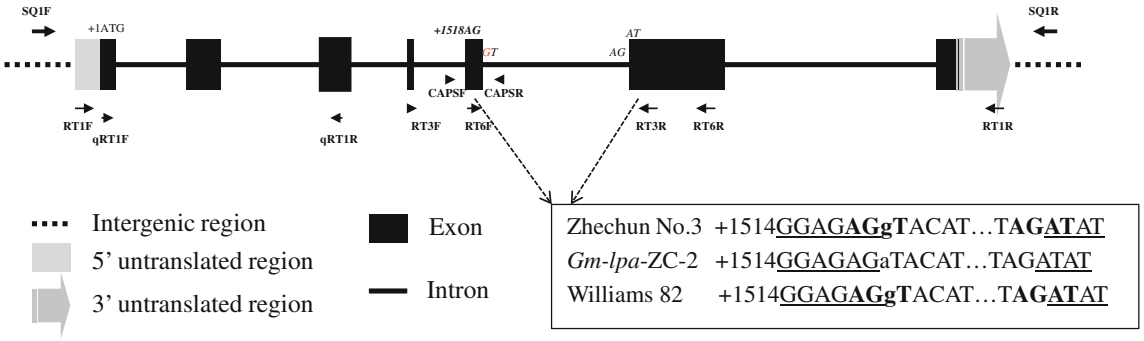

Fig. 1 Diagram of soybean ortholog of the *IPK1* gene, *GmIPK1*. The position of primers for whole gene amplification (SQ1F/R), CAPS marker (CAPSF/R), cDNA amplification (RT1F/R), cDNA genotyping (RT3F/R and RT6F/R) and quantitative RT-PCR (qRT1F/R) is illustrated. *The box* at the *right bottom* shows nucleotides around the fifth exon–fifth intron–sixth exon region where the G → A mutation (in *lowercase*) is located (exon nucleotides *underlined*). The consensus sequences at the 5′ donor and 3′ acceptor splicing sites are highlighted in *bold*. Nucleotides are numbered from +1 of the ATG code

Table 1 PCR primers used for genomic DNA and cDNA amplification of *GmIPK1* (*Glyma14g07880*)[a]

Primer ID	Application	Product size (bp)	Primer sequence (5′–3′) (F: forward; R: reverse)
SQ1	Amplification of *GmIPK1* for sequencing	3,374	F: CCACTCACAGAAAGACAGCACA R: CAAACCAACGCACCCCAT
RT1	Amplification of full-length *GmIPK1* cDNA for sequencing	1,449	F: GGAAGCACGAGGTGAAAC R: CTGACAGCCCCAACATAC
RT3	PCR of *GmIPK1* cDNA fragment	306	F: CAGTCAAGGATCTAGCCTCTGC R: TTTTTGCAACACCTCCCAGT
RT6	PCR of *GmIPK1* cDNA fragment	630	F: CAAATGCGGATTTCTTCCTC R: TCCAGAATCCTCCTCATTCC
qRT1	(Semi-) quantitative RT-PCR of *GmIPK1*	452	F: AAAGGATTGCTCCGTTGA R: GAGGACTGGGAGGTGTTG
CAPS	Development of CAPS marker for *GmIPK1* mutation	350	F: CTCAGCTTCACCCCTTTC R: CTAACTCAGATTTAATGCC
Glyma04g03240F/R	Semi-quantitative RT-PCR of *Glyma04g03240*	166	F: ATATACCTCTTTGCATTCTGCTACA R: ACTCAAGATATATGTTACTGTAT
Glyma06g03310 F/R	Semi-quantitative RT-PCR of *Glyma06g03310*	205	F: TGATTTTAAGGGAGAAGGACGCAAA R: ACGTCTTTCCAGAGGAGGCGTTCGA

[a] All primers were designed based on the genomic and transcript sequences of Williams 82 available in the databases of Phytozome V7 (http://www.phytozome.net/) and used for the design of all primers

1988) and adjusted to a final concentration of ~50 ng/μl after quantification as previously described (Yuan et al. 2007). For routine microsatellite markers, primer sequence information was obtained from the Soy Base Web site (http://soybase.org/resources/ssr.php). All other primers were designed online using the Primer Premier 5 software program (http://www.premierbiosoft.com/primerdesign/index.html) based on the genome and transcript sequences of soybean cultivar Williams 82 (Phytozome V7.0) (http://www.phytozome.net/cgi-bin/gbrowse/soybean). All primers used for genomic DNA and cDNA amplification were synthesized in Shanghai Sangon Biological Engineer Technology and Services Co., Ltd. (Shanghai, China) and are given in Table 1. PCR amplicons were separated in 1.2 % agarose gel electrophoresis and target fragments were cut and purified using the Axy-Prep DNA Gel Extraction Kit (Vitagen, Hangzhou, China)for sequencing in Shanghai Invitrogen Biotech Co., Ltd. (Shanghai, China). Sequence alignments were performed using ClustalX (http://www.ebi.ac.uk/Tools/) and BioEdit7.0 program.

Table 2 Genotype, inorganic P phenotype and phytic acid content in seeds and vegetative tissues

Materials	*GmIPK1* genotype[a]	Seed inorganic P phenotype[b]	Phytic acid	
			Tissue[c]	Content (mg g^{-1})
Zhexiandou No. 4	WT	Colorless	Seed	22.23 ± 0.95
	WT	–	Root	0.11 ± 0.03
	WT	–	Stem	Below detection level
	WT	–	Leaf	Below detection level
Gm-lpa-ZC	LPA	Blue	Seed	9.75 ± 0.12
	LPA	–	Root	0.12 ± 0.04
	LPA	–	Stem	Below detection level
	LPA	–	Leaf	Below detection level
Gm-lpa-ZC × Zhexiandou No. 4 $F_{2:3}$ lines				
Line #1	WT	Colorless	Seed	9.78 ± 0.37
Line #3	WT	Colorless	Seed	7.25 ± 0.25
Line #9	WT	Colorless	Seed	8.63 ± 0.33
Line #65	WT	Colorless	Seed	6.16 ± 0.47
Line #67	WT	Colorless	Seed	7.82 ± 0.24
Line #50	LPA	Blue	Seed	16.87 ± 0.53
Line #51	LPA	Blue	Seed	18.20 ± 1.07
Line #54	LPA	Blue	Seed	19.93 ± 0.66
Line #57	LPA	Blue	Seed	16.50 ± 0.44
Line #59	LPA	Blue	Seed	15.58 ± 1.27

WT wild type, *LPA* low phytic acid type

[a] Determined through allele-specific CAPS marker analysis of *GmIPK1* (see Fig. 2)

[b] Determined colorimetrically according to Wilcox et al. (2000)

[c] Root, stem and leaf tissues were dissected from 40-day-old seedlings

Results

Characterization of ZC-*lpa* mutant and its progeny

In colorimetric assays, ZC-*lpa* showed a typical blue color, while the WT cultivars (e.g., Zhexiandou No. 4 and Zhechun No. 3) appeared to be colorless, which represent a high and low Pi in seeds, respectively (Table 2). For the progeny test, if all six $F_{2:3}$ seeds showed a high Pi level, the corresponding F_2 plants were classified as homozygous LPA type, while those all having normal Pi were classified as homozygous WT, and the remaining classified as heterozygous. In such a way, 480 LPA and 500 WT homozygous plants were identified, respectively, in the F_2 population of ZC-*lpa* × Zhexiandou No. 4 (Table 3). PA measurement of randomly selected homozygous LPA and WT $F_{2:3}$ lines and the two parents showed that seeds with the high Pi phenotype corresponded well to a low PA content (Table 2). For vegetative tissues, a small but similar amount of PA was detected in the roots of ZC-*lpa* and Zhexiandou No. 4, while the level of PA in leaf and stem was below detection.

Fine mapping of the ZC-*lpa* mutation

Microsatellite markers Satt416 and Satt168 which were previously identified to be linked with the ZC-*lpa* mutation (Yuan et al. 2007) were non-polymorphic between ZC-*lpa* and Zhexiandou No. 4. Because Satt168 is known at the genomic position of ~8.2 Mbp (Phytozome, V7) and the ZC-*lpa* mutation is positioned toward to the chromosome end, new microsatellite markers were searched, each 10 kb, in the genomic region of 4.9–8.2 Mbp. Among the 330 microsatellites identified in this region, 9 were polymorphic between ZC-*lpa* and Zhexiandou No. 4 and were used for genotyping of 980 homozygous F_2 plants, which were identified by Pi assay of $F_{2:3}$ seeds (Table 3). The decreasing number of recombinant plants toward PSM226 (Table 3) indicated that the LPA mutation was closest to PSM225 and PSM226, with a genetic distance of ~0.8 cM. Attempts to further delimit the LPA mutation did not succeed due to failure of identifying new polymorphic microsatellite markers between ZC-*lpa* and Zhexiandou No. 4.

Identification and analysis of soybean IPK1 orthologs

Because ZC-*lpa* has the feature of mutants with a defective inositol polyphosphate kinase, a search was performed for soybean IPK1 genes in the NCBI databases. One soybean deduced protein sequence (ABU93829.1) and one mRNA sequence (1,371 bp, EU033956.1), annotated as a putative IPK1, were identified, both submitted by Stiles AR and Grabau EA in 2007. The cDNA sequence was blast against the soybean draft genome sequences in the Phytozome databases and seven chromosome regions were identified with different degrees of similarity. Among them,

Table 3 Primer sequences and genomic positions of polymorphic microsatellites and genotyping of homozygous low phytic acid and wild-type F_2 plants of *Gm-lpa*-ZC × Zhexiandou No. 4

Microsatellite markers	Genomic position (bp)[a]	Primer sequence (5′–3′; F: forward; R: reverse)[a]	Number and microsatellite genotypes of F_2 plants					
			Homozygous LPA plants[b]			Homozygous WT plants[b]		
			I	II	III	I	II	III
PSM38	7 819 097-7 819 166	F: TCGGATGATGTTGAAGTC	425	9	46	12	441	47
		R: GTTTCACATAAAGCAGTT						
PSM96	7 232 803-7 232 884	F: TTATTTGACCGAGTTGTG	424	8	48	10	442	48
		R: GATCCGTCTTGCCTTATT						
PSM117	7 029 743-7 029814	F: ATGTACTTGAACCGTAATG	441	4	35	7	454	39
		R: GACTTGAAATGCCTCTTA						
PSM126	6 933 046-6 933 111	F: GCAACTTGCTATCGAAAT	448	2	30	3	464	33
		R: AAGGTCTCCAGGACTAAA						
PSM163	6 569 065-6 569 203	F: AGTTAGATCCAAAATCAC	455	1	24	3	473	24
		R: GTTGCTAATGCTGTCATA						
PSM219	6 000 374-6 000 437	F: TAGGCTGGCCGCTGTAAT	461	0	19	1	477	22
		R: TCGGGTTGACTTGGAAAA						
PSM224	5 957 861- 957 870	F: GTAAATTGTAACATCCTA	467	0	13	1	484	15
		R: TTCTTATACTAATCACCT						
PSM225	5 955 224-5 955 233	F: ATTGCTTAACTTGCTTTG	466	0	14	0	485	15
		R: TCTCCTTCCAATTAGTGC						
PSM226	5 945 758-5 945 765	F: TGGCAGTGTAGGTAGTGG	468	0	12	0	490	10
		R: GAGCAGGTAATTGGTGGA						

[a] The genomic positions of simple sequence repeat in each microsatellite marker were based on the genome of Williams 82; microsatellite markers were identified and primers were designed according to Phytozome V7 (http://www.phytozome.net/)

[b] I, II and II, respectively, stand for genotypes of homozygous *Gm-lpa*-ZC, homozygous Zhexiandou No. 4 and heterozygous ones at each microsatellite locus

Glyma14g07880 was most similar to the query cDNA sequence and shared 98.7 % identical nucleotides, while the similarity of EU033956.1 to the other six regions was rather low. Because only *Glyma14g07880* was located in the mapped region, it is considered as a candidate gene for the ZC-*lpa* mutation.

According to Phytozome V7, *Glyma14g07880* is physically positioned at 5,949,382–5,952,505 bp of chromosome 14, which is nicely located between SSR PSM225 (5.955 Mbp) and PSM226 (5.945 Mbp). *Glyma14g07880* is annotated to be an inositol penta*kis*phosphate 2- kinase gene (hereafter *GmIPK1*) and has seven exons and six introns (Fig. 1) with a transcript (*Glyma14g07880.1*) of 1,541 nucleotides. For identifying mutation(s) in *GmIPK1*, a fragment of ~3.3 kb encompassing the whole *GmIPK1* gene was PCR amplified using primer pair SQ1 (Fig. 1). Fragments of expected size were cloned for both Zhechun No. 3 and ZC-*lpa* (Fig. 1S), but sequencing revealed a single G → A mutation in ZC-*lpa* compared with Zhechun No. 3 at the position of 1,520 bp, while the *GmIPK1* sequences of Zhechun No. 3 and Williams 82 were identical (Fig. 2S).

The G → A mutation abolished the restriction site G′TAC of Csp61 in ZC-*lpa* (ATAC), hence enabling the development of a CAPS marker for differentiating plants with different genotypes at this locus (Fig. 2). Genotyping of homozygous LPA and WT F_2 plants of ZC-*lpa* × Zhexiandou No. 4, identified above based on $F_{2:3}$ seed Pi level assay, indicated that the CAPS marker was completely co-segregated with Pi phenotype and hence strongly suggested it was this G → A mutation that caused the LPA phenotype of ZC-*lpa*.

Transcription of *GmIPK1*

Based on information of *GmIPK1* available in the Phytozome databases, this point mutation is located in the 5′ terminal of intron 5; hence, it would disrupt the highly conserved 5′ donor splice-site sequence (Fig. 1). The effect of this mutation on pre-mRNA splicing was first analyzed by

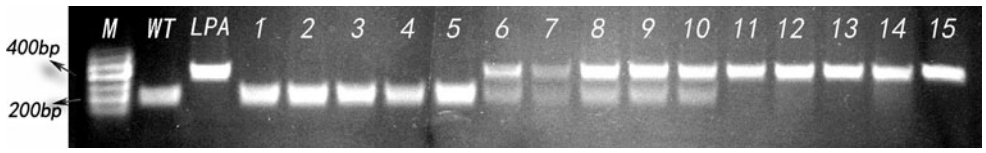

Fig. 2 Genotyping of F_2 plants of *Gm-lpa*-ZC × Zhexiandou No. 4 and their parent for the *GmIPK1* gene using CAPS marker (see Table 1). PCR products were digested by Csp61 and separated on 1.5 % agarose gel. *Lane M* DNA ladders; *WT* and *LPA* represent Zhexiandou No. 4 and *Gm-lpa*-ZC, respectively. *Lanes 1–5* stand for homozygous wild-type F_2 plants (corresponding to Line #1, #3, #9, #65 and #67, respectively, in Table 2). *Lanes 6–10* represent heterozygous plant lines: #2, #8, #39, #42 and #43. *Lanes 11–15* represent homozygous LPA lines (corresponding to Line #50, #51, #54, #57 and #59, respectively, in Table 2)

RT-PCR using primer pairs RT3F/R and RT6F/R. No fragment was amplified from ZC-*lpa* but from Zhechun No. 3 by RT6F/R (data not shown) while the amplicon of ZC-*lpa* by RT3F/3R was ~100 bp shorter than that of Zhechun No. 3 (Fig. 3a). These results pointed that the G → A mutation disrupted the proper splicing and the fifth exon in the mRNA might have been excluded. To test the relationship of shortened cDNA and the LPA phenotype in the progeny of ZC-*lpa*, homozygous LPA ($n = 95$) and WT ($n = 104$) F_2 plants of ZC-*lpa* × Zhexiandou No. 4 were genotyped for *GmIPK1* using RT-PCR with primer pair RT3F/R. Results confirmed that the LPA phenotype co-segregated with shortened cDNA of *GmIPK1* (data not shown).

To have a full view of gene transcription, the full-length cDNA of *GmIPK1* was amplified using primer pairs of RT1F/R (Fig. 1S). Sequencing showed that the entire fifth exon (111 bp) of *GmIPK1* was excluded in ZC-*lpa* compared with Zhechun No. 3 (Fig. 3c), while Zhechun No. 3 and Williams 82 have identical full-length cDNA sequences (Fig. 3S).

Semi-quantitative RT-PCR analyses revealed that *GmIPK1* was expressed in leaves, stems and roots of 14-day-old seedlings and flowering inflorescences, but in much lower levels than in developing seeds (e.g., 20 DAF, Fig. 3b). No obvious differences were observed between ZC-*lpa* and Zhechun No. 3 among those tissues (Fig. 3b).

Quantitative real-time RT-PCR analyses demonstrated that *GmIPK1* expression increased with seed development from 7 to 24 DAF and started decreasing later on in both Zhechun No. 3 and ZC-*lpa* (Fig. 3d). The relative expression levels of ZC-*lpa* were lower than those of Zhechun No. 3 at the early development stages with RQ = 0.72–0.87 at 7–17 DAF, but higher at later stages (RQ = 1.11–1.74). The relatively higher expression level of *GmIPK1* at later stages versus that at 7 DAF in ZC-*lpa* compared with those of Zhechun No. 3 (Fig. 3d) is hence mainly due to its lower expression level at 7 DAF in ZC-*lpa*.

Homologs of *GmIPK1* and their expression in developing seeds

Blast of the soybean genome indicated there were two homologous genes of *GmIPK1*, namely *Glyma04g03240* and *Glyma06g03310*. Semi-quantitative RT-PCR analyses showed that the expression level of both genes was much lower than *Actin* in developing seeds in general and *Glyma06g03310* in particular (Fig. 4). For *Glyma04g03240*, little expression was observed in seeds of 10 DAF, but the expression became apparent in seeds of 15 DAF and remained almost unchanged afterward; no obvious differences in expression were observed between Zhechun No. 3 and ZC-*lpa* (Fig. 4). For *Glyma06g03310*, its temporal expression mode in developing seeds was quite different from that of *Glyma04g03240*: it was mainly expressed in seeds of 15 DAF, only little expression was observed in seeds of 10 DAF and of 20–42 DAF in Zhechun No. 4 (Fig. 4). The overall expression pattern of *Glyma06g03310* in ZC-*lpa* was not different from Zhechun No. 3, but increases of expression were observed in seeds of 10, 15 and 20 DAF, particularly in seeds of 15 DAF (Fig. 4).

No experiment was performed for the direct comparison of expression between *GmIPK1* and its two homologs, but semi-quantitative RT-PCR of *GmIPK1* in seeds of 20 DAF was performed using *Actin* as internal control (Fig. 3b). By comparing results of Figs. 3b and 4, it is safe to conclude that *GmIPK1* had an expression level higher than its two homologs in seeds of 20 DAF. Since it expressed even at higher levels in seeds at stages later than 20 DAF (Fig. 3d), it should be safe to conclude that *GmIPK1* had higher expression levels in developing seeds in general than its two homologs, particularly in seeds older than 20 DAF.

Discussion

The LPA trait of the ZC-*lpa* mutant has the potential for breeding high-yielding LPA soybean cultivars, because its LPA mutation has no negative effect on other agronomic traits (Yuan et al. 2007) but has beneficial effects on some quality characters (Frank et al. 2009a, b; Yuan et al. 2009). In the present study, a single G → A mutation of the soybean IPK1 ortholog gene, *Glyma14g07880* (*GmIPK1*), was detected and its entire fifth exon was excluded in ZC-*lpa*. The G → A mutation and the exclusion of the fifth exon in the *GmIPK1* transcripts are highly likely to be the underlying genetic basis of the LPA phenotype of *ZC-lpa*.

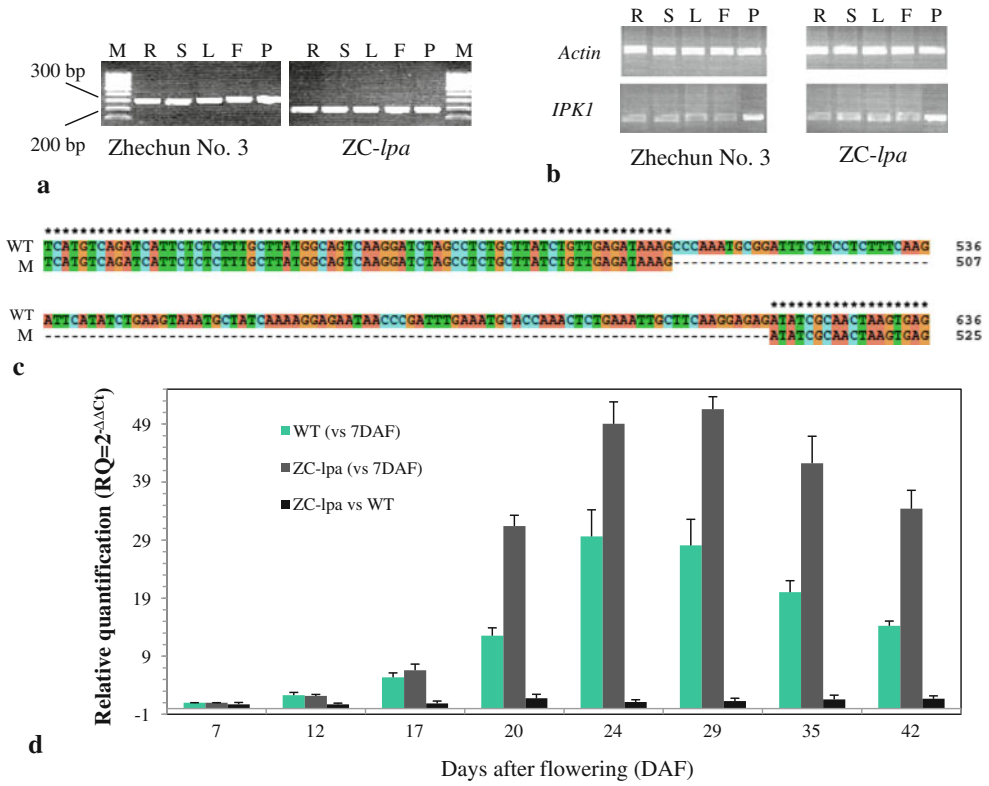

Fig. 3 Expression of *GmIPK1* gene in various tissues of the low phytic acid mutant *Gm-lpa*-ZC-2 (ZC-*lpa)* and it wild-type progenitor Zhechun No. 3 (WT). **a**, **b** Amplification of *GmIPK1* cDNA via RT-PCR using primer pair RT3F/R (**a**, 40 cycles) and RT1F/R (**b**, semi-quantitative RT-PCR with *Actin* as control, 28 cycles), respectively. *M* DNA ladder; *R*, *S*, *L*, *F*, *P* stand for root, stem, leaf, flower and seed at 20 days old, respectively. **c** Alignment of partial *GmIPK1* cDNA sequences between WT and ZC-*lpa* (M) across exon 4 to exon 6; the entire fifth exon is lost in ZC-*lpa* as shown with *dotted line*. **d** Quantitative real-time PCR analysis of *GmIPK1* in developing seeds; relative expression levels (RQ = $2^{-\Delta\Delta C_t}$) are shown for both WT and ZC-*lpa* compared with their respective expression levels at 7 DAF (vs. 7 DAF), as well as for ZC-*lp*a versus WT at each developing stage, following normalization with *Actin* gene control. Standard deviation is indicated by *error bars*

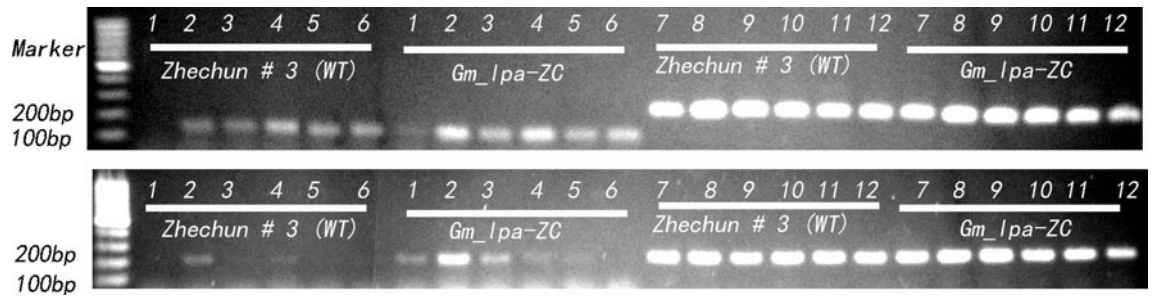

Fig. 4 Semi-quantitative RT-PCR analyses of *Glyma04g03240* (*upper panel*, *lanes 1–6*, 25 cycles) and *Glyma06g03310* (*lower panel*, *lanes 1–6*, 30 cycles) of soybean cultivar Zhechun No. 3 and its low phytic acid mutant *Gm-lpa*-ZC using gene *Actin* as control (*lanes 7–12*). *Lane numbers* of *1*, *2*, *3*, *4*, *5* and *6* for *Glyma04g03240* and *Glyma06g03310* and of 7, 8, 9, 10, 11 and 12 for *Actin* represent seeds at 10, 15, 20, 27, 35 and 42 days after flowering (DAF), respectively

A CAPS marker also was developed for the *GmIPK1* mutation for marker-assisted selection of the LPA trait in breeding programs.

Soybean orthologs of IPK1

A search of the soybean genome (Phytozome, V7) identified three IPK1 orthologs, namely *Glyma14g07880* (*GmIPK1)*, *Glyma04g03240* and *Glyma06g03310*. Although quite different at the DNA level, their transcripts are similar to each other encoding peptides of similar length (456, 457 and 457 aa, respectively; Fig. 4S) and with high degree of identity: Glyma04g03240 and Glyma06g03310 share 96.5 % identical aa, while having 80.3 and 79.4 % aa identical to GmIPK1, respectively. The number of IPK orthologs in soybean is smaller than in *Arabidopsis*, where a number of

IPK1 genes have been identified (Sweetman et al. 2006), but it is higher than in maize where only two duplicated members (*ZmIPK1A* and *ZmIPK1B)* were detected (Sun et al. 2007).

In the present study, *Glyma14g07880* (*GmIPK1*) has been proven to be expressed in all tissues examined (Fig. 3b), but preferentially in developing seeds of 20 DAF and older (Fig. 3d). An electronic search for the expression pattern of *GmIPK1* in the database of SoySeq (http://www.soybase.org/soyseq/RNA) showed similar results: *GmIPK1* has variable levels of expression in different tissues with digital expression counts (DEC) ranging from 4 to 21 in vegetative tissues and from 61 to 225 in developing seeds of 25 DAF and older. However, the increase of expression (as reflected by the DECs) in developing seeds recorded in the database was much slower than that observed in the present study. For example, the DEC is only 5 for seeds of 21 DAF and the expression peak (of DEC 225) was recorded for seeds of 35 DAF in the database, but in the present study the expression of seeds 20 DAF already reached to half of the maximum and the expression peaks were observed in seeds of 24–29 DAF (Fig. 3d). These discrepancies may be explained by the differences in growth conditions (higher temperature and hence faster seeding filling in the present study) and different cultivars used in the two experiments.

The overall expression level of both *Glyma04g03240* and *Glyma06g03310* in developing seeds was generally quite lower (Fig. 4). Interestingly, the expression of these two genes seems to be complementary: *Glyma06g03310* was mainly expressed in seeds of 15 DAF, while *Glyma04g03240* had low but consistent expression in developing seeds of 15 DAF and older (Fig. 4). Sharp expression increase of *Glyma06g03310* was observed in ZC-*lpa* seeds of 15 DAF, and a general increase of expression in ZC-*lpa* seeds of 12 DAF and older compared with Zhechun No. 3 (Fig. 4); however, further investigations are needed for clarifying its implication. Comparison of separately performed (semi-)quantitative RT-PCR analyses using *Actin* as a common control indicated that *GmIPK1* had higher expression level than its two homologs in seeds of 20 DAF (Figs. 3b, 4) and older (Fig. 3d).

The high-level expression of *GmIPK1* in developing seeds and low level in vegetative tissues suggest that GmIPK1 may function mainly in seed PA biosynthesis. The low but similar PA contents detected in roots of ZC-*lpa* and Zhechun No. 3 (Table 2) suggested that GmIPK1 did not play a major role in PA biosynthesis in roots.

The low-level expression of *Glyma04g03240* and *Glyma06g03310* indicated that they could also contribute to PA biosynthesis in seeds, but to a lesser degree than GmIPK1, which may also explain the remaining PA biosynthesis in ZC-*lpa* seeds.

The main IPK1 motifs and effect of ZC-*lpa* mutation

The 1,3,4,5,6-penta*kis*phosphate 2-kinase (IPK1) is a family of enzymes that catalyzes the synthesis of $InsP_6$ from $InsP_5$ and ATP; it is a distant IPK member and is the only enzyme that transfers a phosphate group to the axial 2-OH of the *myo*-inositide (Gozalez et al. 2010). The molecular structural features of AtIPK1 have been fully deciphered recently (Gozalez et al. 2010). It has six important motifs that are highly conserved among plants: Motif I, RxxMHQxLK Motif, Motif II, LDxLDIEGx4Y Motif, Motif III and Motif IV (Fig. 4S). The single G → A point mutation in ZC-*lpa* caused a 37 aa deletion and disrupted Motif I and RxxMHQxLK Motif (Fig. 4S), which would in no doubt affect the functionality of GmIPK1 and thus reduced the biosynthesis of PA in ZC-*lpa*.

The reduction of PA in ZC-*lpa* is accompanied by the increase of Pi and lower inositol polyphosphates such as $InsP_5$, $InsP_4$ and $InsP_3$ (Yuan et al. 2007; Frank et al. 2009b). This is consistent with the function of GmIPK1: when less is transformed into $InsP_6$, more $InsP_5$ is accumulated, and the same for $InsP_4$ and $InsP_3$. Such phenotype had also been observed in maize when *ZmIPK* was mutated (Shi et al. 2003).

ZC-*lpa* seeds still accumulate PA at about 50 % of the WT level. The remaining ability to produce PA in the mutant might be explained by the following. First, the mutant GmIPK1 may still have some functionality though two conserved motifs were disrupted. Second, the other two IPK1 genes, *Glyma06g03310* and *Glyma04g03240*, may also play a role in seed PA biosynthesis. As observed in the present study and reported in the SoySeq database, both genes were expressed at certain stages of developing seeds. The significant increase of expression of *Glyma06g03310* in developing seeds of 15 DAF of *ZC-lpa* further indicates that they could contribute to the 2-kinase activity, particularly when *GmIPK1* is not properly functioning. Third, other kinase genes and alternative pathway(s), yet unknown, may exist for PA biosynthesis. For example, a barley multifunctional inositol phosphate kinase of the ATP-grasp superfamily can phosphorylate $Ins(1,2,3,4,5)P_5$ into PA (Josefsen et al. 2007).

Effect of splice-site mutation on pre-mRNA splicing

Four intron-defining splicing signals are important for pre-mRNA splicing (see review, Reddy 2007). These include (a) a consensus sequence at the 5′ donor site with a conserved GU dinucleotide, (b) another consensus sequence at the 3′ acceptor site with a conserved AG dinucleotide, (c) a polypyrimidine tract at the 3′ end of the intron, and (d) a branch point (CURAY, where R is purine and Y is

pyrimide). Depending on the nature of splice-site mutations and in certain cases the existence of an alternative splicing site in neighboring exon and intron, splice-site mutations could result in exon skipping, new exon generation or intron retention (Nakaik et al. 1994). There have been dozens of splice-site mutations reported in plants, but to our knowledge all 5′ donor splice-site (GT) mutations are substitutions of the "G" by other nucleotides, predominantly by "A". In soybean, a few examples of splice-site mutations have been reported. For example, a G → A transition at the 5′ donor splice-site of the sixth intron of *GmFAD3A* (Bilyeu et al. 2005) and of the fifth intron of *GmFAD 3B* (Reinprecht et al. 2009), and a in the low linolenic acid mutant line RG10, and a G → A transition at the 3′ acceptor splice-site of the fourth intron of *GmFAD 3B* (Bilyeu et al. 2006), all led to a low linolenic acid concentration in soybean seeds. Also of interest is that Sun et al. (2007) reported that there are a variety of alternative splicing products of the *ZmIPK1A* gene in maize leaves and seeds, but they are not related to any disruption on PA biosynthesis.

While the majority of mutations induced by chemical mutagenesis (e.g., EMS) are point mutations (Cooper et al. 2008), most if not all mutations generated by gamma rays are known to be deletions of small (one to a few nucleotides, Xu et al. 2009) or large (e.g., ∼1.4 kb, Zhao et al. 2008) size. The *lpa-ZC* mutant was identified in the M_2 population of Zhechun No. 3 after gamma rays mutagenesis (Yuan et al. 2007); hence, the identification of a G → A was somehow not expected. However, a previous study also identified a C → T substitution that underlies the LPA phenotype of KBNT *lpa1-1* (Kim et al. 2008b), which was developed using gamma radiation (Larson et al. 2000). Our finding further indicated that gamma radiation might also result in nucleotide changes (transition or substitution), though more carefully designed experiments are needed before a solid conclusion can be made.

LPA germplasm has now become available in main crop species and several LPA genes have been either cloned or mapped; therefore, breeding of yield-competitive LPA varieties has become a top priority in this field. In soybean, ZC-*lpa* is the only known mutant that has no negative effect on seed viability and yield traits and thus is expected to be an excellent source of the LPA trait (Yuan et al. 2007). Identification of the most likely causative gene and the basic mutation, and consequently the development of the CAPS marker, will doubtlessly facilitate its use in such breeding programs.

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