Novel allelic variant of \textit{Lpa1} gene associated with a significant reduction in seed phytic acid content in rice (\textit{Oryza sativa} L.)

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Abstract

In plants, \textit{myo-inositol-1,2,3,4,5,6-hexakisphosphate} (InsP\textsubscript{6}), also known as phytic acid (PA), is a major component of organic phosphorus (P), and accounts for up to 85\% of the total P in seeds. In rice (\textit{Oryza sativa} L.), PA mainly accumulates in rice bran, and chelates mineral cations, resulting in mineral deficiencies among brown rice consumers. Therefore, considerable efforts have been focused on the development of low PA (LPA) rice cultivars. In this study, we performed genetic and molecular analyses of \textit{OsLpa1}, a major PA biosynthesis gene, in Sanggol, a low PA mutant variety developed via chemical mutagenesis of Ilpum rice cultivar. Genetic segregation and sequencing analyses revealed that a recessive allele, \textit{lpa1-3}, at the \textit{OsLpa1} locus (Os02g0819400) was responsible for a significant reduction in seed PA content in Sanggol. The \textit{lpa1-3} gene harboured a point mutation (C623T) in the fourth exon of the predicted coding region, resulting in threonine (Thr) to isoleucine (Ile) amino acid substitution at position 208 (Thr\textsubscript{208}Ile). Three-dimensional analysis of \textit{Lpa1} protein structure indicated that \textit{myo-inositol} 3-monophosphate [Ins(3)P\textsubscript{1}] could bind to the active site of \textit{Lpa1}, with ATP as a cofactor for catalysis. Furthermore, the presence of Thr\textsubscript{208} in the loop adjacent to the entry site of the binding pocket suggests that Thr\textsubscript{208}Ile substitution is involved in regulating enzyme activity via phosphorylation. Therefore, we propose that Thr\textsubscript{208}Ile substitution in \textit{lpa1-3} reduces \textit{Lpa1} enzyme activity in Sanggol, resulting in reduced PA biosynthesis.

Introduction

In most cereal crops, \textit{myo-inositol-1,2,3,4,5,6-hexakisphosphate} (InsP\textsubscript{6}), also known as phytic acid (PA), is considered a major source of phosphorus (P) available in the form of phytate, and accounts for 65–85\% of the total P in seeds [1]. Monogastric animals poorly digest PA, as they lack the phytase enzyme, which is responsible for the release of phosphate residues [2]. PA is...
an efficient chelator of mineral cations, such as zinc (Zn$^{2+}$), iron (Fe$^{2+}$), magnesium (Mg$^{2+}$), potassium (K$^{+}$), and calcium (Ca$^{2+}$), in the nutritional tract. Because of these attributes, PA is considered as an antinutrient [3, 4]. Hence, there is a need to develop low PA (LPA) crop cultivars to maximize the nutritional benefits of grains.

Mutants associated with the LPA phenotype have been identified in several crop plants including maize (Zea mays) [5, 6], barley (Hordeum vulgare) [7], soyabean (Glycine max) [8], rice (Oryza sativa) [9], and wheat (Triticum aestivum) [10]. Although, LPA mutants are identified primarily on the basis of percentage reduction of PA and high inorganic P (P$\text{$_i$}$) content in seeds [5, 11], some mutants show a significant accumulation of myo-inositol and inositol phosphate [Ins(1,3,4)P$\text{$_5$}$] intermediates in seeds [12, 13].

Previously, the LPA phenotype of seeds has been associated with reduced agronomic performance of mutant crop plants in the field [5, 14]. It is important to understand the genetic and molecular bases of reduced agronomic performance of LPA mutants for effective utilization in breeding programs. In addition, studies show that climate change and elevated carbon dioxide (CO$\text{$_2$}$) levels negatively affect micronutrient bioavailability and total P in grains [15, 16]. Therefore, developing crop cultivars with increased micronutrient bioavailability in seeds and greater adaptability to environmental variations, by reducing the PA content in grains, is an important priority of breeding programs.

PA is biosynthesized via two different routes: lipid dependent and lipid independent [3, 17]. The lipid dependent pathway operates in all plant organs, whereas the lipid independent pathway is predominant only in seeds [13, 17, 18]. In the first step of PA biosynthesis, D-glucose-6-phosphate is converted to myo-inositol 3-monophosphate [Ins(3)P$_1$] by myo-inositol 3-phosphate synthase (MIPS) [19]. This is followed by the sequential phosphorylation of specific inositol to InsP$_6$, through the action of various inositol phosphate kinases (S1 Fig). However, enzymes involved in lipid independent PA biosynthesis, from Ins(3)P$_1$ seem to be complicated and are not well understood [3]. Nevertheless, PA biosynthetic genes encoding other myo-inositol enzyme and inositol phosphate kinases are well documented in major plants [12, 13, 20, 21]. Additionally, biochemical and functional analyses of PA biosynthetic genes encoding Ins monophosphate kinase could address the missing steps in the lipid independent pathway.

Genetic studies of LPA mutants have shown that a single recessive gene is responsible for the LPA phenotype in rice and other crop plants [21–24]. The first lpa gene encoding inositol 1,3,4-trisphosphate 5/6-kinase (ITPK5/6) was identified in maize, and designated as Lpa2. Subsequently, myo-inositol kinase gene Lpa3, and multidrug resistance protein (MRP) ATP binding cassette (ABC) transporter gene Lpa1 were identified [12, 13, 25]. In addition, reduction of PA content in Arabidopsis atipk2β mutant indicates the inositol 1,4,5-tris-phosphate (IPK2) kinase of lipid dependent pathway is also active the seeds [20]. In rice, several mutants with low seed PA content have been reported [14, 21–23,26–29]. The rice OsLpa1 gene has been reported to associated with the reduction in seed PA content and increase in seed P$_i$ content, with little change in the total P content in seeds [22, 30]. OsLpa1 has been found to share sequence homology with Os09g0572200 (OsLpa1 paralog) suggesting possible overlapping or redundant functions [22].

Genetic studies in rice have confirmed that a mutation in the OsLpa1 locus generates the LPA phenotype in seeds. Molecular characterization of LPA mutants has previously revealed three alleles of the OsLpa1 locus, including KBNT lpa 1–1, DR1331–2, and Os-lpa-XQZ-1, responsible for the low PA phenotype of seeds [22, 30]. In the present study, we report a novel allele of OsLpa1, OsLpa1-3, responsible for a significant reduction in the seed PA content in a new lpa rice mutant, Sanggol developed at Kangwon National University, Republic of Korea [31]. Sequence analysis of OsLpa1-3 revealed a point mutation in the gene coding sequence. Our data suggest that this mutation is responsible for the LPA phenotype of Sanggol mutant.

Competing interests: The authors have declared that no competing interests exist.
Material and methods

Plant material

The low PA mutant, Sanggol derived from a japonica rice cultivar Ilpum mutagenized with N-methyl-N-nitrosourea (MNU) [31]. Ilpum was used as the wild type in comparing phenotypic data. Sanggol was crossed with Ilpum to develop F₂ population. Segregation analysis was performed using the F₂ population. Both parent cultivars and F₂ individuals were grown in experimental fields of Seoul National University, Republic of Korea.

Agronomic trait analysis

To characterize agronomic traits, 15 phenotypic observations were recorded during various stages of plant growth, according to the Standard Evaluation System (SES) for rice, 2014. Yield data was obtained from “3.6 m X 3.6 m” plot size. All agronomic data were analyzed using the Student’s t-test in SPSS 16.0 (https://www.ibm.com/analytics/spss-statistics-software) to determine significant differences among low PA mutant, Sanggol, and wild type, Ilpum.

Analysis of Pᵢ and PA content in seeds

Concentrations of Pᵢ and PA in seeds were examined using P³¹ nuclear magnetic resonance (P³¹ NMR) spectroscopy [32], with slight modifications.

Sample preparation. Fine powdered samples (1 g dry weight) of brown rice were thoroughly mixed with 10 mL of 2.4% HCl in 14 mL Falcon tubes. Samples were incubated at room temperature for 16 h on an HB-201SF shaker (HANBAEK Scientific Co) at 220 rpm, and subsequently centrifuged at 1,500 × g (combi 514R, Hanil science Inc.) at 10˚C for 20 min. Crude extracts were transferred to a new 14 mL Falcon tube containing 1 g NaCl, and incubated at 25˚C for 40 min on a shaker at 220 rpm to dissolve NaCl. Samples were allowed to settle at 4˚C for 60 min, and then centrifuged at 1,500 × g at 10˚C for 20 min.

³¹P NMR. For ³¹P NMR spectroscopy, samples were prepared by mixing 450 μL of NaCl treated acid extract with 450 μl of buffer containing 0.11mM EDTA-disodium salt and 0.75 mM NaOH, 40 mg NaOH, and 100 μL D₂O in 1.5 mL microtubes. Sample and standard peaks were obtained on a 600 MHz spectrometer using Advance 600 P³¹ NMR system (Bruker, Germany). PA sodium salt and 85% phosphoric acid were used as external standards for peak identification and further analysis [33, 34]. For internal calibration, 1 mM of phenylphosphonic acid was included in 100 μL D₂O during NMR measurements. All standards were purchased from Sigma-Aldrich, USA.

To determine significant differences in seed PA and Pᵢ contents among parents and F₂ individuals, data were analyzed using the Student’s t-test in SPSS 16.0 (https://www.ibm.com/analytics/spss-statistics-software). Additionally, correlation analysis was performed to assess significant association between seed PA (%) and percentage of grain chalkiness (GC) using parents and 20 homozygous F₂ individuals consisted of mutant, and wild type in SPSS 16.0.

Expression analysis of PA biosynthetic genes

Genes involved in PA biosynthesis and transport were identified from the RAB-DB and from recent studies [25, 35, 36]. The rice microarray database, RiceX-Pro, shows different expression patterns of most of the PA biosynthetic genes in various tissues and organs [37]. To confirm the expression pattern of PA biosynthetic genes, spikelets were harvested from the wild type Ilpum at 5 days after flowering (DAF), and total RNA was extracted using RNaIso Plus (Takara Bio, Japan). The extracted RNA samples were treated with RNase-free recombinant DNase I (Takara Bio, Japan) to eliminate genomic DNA contamination, and first-strand
cDNA was synthesized using M-MLV reverse transcriptase (Promega, USA). The fragments of size 200-550bp were amplified from cDNA samples by reverse transcription polymerase chain reaction (RT-PCR) using myo-inositol 3-phosphate synthase-1 (OsMIPS1), myo-inositol 3-phosphate synthase-2 (OsMIPS2), myo-inositol monophosphate-1 (OsIMP1), myo-inositol monophosphate-2 (OsIMP2), myo-inositol kinase (OsMIK1), low phytic acid 1 (OsLpa1), low phytic acid 1-paralog (OsLpa1-P), inositol 1,3,4-trisphosphosphate 5/6-kinase 1 (OsITPK1), inositol 1,3,4-trisphosphosphate 5/6-kinase 2 (OsITPK2), inositol 1,3,4-trisphosphosphate 5/6-kinase 3 (OsITPK3), inositol 1,3,4,5-trisphosphate 5/6-kinase 4 (OsITPK4), inositol 1,3,4-trisphosphophate 5/6-kinase 5 (OsITPK5), inositol 1,3,4-trisphosphophate 5/6-kinase 6 (OsITPK6), inositol 1,4,5-trisphosphate 1 (OsIpk1), GLFG lethal 1 (OsGLE1), inositol 1,4,5-trisphosphate 2 (OsIpk2), and multidrug resistance protein 13 (OsMRP13) genes-specific primers (Table 1) with the following conditions: initial denaturation at 95˚C for 2 min, followed by 32 cycles of denaturation at 95˚C for 20 s, annealing at 58˚C for 40 s, and extension at 72˚C for 1 min, and a final extension at 72˚C for 5 min. The Actin gene was used as an internal control.

**Sequence analysis**

Genomic DNA and RNA were isolated from young leaves and spikelets of low PA mutant, Sanggol, respectively. cDNA was synthesized with mRNA derived from young leaves and spikelets. Fragments of size 300bp-2000bp were amplified from the coding region and untranslated region (UTR) of 16 PA genes of lipid dependent and independent pathways using gene-specific primers, designed with Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (S1 Table). The PCR products were purified using the DNA Purification Kit (Inclone, Korea), and analyzed with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA). In addition, genome sequences of all 16 PA genes in wild type, Ilpum were retrieved from the genome analysis center, National Instrumentation Center of Environmental Management (NICEM), Seoul National University. (http://nature.snu.ac.kr/rice/). Sequence alignment analysis were performed using the Codon Code Aligner software (Codon Code Corporation, USA).

**Candidate gene analysis**

To confirm nucleotide polymorphisms in candidate genes, validation primers were designed using Primer3 for cDNA sequencing (Table 2). The PCR products were purified using the DNA Purification Kit (Inclone, Korea), and analyzed with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA). Sequences were aligned using the Codon Code Aligner software (Codon Code Corporation, USA). Simultaneously, BLAST search was performed using the predicted amino acid sequences of the candidate genes in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and deleterious amino acid substitutions were predicted using Provean web server with proven scores [38].

**Expression analysis of Lpa and lipid dependent PA biosynthesis genes in a low PA mutant, Sanggol and wild type, Ilpum**

Total RNA was extracted from leaves at 15 days after germination (DAG) to analyze the expression of Lpa and lipid dependent pathway genes, and 5 DAF from spikelets to analyze the expression of OsLpa1 in lpa mutant, Sanggol and wild type, Ilpum. For the expression analysis of OsLpa1 paralog and OsIpk2 genes, total RNA was extracted only from spikelets at 5 DAF. RNA extraction was performed as described above, and cDNA was synthesized with mRNA derived from spikelets of 5 DAF. The CDNA was subjected to RT-PCR using gene-specific primers (Table 3). The Actin gene was used as an internal control.
**Derived cleaved amplified polymorphic sequence (dCAPS) analysis**

Genomic DNAs were isolated from all 96 F2 plants derived from the cross between Sanggol and Ilpum, and subjected to dCAPS analysis. The F2 genotyping primers (Table 2) were

### Table 1. RT-PCR primers used to amplify PA biosynthetic genes.

| Gene ID   | Gene Name | Primer name | Sequence (5'→3') |
|-----------|-----------|-------------|------------------|
| Os03g0192700 | OsMIPS1  | OsMIPS1F     | AGTTGGAACAGGTGGTGGTTG |
| Os10g0369900 | OsMIPS2  | OsMIPS2F     | GAAGAGCAAGTGAGCAAGG |
| Os03g0587000 | OsIMP1   | OsIMP1F     | GTGGATTGAGCAGCAGGAC |
| Os02g0169900 | OsIMP2   | OsIMP2F     | ATCACCACAAATCGGAGG |
| Os07g0507300 | OsMIK1   | OsMIK1F     | TCTACTGGGACAGTGGAGAG |
| Os02g0819400 | OsLpa1   | OsLPA1F     | TATGGGGACACTGGAGATG |
| Os09g0572200 | OsLpa1-P  | OsLPA1-P F  | GGGCTGATGTTCCACCTAAT |
| Os10g0103800 | OsITPK1  | OsITPK1F     | ACAAGAGATGAGCAGAAAGT |
| Os03g0230500 | OsITPK2  | OsITPK2F     | TCTGTCCTCAGGAAATGT |
| Os03g0726200 | OsITPK3  | OsITPK3F     | CACACTCTCAAGAAGCT |
| Os02g0466400 | OsITPK4  | OsITPK4F     | ACCGAGGGGTTGGCACTAA |
| Os10g0576100 | OsITPK5  | OsITPK5F     | CACGTCCTCAAGAAGCT |
| Os09g0518700 | OsITPK6  | OsITPK6F     | GCAAAACGAGGTGCAAGATA |
| Os04g0661200 | OsIPK1  | OsIPK1F     | CAACCAGCCCACAAGCTGAT |
| Os02g0596100 | OsGLE1  | OsGLE1F     | AGACGGGCGGCTTGGCAC |
| Os02g0523800 | OsIpK2  | OsIPK2F     | TCTTCTCAAGCCCTCCTCA |
| Os03g0142800 | OsMRP13  | OsMRP13F     | CCTATGTGCTATGGTGGAGAG |
| Os03g0836000 | Actin   | Actin 4     | AGCGAGTTGACGACGAGCA |

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### Table 2. Primers used for validating cDNA sequences and genotyping the F2 population.

| Analysis      | Primer name | Sequence (5'→3') | Amplicon size |
|---------------|-------------|------------------|---------------|
| cDNA validation | Lpa1-3 F    | GCCATGCTTCAAGATTCACG | 1,186 bp |
|               | Lpa1-3 R    | TGAACATCCTCTGGGAAACC | |
| Genotyping    | Lpa1-3 F    | AGCATGCTTCAAGATTCACG | Homozygous wild type (192 bp), homozygous mutant (174 bp), heterozygous (both 192 bp and 174 bp) |
|               | Lpa1-3 R    | AGCATGCTTCAAGATTCACG | |

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designed using dCAPS 2.0 (http://helix.wustl.edu/dcaps/) to validate a single nucleotide substitution (C to T) in the *OsLpa* gene in Sanggol, which generates a TaqI restriction site (TCGA) in the amplified PCR product. PCR was performed using the following conditions: initial denaturation at 95˚C for 2 min, followed by 32 cycles of denaturation at 95˚C for 20 s, annealing at 58˚C for 40 s, and extension at 72˚C for 30 s, and a final extension at 72˚C for 1 min. The amplified PCR product was digested with TaqI restriction endonuclease (Promega, USA), and separated on 3% agarose gel.

### Multiple sequence alignment and phylogenetic analysis

Amino acid sequences of the Lpa superfamily were obtained from the NCBI protein database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), and subjected to multiple sequence alignment using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). Multiple sequence alignment editing, visualization, and analysis was performed using Jalview 2.10.4 (http://www.jalview.org/). The Lpa and other superfamily proteins obtained from the NCBI protein database were used for phylogenetic analysis. Neighbour-joining tree was constructed using MEGA 7 [39] with 1,000 bootstrap replicates.

### Biocomputational analysis

A three-dimensional (3D) model of Lpa1 protein was produced under the intensive mode of the Phyre2 server [40] (www.sbg.bio.ic.ac.uk/phyre2/html/). The ligand and cofactor were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) for protein ligand analysis. Furthermore, auto docking and 3D model were analyzed using the CLC drug discovery workbench 4.0 (QIAGEN, Denmark). Putative phosphorylation sites were predicted with the GPS 3.0 server (http://gps.biocuckoo.org/) using high cut-off values ranging from 1.36 to 17.72.

### Results

#### Agronomic characterization of low PA mutant, Sanggol and wild type, Ilpum

Analysis of agronomic traits demonstrated a significant reduction in plant height (cm), number of productive tillers, culm length (cm), the first internodal length (cm), 1,000-grain weight (g), number of spikelets per panicle, number of panicles per plant, and yield components of a

| Gene ID     | Transcript       | Primer name | Primer sequence (5’—3’) | Amplicon size |
|-------------|------------------|-------------|--------------------------|---------------|
| Os02g0819400 | OsLpa1.1         | *lpa1.1 F*  | TATGGGACTACCGGATGC       | 192 bp        |
|             |                  | *lpa1.1 R*  | GAGCAACTGCACAGGCTG       |               |
| OsLpa1.2    |                  | *lpa1.2 F*  | TATGGGACTACCGGATGC       | 445 bp        |
|             |                  | *lpa1.2 R*  | GAGCAACTGCACAGGCTG       |               |
| OsLpa1.3    |                  | *lpa1.3 F*  | ATCTTCCGGATACGGCAT     | 200 bp        |
|             |                  | *lpa1.3 R*  | TGCCAGCTAATTTCTCCTATC   |               |
| Os09g0572200 | OsLpa1 paralog   | *OsLPA2F*   | CGGCTGATGCTCCACCATAT    | 236 bp        |
|             |                  | *OsLPA2R*   | TGAGCGCTTCTCTCAATGTG    |               |
| Os02g0523800 | OsIpk2-0         | *OsIPK2F*   | CTCTTCTACAGGCCCCTCA     | 291 bp        |
|             |                  | *OsIPK2R*   | GAGCCACTTGGGACGTA       |               |
| Os03g0836000 | OsActin          | *Actin 4*   | AGCCAGTCAGTACAGAGCA     | 194 bp        |
|             |                  | *Actin 5*   | GAGCAATTTCAATGCACAGCA   |               |

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low PA mutant, Sanggol compared with a wild type, Ilpum (Table 4 and Fig 1A). By contrast, the number of days to 50% flowering was significantly higher in Sanggol than in Ilpum, indicating delayed flowering in the mutant. In addition, Sanggol exhibited significantly higher percentage of chalky grains compared with the wild type. However, no significant differences were observed between mutant and wild type in morphological characteristics, such as secondary internodal length, grain length, grain width, panicle length, and spikelet fertility (Fig 1B and Fig 1C). Overall, these data indicate that a low PA mutant, Sanggol shows poor agronomic performance with respect to the flowering time, yield, and yield components compared with the wild type, Ilpum.

### Determination of PA and P<sub>i</sub> content in seeds

To quantify PA and P<sub>i</sub> content in seeds, brown rice extracts of Sanggol and Ilpum were analyzed via 31P NMR spectroscopy. Results showed that PA contents were significantly reduced (49% reduction), and P<sub>i</sub> content was significantly increased in the seeds of Sanggol compared with Ilpum (Table 5). The 31P NMR analysis showed peaks analogous to standard (Fig 2A) for P<sub>i</sub> and PA peak identification. Similarly, P<sub>i</sub> and PA analogous peaks were observed for wild type (WT) (Fig 2B), and mutant (lpa) (Fig 2C).

Additionally, PA and P<sub>i</sub> amounts were also quantified among 96 F<sub>2</sub> individuals using 31P NMR spectroscopy. Segregation analysis revealed that 77 F<sub>2</sub> plants showed the wild type phenotype, whereas 19 F<sub>2</sub> plants showed the mutant phenotype (Table 6), and the phenotype segregation fitted a 3:1 ratio, suggesting that a single recessive allele control the low PA in the seeds of the lpa mutant, Sanggol.

Additionally, correlation analysis among PA (%) and GC (%) using parents and 20 homozygous F<sub>2</sub> individuals revealed that GC had negative significant correlation with PA (r =

![](Fig 1. Phenotypic comparison between low PA mutant, Sanggol (lpa) and wild type (WT), Ilpum. (A) Whole plant. (B) Spikelet. (C) Mature grain.)
Further, homozygous *lpa* individuals in $F_2$ population, and *lpa* mutant, Sanggol showed higher GC (%) compared with homozygous wild types in $F_2$, and wild type parent, Ilpum. Statistical analysis using Student’s $t$-test revealed significant differences for GC (%) between homozygous wild type and *lpa* individuals in $F_2$ population (S5 Fig).

Table 5. Seed PA and $P_i$ content in Sanggol and Ilpum.

| Cultivar | PA $P$ (mg g$^{-1}$) | $P_i$ (mg g$^{-1}$) | Total $P$ (mg g$^{-1}$) |
|----------|----------------------|---------------------|-------------------------|
| Ilpum    | 5.7 ± 0.34           | 0.1 ± 0.04          | 5.85 ± 0.34             |
| Sanggol  | 2.9 ± 0.69           | 1.8 ± 0.10**        | 5.21 ± 0.62             |

Data represent mean ± standard error ($n = 3$). Asterisks indicate the level of significance (*, $P < 0.05$; **, $P < 0.01$) between Sanggol and Ilpum.

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Fig 2. $^{31}P$ NMR spectrum of standard, Ilpum (WT), and the mutant, Sanggol (*lpa*) (A) Reference standard peaks. (B) Wild ‘WT’ (C) *lpa* ‘Mutant’.

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Expression of PA biosynthetic gene and sequence analysis

To identify the gene responsible for reduced PA content in seeds, the candidate gene approach was followed. In rice, PA biosynthesis and accumulation begins after flowering [41, 42], and continues until 25 DAF during seed development [43]. Therefore, we extracted total RNA from 'Ilpum' spikelets at 5 DAF, and subjected it to RT-PCR analysis. Results showed that 15 genes in the PA biosynthesis pathway were expressed at 5 DAF (Fig 3). Further, we amplified and sequenced 16 genes involved in PA biosynthesis from low PA mutant, Sanggol and wild type, Ilpum (S1 Table).

Sequence analysis of PA biosynthetic genes revealed a single nucleotide polymorphism (SNP) in the OsLpa1 gene of lpa mutant, Sanggol (Fig 4A); none of the other PA biosynthetic genes showed mutations in lpa mutant, Sanggol. Previously, the OsLpa1 locus has been mapped to chromosome 2 [11], and narrowed down to a region less than 150 kb using

![Image of RT-PCR analysis](https://doi.org/10.1371/journal.pone.0209636.g003)

Table 6. Segregation and co-segregation analysis of seed PA content among 96 F2 individuals derived from a cross between low PA mutant, Sanggol and wild type, Ilpum.

| Cross            | No. of F2 plants | PA phenotype | dCAPS genotyping |
|------------------|------------------|--------------|------------------|
|                  |                  | High PA     | Low PA | Expected | χ² | P-value† | W | H | M | Expected | χ² | P-value† |
| Sanggol /Ilpum   | 96               | 77           | 19     | 3:1      | 1.38 | 0.23 | 26 | 19 | 51 | 1:2:1     | 1.39 | 0.49 |

†Not significant (P > 0.05).

*Wild: homozygous wild type, H: heterozygous, M: homozygous mutant.

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![Image of RT-PCR analysis](https://doi.org/10.1371/journal.pone.0209636.g003)
Further, the OsLpa1 has been characterized in lpa mutants of rice [22, 30]. The OsLpa1 gene encodes three expressed splice variants in rice [22, 35]. Sequence analysis of the OsLpa1 locus (position +1 to 2,058 bp; Genbank accession number: MH707666) showed a SNP (C623T) in the fourth exon of the largest splice variant, designated as OsLpa1-3.1, in lpa mutant, Sanggol. This SNP corresponds to C53T located in the first exon of splice variants of OsLpa1-3.2 and OsLpa1-3.3 (S2 Fig). Further, sequence analysis of OsLpa1-3.1 cDNA confirmed the presence of lpa1-3 allele in lpa mutant, Sanggol (Fig 4B).

To determine the expression of OsLpa1 splice variants in lpa mutant and wild type, we performed RT-PCR analysis of OsLpa1 gene at 15 DAG using total RNA isolated from leaves and spikelets at 5 DAF. Expression analysis revealed that both OsLpa1-3.1 and OsLpa1-3.2 were expressed at 15 DAG, with slightly different expression patterns, whereas OsLpa1-3.3 showed
no expression at 15 DAG in both lpa mutant, Sanggol and wild type, Ilpum (Fig 5A), indicating that OsLpa1-3.1 and OsLpa1-3.2 may play an important role in seedling growth. At 5 DAF, OsLpa1-3.1 showed strong expression in both lpa mutant, Sanggol and wild type, Ilpum; however, OsLpa1-3.3 exhibited low expression in both mutant and wild types, and OsLpa1-3.2 exhibited no expression in either types, suggesting OsLpa1-3.1 as a candidate transcript responsible for the low PA phenotype of Sanggol mutant. Protein analysis of Lpa1 amino acid sequence predicted deleterious amino acid substitution changes threonine (Thr) to isoleucine (Ile) in OsLpa3.1 (Thr208Ile), with a -5.715 proven score. Similarly, deleterious amino acid substitution changes were observed in OsLpa3.2 and OsLpa3.3 (Thr18Ile), with -5.482 proven scores.

Additionally, existence of the OsLpa1 paralog, reported previously by Kim et al. [22], was investigated at 5 DAF in lpa mutant, Sanggol and wild type using RT-PCR. The OsLpa1 paralog exhibited strong expression in both lpa mutant, Sanggol and wild type (Fig 5B), suggesting that sequence variation in the coding region of OsLpa1 was responsible for the low PA content.

Fig 5. RT-PCR analysis of the Lpa gene family in Sanggol and Ilpum. (A) Expression of OsLpa1 gene at 15 DAG and 5 DAF. (B) Expression of OsLpa1 gene paralog at 5 DAF.

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of Sanggol seeds. In addition, reduction of PA content in Arabidopsis atipk28 mutant indicates the IPK2 kinase of lipid dependent pathway is active the seeds [20]. We also ruled out the possibility for seed PA biosynthesis similar to Arabidopsis in low PA mutant, Sanggol. However, our RT-PCR results showed no expression of Osipk2, a key PA biosynthesis gene in the lipid dependent pathway (data not shown), suggesting that the lipid dependent pathway is not active in low PA mutant, Sanggol or wild type, Ilpum.

Next, we performed multiple sequence alignment of Lpa1 amino acid sequences of Sanggol and other major plant species. Results revealed an amino acid substitution in the conserved kinase domain in lpa mutant, Sanggol (Fig 6A), thus showing the impact of a SNP in gene coding of Lpa1 for low phytic acid in rice.

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Fig 7. dCAPS analysis of lpa1-3 allele in the F2 population derived from a cross between Sanggol and Ilpum. P1, ‘Ilpum’; P2, ‘Sanggol’; W, homozygous wild type; M, homozygous mutant; H, heterozygous.

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sequence. The kinase domain of Lpa1 shows weak homology with that of 2-phosphoglycerate kinase (2-PGK) found in hyperthermophilic methanogens [22]. However, there is structural similarity among the substrates and products of 2-PGK and Lpa1 [45].

Phylogenetic analysis revealed a strong relationship among the kinase proteins in the glycolysis and PA biosynthesis pathways. In contrast, Lpa proteins are clustered with other 2-phosphoglycerate kinases (2-PGK) proteins (Fig 6B).

Co-segregation analysis of low PA phenotype with lpa1-3 allele

A dCAPS assay was applied to determine the co-segregation of lpa1-3 allele with the low PA phenotype (Fig 7). A pair of dCAPS markers amplified a 192 bp PCR product. Digestion of this PCR product with TaqI yielded a 174 bp fragment in Sanggol, but an uncut fragment (192 bp) in Ilpum. Genotyping the F₂ individuals using this dCAPS marker showed a segregation ratio, which was consistent with the expected ratio of 1:2:1 (Table 3). In addition, the dCAPS marker genotype co-segregated with the low PA phenotype in the F₂ population. Statistical analysis using Student’s t-test revealed significant differences in the seed PA (S3 Fig) and P₁ (S4 Fig) contents of Ilpum, Sanggol, and F₂ individuals.

Biocomputational analysis

Structural analysis of Lpa1 using molecular docking of ligand and cofactors showed that Ins(3)P₁ could bind to the active site of Lpa1 protein, with ATP as a cofactor for catalysis (Fig 8A). Detailed view of the 3D protein model showed that Thr residue at amino acid position 208 was located in the kinase loop (Fig 8B) on the outer surface of the protein, adjacent to the entry site of the binding pocket, thus indicating its potential involvement in regulating the enzyme activity of Lpa1 protein. In addition, GPS 3.0 predicted Thr208 residue as a putative phosphorylation site, with a score of 9.66 above the cut-off value of 8.31. In previous studies, biochemical characterization of the regulatory mechanisms of various other metabolic enzymes has shown that amino acid substitutions are responsible for the reduction in enzyme activity of mutant proteins compared with wild type proteins [46, 47]. Altogether, our results suggest that Thr208Ile amino acid substitution regulates the enzyme activity of Lpa1 protein via phosphorylation in lpa mutant, Sanggol.
Discussion

To date, several genes controlling PA biosynthesis have been reported in major crop plants [13, 20, 25, 28, 48–50]. The biosynthesis of PA proceeds via two major routes: a lipid dependent pathway, which operates in all plant tissues, and lipid independent pathway, which operates predominantly in seeds [3, 17]. In rice, molecular characterization of genes encoding MIPS, MIK, Lpa1, ITPK5/6, and IPK1 has revealed association with the low PA phenotype [21–25]. The first step of PA biosynthesis involves the conversion of D-glucose-6-phosphate to Ins(3)P₁ by MIPS [19], which is followed by a series of phosphorylation steps, leading to the formation of InsP₆ (S1 Fig). However, biochemical pathways leading to the conversion of Ins(3)P₁ to InsP₄, and the enzymes involved are very complex and not yet fully understood in plants [3].

Understanding the genetic basis of low PA phenotype is important for developing cultivars with low PA content in seeds. Therefore, we obtained the low PA mutant, ‘Sanggol’ from Kangwon National University, Republic of Korea [31, 46]. In this study, Sanggol showed relatively poor agronomic performance compared with the wild type, Ilpum (Table 4). These results are in agreement with previous studies showing superior agronomic performance of wild type compared with the low PA mutants [5, 14]. Edwards et al. [51] report an association between *Lpa*₁ locus and grain chalkiness in rice. Similarly, GC (%) had negative significant correlation with PA, indicating that the low PA phenotype interacts with grain chalkiness. Thus, results of this study and previous studies suggest that the *lpa* allele plays an important role in determining the yield potential and seed quality of rice.

Phenotypic analysis using P³¹ NMR spectroscopy showed a significant reduction in PA content and an increase in Pᵢ content in Sanggol seeds (Table 5). Expression analysis of PA biosynthetic genes in spikelets of the wild type Ilpum at 5 DAF indicated that 15 genes from the lipid independent pathway were possibly responsible for the low PA content in Sanggol (Fig 3). Our data showed that a point mutation in the *OsLpa1* locus was associated with low PA content in Sanggol seeds. Previous studies have also shown that rice low PA mutants exhibit a reduction in seed PA content because of SNPs [25, 30]. Candidate gene sequencing (Fig 4) and co-segregation analysis (Fig 7 and Table 6) confirmed that a new single recessive allele of *Lpa1*, designated as *lpa1*-3, was responsible for the low PA phenotype of *Ipa* mutant, Sanggol because of a C/T SNP located at nucleotide position 623 in *OsLpa1*, resulting in a single amino acid substitution (Thr208Ile). In a previous study, the *japonica* mutant ‘KBNT *lpa1*-1’ exhibited a 28% reduction in seed PA content because of a SNP (C/G to T/A), resulting in a nonsense mutation at amino acid position 409 whereas the DR1331-2 (*lpa1*-2) mutant showed a 48% reduction in seed PA content because of a single nucleotide deletion (T/A) at position 313, causing a frame shift mutation [22]. In addition, molecular characterization of the *indica* mutant ‘Os-lpa-XQZ-1’ shows the deletion of a 1,475 bp fragment in *lpa1*-1, resulting in a 38% reduction in seed PA content [30].

The *OsLpa1* gene encodes three splice variants, all of which are expressed in seeds, suggesting that these variants play different roles in rice seed development [22, 35]. However, RT-PCR analysis of *OsLpa1* locus revealed that *OsLpa1*-3.1 expression exhibited both vegetative and seed specificity, which indicates a major role of *OsLpa1*-3.1 in PA biosynthesis; however, *OsLpa1*-3.2 and *OsLpa1*-3.3 showed significant and dynamic changes at 15 DAG and 5 DAF, respectively (Fig 5A), suggesting that these variants may play important roles in seedling growth and seed development, respectively. This finding is consistent with a previous study in rice [52]. Additionally, we investigated the expression of *OsLpa1* paralog on chromosome 9 (Os09g0572200), that encodes a protein homology to *OsLpa1*-3 and a IPK2 kinase is specific for the lipid dependent pathway, *OsIpk2* (Os02g0523800), in spikelets at 5 DAF, to provide an
alternative explanation for the low level of PA in lpa mutant seeds. However, expression analysis OsLpa1 paralog gene suggests possible overlapping functions for PA biosynthesis in lpa mutant, Sanggol (Fig 5B).

According to a previous study, OsLpa1 shows a weak homology to P-loop kinase domain of 2-PGK found in Methanothermus fervidus [22]. 2,3-bisphosphoglycerate (2,3-BPG), derived from 2-PGK, is a strong inhibitor of inositol polyphosphate 5-phosphatasers [53]; thus, removing this inhibition may degrade inositol polyphosphate intermediates, causing a reduction in seed PA content in low PA mutants [22, 54]. Based on the structural similarity among substrates and products of OsLpa1 and 2-PGK, it is possible that Lpa1 protein functions as a kinase [3]. Further, sequence analysis search of OsLpa1-3 showed that P-loop NTPase domain super-family is characterized by two ATP/GTP binding motifs, i.e. Walker A [GxxxxGK(S/T)] and Walker B [hhhhEG] thus, indicated OsLpa1-3.1 has both Walker A and Walker B motifs, OsLpa1-3.2 and OsLpa1-3.3 doesn’t contain Walker A motif and mutation residue (Thr18) located in N-terminal, suggesting OsLpa1-3.2 and OsLpa1-3.3 could be non-functional proteins. However, existence and possible involvement of OsLpa1-3.2 and OsLpa1-3.3 in PA biosynthesis remains to be determined.

Additionally, our results revealed a single amino acid substitution (Thr208Ile) in the kinase domain of Lpa1 in Sanggol. It is possible that the Lpa1 encodes an Ins(3)P1 kinase, which is phylogenetically clustered separately with other kinases in glycolysis and PA biosynthesis. From the molecular docking analysis, it is evident that Ins(3)P1 binds to the Lpa1 protein, with ATP as a cofactor for catalysis (Fig 8A). Overall, these results suggest that Lpa1 protein functions as a kinase, and is probably involved in the conversion of Ins(3)P1 to myo-inositol 3,4-bisphosphate [Ins (3,4) P2].

In Arabidopsis, aspartic acid (Asp) to alanine (Ala) substitutions at amino acid positions 98 and 100 (Asp98Ala and Asp100Ala) in two genes encoding inositol polyphosphate kinases result in inactive enzymes and LPA phenotypes [55]. Similarly, analysis of phosphorylation deficient mutants in yeast and human shows decreased MIPS activity compared with wild type because of amino acid substitutions at phosphorylation sites [48]. Several studies of various kinases and other metabolic enzymes show reduced enzyme activity of the mutant protein because of Thr and other amino acid substitutions at phosphorylation sites [47, 56–60]. Therefore, we speculate that a point mutation (C623T) causing Thr208Ile amino acid substitution in the loop adjacent to the entry site of the binding pocket of OsLpa1 is responsible for the altered enzyme activity of OsLpa1-3.1, resulting in reduced PA biosynthesis in Sanggol mutant seeds. Additionally, enzyme activity analysis is necessary to confirm the association of Thr208Ile substitution with the reduction in seed PA content in low PA mutant, Sanggol.

Though lpa rice lines are accompanied with negative impact on plant performance, studies in barley and soyabean showed that lpa mutants, barley lpa 1–1, and soyabean Gm-lpa-ZC-2, have not associated with any yield reduction [49, 61]. These results suggest the possibility of developing lpa lines when much attention is given simultaneously to both yield performance and low PA, particularly through the modulation of metabolic cross talk between P and grain yield. The results of Sanggol mutant reported in this study will facilitate the development of low PA lines with increased seed micronutrient bioavailability, high P uptake, and enhanced agronomic performance, through marker assisted introgression of the lpa1-3 allele into high yielding elite rice varieties.

Supporting information

S1 Fig. Schematic representation of the phytic acid (PA) biosynthetic pathway. Glu6p, glucose-6-phosphate; Ins, myo-inositol; PtdIns, phosphatidyl inositol; MIPS, myo-inositol-
3-phosphate synthase; IMP, myo-inositol monophosphatase; MIK, myo-inositol kinase; LPA1, low phytic acid 1; ITPK, inositol 1,3,4-triphosphate 5/6-kinase; IPK1, inositol 1,3,4,5,6 pentakisphosphate 2-kinase; PIS, phosphatidyl inositol phosphate synthase; PI4K, phosphatidyl inositol 4-kinase; PIP5K, phosphatidyl inositol 4 phosphate 5-kinase; PLC, phospholipase C; IPK2, inositol 1,4,5-tris-phosphate kinase; MRP, multidrug resistance protein.

S2 Fig. Gene structure of OsLpa1-3.2 and OsLpa1-3.3 splice variants. Mutations (C53T) in the first exon of OsLpa1-3.2 and OsLpa1-3.3 are indicated with red lines. Empty boxes represent 5’ and 3’ untranslated regions (UTRs), black box represents the coding region, and lines between boxes indicate introns. ATG (start codon) and TGA (stop codon) are shown.

S3 Fig. Statistical analysis of seed PA content among F2 plants derived from a cross between the lpa mutant, Sanggol and wild type, Ilpum. Data were analyzed using the Student’s t-test. M, mutant; W, wild type.

S4 Fig. Statistical analysis of inorganic phosphorous (P_i) content in seeds of F2 plants. Data were analyzed using the Student’s t-test. M, mutant; W, wild type.

S5 Fig. Statistical analysis of GC (%) in seeds of F2 plants. Data were analyzed using the Student’s t-test. M, mutant; W, wild type.

S1 Table. Primers used to sequence 16 PA biosynthetic genes in the lipid dependent and independent pathways.

Author Contributions

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