Phosphorus Partitioning of Soybean Lines Containing Different Mutant Alleles of Two Soybean Seed-Specific Adenosine Triphosphate-Binding Cassette Phytic Acid Transporter Paralogs

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Abstract
Seed phytate is a repository of P and minerals in soybean [Glycine max (L.) Merr.] seeds that limits P and mineral bioavailability for monogastric animals (e.g., humans, swine [Sus scrofa], and poultry [especially chicken, Gallus domesticus]) due to insufficient digestive tract phytase activity. We previously identified epistatic recessive mutations affecting two paralogous adenosine triphosphate-binding cassette phytic acid transporter genes (one a nonsense mutation in Lpa1 and the other a missense mutation in Lpa2) as the molecular genetic basis in the ethyl methanesulfonate (EMS)-induced mutant low phytate soybean line M153. An additional mutant low phytate line, M766, contained one single nucleotide polymorphism within the ninth intron of the Lpa1 locus as well as a nonsense mutation in Lpa2. The objectives of this research were to clarify the genetics underlying the low phytate phenotype in line M766 and to determine P partitioning in new combinations of mutant alleles from M766 and M153. Inheritance of nonsense alleles affecting both low phytic acid (Lpa) genes (one from M153 and one from M766) led to the production of viable seeds that contained transgressive reductions in total seed phytate and significantly higher levels of inorganic phosphate than has been reported for nontransgenic soybean material and will allow efficient molecular selection of soybeans with even greater reductions of phytate for improved quality soybean meal.

Phytic Acid (myo-inositol-1,2,3,4,5,6-hexa-kisphosphate) is a storage form of P in plant seeds. Monogastric animals (including humans, poultry, and swine) lack sufficient levels of phytase to mobilize this P (Raboy, 2007b). In addition, the highly negative overall charge of phytic acid causes it to spontaneously precipitate cationic species, including a number of critical micronutrients such as Fe2+ and Zn2+ (Raboy, 2002). Phosphorus is an essential animal macronutrient, and feedstocks for nonruminants are routinely supplemented with nonsustainable inorganic rock phosphate for optimal growth and weight gain (Waldroup et al., 2000). In addition to slightly increasing the cost of feed mixtures, excess phosphate can accumulate in the environment due to use of manures as a ready source of fertilizer (Raboy, 2002, 2007b). Unused P has the potential to accelerate eutrophication when released into fresh water streams and lakes (Correll, 1998; Raboy, 2007a). In soybean, phytic acid typically makes up the majority of the total seed P (~72%) (Raboy et al., 1984) although this varies based on the nutritional status of the maternal plant. The use of seeds with lowered phytic acid in animal feed mixtures has the potential to reduce or potentially eliminate the need for supplementation with exogenous inorganic phosphate (Raboy, 2007b) as well as increasing the bioavailability of critical micronutrients (Zhou et al., 1992).
For these reasons, there has been a great deal of interest in both the applied goal of reducing the level of phytic acid accumulation in seeds as well as elucidating the biosynthetic pathways for phytic acid production. In soybean, mutagenesis has been used to develop several independent mutant lines (and in one case a spontaneously occurring mutant) with significant reductions in phytic acid content and a concomitant increase in bioavailable inorganic phosphate (Hitz et al., 2002; Maroof and Buss, 2011; Yuan et al., 2007) and (ii) Buss, 2011; Wilcox et al., 2000). Mutations affecting two different classes of genes have identified as causative: (i) a myo-inositol phosphate synthase (MIPS) gene (Hitz et al., 2002; Maroof and Buss, 2011; Yuan et al., 2007) and (ii) two phytic acid specific adenosine triphosphate-binding cassette transporter paralogs, which have been named low phytic acid (Lpa) genes (Gillman et al., 2009; Maroof and Buss, 2011; Shi et al., 2007; Nagy et al., 2009). Deleterious impacts on germination and/or field emergence have been observed as consequences of the presence of either of these two biochemically and genetically distinct sources of low phytic acid soybean (Anderson and Fehr, 2008; Maupin and Rainey, 2011; Meis et al., 2003). Unfortunately, the variable environmental effect has so far precluded a clear understanding of the physiological basis of the germination defect, aside from being associated with the presence of mutant alleles.

Mutations in a single MIPS gene result in modest reductions in phytic acid content as compared to wild-type lines as well as an additional beneficial reduction in indigestible raffinose family oligosaccharides (Hitz et al., 2002). As such, mutations affecting MIPS would be the more logical a priori target for breeding efforts. However, MIPS mutant lines have higher levels of the antinutritional compound phytic acid and less bioavailable P and feature more significant germination defects as compared to Lpa mutant lines (Maupin and Rainey, 2011). Indeed, complete silencing of the MIPS gene has been shown to be embryo lethal in soybean (Nunes et al., 2006).

In contrast, silencing of the soybean Lpa genes failed to result in either embryo or seedling lethality in a large number of transgenic events in maize (Zea mays L.) and soybean (Shi et al., 2007), and the germination issues appear to have a lower overall effect (Maupin and Rainey, 2011), which can at least partially be ameliorated by appropriate genetic selection (Anderson and Fehr, 2008; Spear and Fehr, 2007). Commercial cultivars with the reduced phytic acid trait derived from mutant alleles of the two Lpa genes are in the process of being developed (Vince Pantalone, personal communication, 2012).

In our previous work (Gillman et al., 2009) we identified a nonsense mutation in one Lpa paralog (Glyma03g32500:lpal-a) (for clarity of presentation in this study; nonsense mutations will be indicated by presence of bold type) and a missense R1039K substitution affecting an ancestrally invariant residue within the second paralog (Glyma19g35230:lpal-a) in ethyl methanesulfonate (EMS)-induced mutant line M153 (Wilcox et al., 2000). M153 was later used to develop a partially adapted breeding line CX1834 (Oltmans et al., 2004). Our genetic analysis revealed a rational biochemical basis for the duplicate dominant epistatic interaction, based on the combination of two independent deleterious mutations (one nonsense and one missense) in two of the genes encoding Lpa paralogous genes (Gillman et al., 2009). We also sequenced these genes in a “sister” low phytic acid EMS-induced mutant line, M766, created in the same mutagenesis experiment as M153/CX1834 (Wilcox et al., 2000). M766 contained a single nucleotide polymorphism (SNP) in intron 9 compared to the ‘Williams 82’ reference sequence and is designated herein as lpal-b (Gillman et al., 2009).

M766 bears a nonsense mutation in Glyma19g35230 (lpal-b), which coincidentally affects the exact same codon (R1039†; Fig. 1) as the missense mutation in M153. Thus, the current collection of variant Lpa alleles is two null alleles, one of lpal-a from M153 and one of lpal-b from M766, one missense allele of lpa2-a from M153, and one lpal-b allele from M766 that does not differ in the coding sequence from the reference Williams 82 allele but that contains a single SNP within intron 9 of the gene.

In this study, we report on genetic analysis of F2 and F1 progeny of two different crosses using the low phytate EMS mutant line M766: (i) low phytate line TN09-239 (which possesses a nonsense lpa1-a mutation and a missense lpa2-a mutation originally derived from M153) × M766 (which contains the unique polymorphic allele lpal-b and a nonsense lpa2-b) and (ii) normal phytate line Williams 82 × M766.

Materials and Methods

Population Development

M766 (lpal-b/lpa2-b) (Wilcox et al., 2000) was crossed to Williams 82 (Bernard and Cremeens, 1988) and TN09-239 during the summer of 2009. Williams 82 possesses typical levels of inorganic phosphate and phytic acid whereas TN09-239 (lpal-a/lpa2-a) is a BC, low phytic acid line developed by backcrossing CX18341-2 to ‘5601T’ (Pantalone et al., 2003), which has been developed by Vince Pantalone at the University of Tennessee. The F1 seed were produced in a growth chamber with a day/night cycle of 28°C day for 13.5 h followed by 22°C night for 10.5 h. Nutrients were provided by Osmocote beads (Scotts Company). Putative F1 plants were verified by lpa2-llpa2-b assays as previously described (Gillman et al., 2009). For a subset of the verified F1 plants, the F2 progeny seed from two plants (M766 × Williams 82; n = 65) or one plant (M766 × TN09-239; n = 63) were harvested and lyophilized, and individual seeds were ground with a mortar and pestle.

Additional F2 seed (from verified F1 plants) was hand planted at the Bradford experimental field location near Columbia, MO, in the spring of 2010 to produce F3 seed in a field environment. Leaf presses of the F3 plants were made in the field with FTA cards (Whatman) for DNA isolation and genotyping assays. Based on lpal/ lpa2 marker analysis, a subset of F3 plants were selected...
for further analysis. Seed from F₂ or F₃ plants were harvested when pods were mature, and five F₃ seed were lyophilized and individually ground with a mortar and pestle. Mature, lyophilized seed powder was subdivided and used for three distinct analyses: (i) DNA isolation for marker genotyping, (ii) determination of free inorganic phosphate by Chen’s method (Gillman et al., 2009; Wilcox et al., 2000), and (iii) inductively coupled plasma mass spectrometry (ICP-MS) analysis to determine total P and other ionomic constituents. The F₃ seed was also analyzed for phytic acid content by high performance liquid chromatography (HPLC) (Chen and Li, 2003).

**Determination of Available Inorganic Phosphate of Soybean Seeds**

Inorganic phosphate content was quantified for 10 to 30 mg of seed tissue as previously described (Gillman et al., 2009; Wilcox et al., 2000).

**Determination of Phytic Acid Content of F₃ Soybean Seeds**

Phytic acid (PA) P was quantified by modified HPLC method (Chen and Li, 2003). Powdered seed samples (0.025 g) were extracted 1 h by shaking at room temperature in 0.5 mL of 0.5 M HCl. After centrifugation for 15 min at 15,000 × g, supernatants were filtered through a 0.22-μm filter, and 100 μL of filtrate was analyzed. Phytic acid P and inositol polyphosphate separations were performed by a linear gradient elution program on a Dionex CarboPac PA-100 guard column and a CarboPac PA-100 analytical column (Dionex) on a Dionex ICS-5000 Ion Chromatography System. The elution gradient was effected by the following program: from start to 30 min ramping from 8 to 92% 0.5 M HCl, from 30 to 35 min running 92% 0.5 M HCl, then ramping down from 92 to 8% 0.5 M HCl during the next 0.1 min, and finishing for 4.9 min running at 92% 0.5 M HCl. A postcolumn derivitization was achieved with a solution of 1 g L⁻¹ Fe(NO₃)₃ in 0.33 M HClO₄ using a 750-μL knitted coil and was followed by detection of A₂₉₅. Flow rates of eluent and postcolumn solution were 1.0 and 0.4 mL min⁻¹, respectively. The purified standard phytic acid (phytic acid dipotassium salt; Sigma-Aldrich Corp.) was eluted at 30 min. Standard curves were calculated from dilutions of PA standards. Results were converted to micrograms phytic acid P per milligrams seed.

**Determination of Soybean Seed Total Ionomne**

Powder was aliquoted into 16 by 100 mm Pyrex test tubes and weighed. Digestion was performed by adding 2.5 mL of concentrated HNO₃ containing In internal standard to the test tubes and incubating overnight at room temperature before heating the samples to 105°C over 2 h and then cooling to room temperature over 2 h. The digested samples were diluted in the test tubes to 10 mL by adding ultrapure water, and a second dilution was made in a
second set of test tubes by taking 900 μL of the first dilutions to 5 mL with ultrapure water. Then 1.2 mL of the second dilutions was transferred to 96-well autosampler plates using an adjustable-width multichannel pipette. Elemental analysis was performed using an ICP-MS (PerkinElmer Elan DRCe; PerkinElmer Inc.) with an Apex Desolvation Nebulizer, FAST sampling valve, and SC4 DX autosampler (Elemental Scientific Inc.). A liquid reference material composed of pooled samples of soybean digests was run every ninth sample to correct for ICP-MS within-run drift. All samples were normalized to the recorded weights.

**DNA Isolation from Seeds**

The DNA was isolated from approximately 20 μg of seed tissue using a DNeasy kit (Qiagen, Inc.) and used in genotyping assays at 5 to 50 ng per reaction.

**M766 lpa1-b Marker Development**

To track the lpa1-b allele in the Williams 82 × M766 segregating population, we developed a SimpleProbe assay (Roche Applied Sciences) corresponding to a silent SNP within intron 9 (T5202A relative to the start codon), which was unique to M766 (Gillman et al., 2009). The M766 intron T5202A SNP was detected through melting curve analysis to measure the disassociation kinetics of a SimpleProbe oligonucleotide (Fluorescein-SimpleProbe chemistry-TTTCT-GTTGCTATTGCTTACTTTTCAATATAGA-Phosphate) designed to match the Williams 82 Glyma03g32500/LPA1 sequence (http://www.phytozone.net/soybean). Asymmetric polymerase chain reaction (PCR) using a 1:5 ratio of primers (forward = ATCCCTGGACGATCAACTTATGCA and reverse = GAGGGCGAGAATCTTCAAATATGC) provided template for SimpleProbe binding. Asymmetric PCR and melting curve analysis were performed as previously described (Gillman et al., 2009) using a Lightcycler 480 II instrument (Roche Applied Sciences).

**lpa1-a and lpa2-a and/or lpa2-b Genotyping Assays**

Genotyping assays for M153 and/or CX1834-derived lpa1-a and lpa2-a alleles were performed as previously described (Gillman et al., 2009) using either DNA isolated from seed tissue or using leaf extract punches from FTA cards (Whatman) according to manufacturer’s recommendations.

**Examination of lpa1-b Messenger Ribonucleic Acid Transcripts by Reverse Transcription Polymerase Chain Reaction**

Pods corresponding to two stages of seed fill (8–10 mm in length and 10+ mm and fully expanded seeds) were collected from field grown plants of Williams 82, M766, and the F_{2,3} line that recreated the lpa1-b/lpa2-b homozygote mutant allele combination (line 6-8-31). Seeds were then separated from pods before being flash frozen and ground in liquid N\textsubscript{2}. Messenger ribonucleic acid (mRNA) was extracted from approximately 50 mg seed powder using the Trizol reagent (Invitrogen Corp.) and purified and on-column deoxyribonuclease (DNase) treated using the Direct-Zol RNA (ribonucleic acid) miniprep kit according to manufacturer’s recommendations (Zymo Research).

Samples of 0.4 μg of DNase treated mRNA were used with the SMARTScribe Reverse Transcriptase kit with a gene specific primer located in exon 10 of Lpa1 (5'-GGAGGGCGAGAATCTTCAATAAT-3') according to manufacturer’s recommendations (Clontech Laboratories, Inc.), and reverse transcription polymerase chain reaction (RT-PCR) was performed using a SYBR green Quantitect PCR kit according to manufacturer’s recommendations (Qiagen) using a forward primer located in exon 9 (5'-TATTGTCAGACATTCTTCAATAAT-3') and a reverse primer located in exon 10 of Lpa1 (5’-GGAGGGCGAGAATCTTCAATAAT-3’) primers. Reverse transcription PCR products were verified to be of appropriate size, purified using a Qiaquick PCR purification kit (Qiagen), and Sanger sequenced at the DNAcore facility at the University of Missouri. The resulting sequence traces were imported into the Geneious software package (version 5.6) (Biomatters, 2012) and manually trimmed and aligned. To verify the putative mixture of spliced and misspliced transcripts, a fluorescently labeled version of the forward primer (carboxytetramethylrhodamine [TAMRA] 5’-TATTGTACATACAGAGTCTTCCAT-3’) was used in RT-PCR, with amplification products purified as described previously, and diluted 1:15- and 1:50-fold before submission to the DNAcore facility at the University of Missouri for size fractionation. The exact sizes of RT-PCR products were determined by comparison to a standard mixture of size standards using the PeakScanner software package (Applied Biosystems, 2006).

**Results and Discussion**

**Development of Populations Segregating for M766-Derived Alleles of LPA1 and LPA2**

To investigate the roles of distinct alleles of the Lpa1 (Glyma03g32500) and Lpa2 (Glyma19g35230) genes in different combinations, two populations were developed by crossing the low phytic acid line M766 (lpa1-b/lpa2-b) with the reference cultivar Williams 82 (Bernard and Cremeens, 1988) and also with the low phytic acid line TN09-239 (lpa1-a/lpa2-a) (Gillman et al., 2009). Wild-type and mutant alleles of Lpa1 and Lpa2 were segregating in the Williams 82 cross while only mutant alleles of Lpa1 and Lpa2 were segregating when M766 was crossed with TN09-239 (Fig. 1).

Sequence differences between wild-type and mutant lpa genes enabled us to track alleles in the two segregating populations. The M766 lpa1-b allele contains a single polymorphism when compared to the reference sequence for Williams 82, located within intron 9 (T5202A), which was used to develop a molecular marker assay. We used a previously developed lpa2 molecular marker assay that correctly distinguished wild-type Lpa2 alleles from both the missense and the nonsense
lpa2 alleles (lpa2-a and lpa2-b, respectively), and that is also able to distinguish the two mutant alleles from each other (Gillman et al., 2009).

Confirmed F\textsubscript{1} seeds were germinated and grown to maturity in a growth chamber. For initial characterization of the P partitioning in the seed, the F\textsubscript{2} progeny of one or two plants from each cross was destructively analyzed. Individual F\textsubscript{2} seeds were ground and subsampled for P phenotypes and genotyped for lpa1/lpa2 on a seed-by-seed basis. Evaluation of Genetic Loci that Influence Seed Phosphate Concentration in M766

Based on previous results with M153 derived lines containing the lpa1-a and lpa2-a mutations (Gillman et al., 2009), we predicted that the loss of function mutation lpa2-b in M766 may reduce the phytic acid transporter activity below the threshold necessary to produce a measurable P partitioning phenotype even in the presence of functional versions of Lpa1 and Lpa2. Alternately, the phytate reduction could be due to an epistatic effect of the lpa1-b allele (containing a SNP within intron 9 [T5202A]) or due to an uncharacterized cis-acting factor in the lpa1-b promoter or enhancer. To evaluate if the lpa1-b allele was linked to the low phytate phenotype, we crossed M766 to a line with typical levels of phytic acid, Williams 82, and examined F\textsubscript{2} seeds for the phenotypic consequences of presence or absence of alleles derived from M766 on seed P partitioning. We made the assumption that Williams 82 contained functional alleles of Lpa1 and Lpa2. Only homozygosity for both the lpa1-b and lpa2-b alleles was found to be associated with significantly elevated levels of inorganic phosphate compared to the Williams 82 parent for the F\textsubscript{2} seeds (Fig. 2). This finding is consistent with our previous findings for CX1834 (Gillman et al., 2009). The necessity for the combination of homozygous lpa1-b and lpa2-b alleles for the high inorganic phosphate phenotype was confirmed by examining the F\textsubscript{3} progeny of an F\textsubscript{2} selection that was homozygous for the lpa2-b allele but was heterozygous for Lpa1 (Fig. 3). The F\textsubscript{3} seeds containing the lpa2-b alleles and one or two functional Lpa1 alleles contained available phosphate levels just above the detection limit of the assay. Seeds homozygous for the combination of the lpa2-b alleles and the lpa1-b alleles had approximately sevenfold more available phosphate, close to the level achieved by M766 (Fig. 3; Supplemental Table S1).

Linkage of the lpa1-b allele with altered P partitioning suggests it is not equivalent to a fully functional allele. However, no changes in the coding sequence or alterations to the promoter (within 240 bp upstream) were detected in the lpa1-b allele from M766. We identified a single T > A SNP 7 bp upstream of the start of exon 10 (Supplemental Fig. S1). Software prediction analysis (Hebsgaard et al., 1996) of this SNP indicated the possibility that a cryptic splice site could have been activated. The result would be the introduction of five additional basepairs of intron sequence and a frameshift mutation starting at exon 10. This hypothesis proved correct, as a portion of transcripts from the lpa1-b locus are misspliced (Supplemental Fig. S1 and S2). The cryptic splice site is activated in only a subset of the total lpa1-b transcripts (Supplemental Fig. S2), which likely explains our finding that the lpa1-b allele is inferior as a means of reducing the antinutritional compound
phytic acid as compared to the nonsense \textit{lpa1-a} mutation found in M153 and/or CX1834 (Gillman et al., 2009).

**Phosphate Partitioning in F$_2$ Seeds from the Cross of M766 $\times$ TN09-239**

Although the combination of alleles found in M766 (\textit{lpa1-b} and \textit{lpa2-b}) is unlikely to be useful in breeding efforts for reducing seed phytic acid, we predicted that the \textit{lpa2-b} nonsense mutation in combination with the \textit{lpa1-a} nonsense mutation could increase inorganic phosphate to a greater extent than possible in CX1834 or derived lines (Gillman et al., 2009).

To address this possibility, a population of plants was developed from the cross of TN09-239 $\times$ M766. F$_2$ seeds produced in a growth chamber environment were analyzed as described above and data from individual seeds were grouped into appropriate genotypic classes for statistical analysis (Fig. 4).

For the two wild-type soybean lines Williams 82 and 5601T, inorganic phosphate was virtually undetectable in seeds. In contrast, both TN09-239 (bearing homozygous mutant alleles \textit{lpa1-a} and \textit{lpa2-a}) and M766 (bearing homozygous alleles of \textit{lpa1-b} and the nonsense mutant alleles of \textit{lpa2-b}) possessed elevated inorganic phosphate: 1.63 ± 0.60 and 3.90 ± 0.24 $\mu$g P mg$^{-1}$ seed, respectively. Curiously, the seeds produced by TN09-239 line in the growth chamber possessed a lower amount of inorganic phosphate as compared to M766 as well as slightly lower levels of total seed P (although this finding was not statistically significant) as compared to M766 seeds (Fig. 4). This result was unexpected and may be due to growing a maturity group V line in an artificial environment optimized for the maturity group III lines M766 and Williams 82.

We investigated the progeny of a cross between M766 and TN09-239 to assess the phenotypic consequences of the combinations of the different mutant versions of \textit{lpa1} and \textit{lpa2}. We had previously hypothesized (Gillman et al., 2009) that the missense \textit{lpa2-a} allele found in M153 and/or CX1834 might only impair functionality rather than abolishing it. If this hypothesis was correct, then the combination of two nonsense mutations affecting the two independently inherited paralogous genes (\textit{lpa1-a} derived from M153 and/or CX1834 in conjunction with \textit{lpa2-b} from M766) could result in even higher levels of available inorganic phosphate in seeds. The combination of the \textit{lpa1-b} allele from M766 with the \textit{lpa2-a} allele from CX1834 (\textit{lpa1-b}/\textit{lpa2-a}) resulted in accumulation of the least available inorganic phosphate (1.88 ± 0.42 $\mu$g P mg$^{-1}$ seed) among the mutant genotypic classes and the novel homozygote combination of the two nonsense mutant alleles, \textit{lpa1-a}/\textit{lpa2-b}, produced the highest available inorganic phosphate of any of the samples examined (5.39 ± 0.65 $\mu$g P mg$^{-1}$ seed) although the relatively high standard deviations precluded definitive conclusions based solely on the F$_2$ generation (Fig. 4). Previous experiments with different germplasm sources of the low phytate phenotype had consistently shown strong negative correlations between the amount of P measured in available phosphate and the amount of P measured in phytate (Bilyeu et al., 2008; Gillman et al., 2009), so the F$_2$ experiments did not include phytate measurements.

**Analysis of F$_3$ Seeds from F$_2$ Genotypic Field-Grown Selections**

More comprehensive analyses were performed on field produced F$_{2,3}$ seeds derived from remnant F$_2$ seeds from the populations. Plants representing all of the possible
homozygous genotypic combinations of lpa1 and lpa2 mutations (lpa1-a/lpa2-a, lpa1-a/lpa2-b, lpa1-b/lpa2-a, and lpa1-b/lpa2-b) were selected and four or five F3 seeds from each plant were analyzed independently to investigate three distinct seed P related traits: available inorganic phosphate by colorimetric analysis, phytic acid content by HPLC analysis, and total cellular P as determined by ICP-MS (Fig. 5; Supplemental Table S2).

Similar to the situation for growth chamber produced seeds, free inorganic phosphate is present in trace, nearly undetectable amounts for seeds from field-grown wild-type soybean lines (Supplemental Tables S1 and S2). All homozygote combinations of mutant alleles for lpa1 and lpa2 resulted in elevation of inorganic phosphate and a concomitant reduction in phytic acid (Fig. 5). The novel combination of lpa1-a mutation from CX1834 with the lpa2-b mutation from M766 resulted in the greatest accumulation of available inorganic phosphate P (3.33 ± 0.40 μg P mg⁻¹ seed) and the least amount of phytic acid P (0.25 ± 0.06 μg P mg⁻¹ seed). The amount of available inorganic phosphate was approximately 1.88 times higher than noted for TN09-239 (1.77 ± 0.57 μg P mg⁻¹ seed) or the F2 selection homozygous for lpa1-a/lpa2-a (1.78 ± 0.25 μg P mg⁻¹ seed) and these differences were statistically significant (p = 0.0024). Phytic acid P was dramatically reduced approximately fourfold for the novel allele combination (0.25 ± 0.06 μg P mg⁻¹ seed) as compared to TN09-239 (0.99 ± 0.16 μg P mg⁻¹ seed) or approximately 3.6-fold reduction when compared to the F2 selection bearing lpa1-a/lpa2-a (0.90 ± 0.19), and these differences were statistically significant (p < 0.0001). In comparison to the wild-type line 5601T (4.12 ± 1.18 μg P mg⁻¹ seed), the reduction in phytic acid was approximately 16.5-fold.

Analysis of F3 Progeny of F2 Selections Heterozygous for One lpa Locus

We investigated the P partitioning in seeds for the progeny of a plant heterozygous for lpa1-a but homozygous for lpa2-b, which confirmed our findings (Fig. 5) that the novel combination lpa1-a/lpa2-b results in greater reductions in phytic acid than is possible using M153 and/or CX1834 derived alleles alone, as evinced by TN09-239. A concomitant increase in the available inorganic phosphate was also noted. Among these selections, there was no significant difference in terms of total P, as detected by ICP-MS (Supplemental Tables S1 and S2). The percentage of potentially available P (total P – phytic acid P) ranged from 42.2 to 44.2% for wild-type samples (Williams 82 and 5601T, respectively) to 87.5% for TN09-239 or the genotypic class representing lpa1-a/lpa2-a (88.5%). The novel combination of lpa1-a/lpa2-b resulted in a remarkable approximately 97.4% of the total P potentially available for digestion, with only a very minute trace amount of indigestible phytic acid P present (0.25 ± 0.06 μg PA mg⁻¹ seed or ~2.6% of total P).

Ionic Impacts of Presence of Low Phytic Acid Alleles

Inductively coupled plasma mass spectrometry analysis yields a considerable amount of data regarding the mineral composition of tissues. A full listing of seed mineral composition results is presented in Supplemental File S1, but some selected examples will be mentioned here. We noted differences for certain mineral species between parental genotypes. For example, Williams 82 possesses a low level of Ni as compared to plants of the other genotypes grown in the same environment. Similarly, both Williams 82 and
TN09-239 appear to have lower levels of Cu as compared to 5601T and M766. M766 also appears to have significantly higher levels of total P content. The genetic factors responsible and/or biological significance (if any) of these mineral compositional differences remain to be determined. In contrast to the results noted for the parental lines, we did not observe any consistent, statistically significant differences among any of the lpa1 and lpa2 genotypic classes for F2 or F3 generations in terms of elemental content. Based on the dramatic reduction in phytic acid content and the apparently unchanged level of mineral species, soymeal derived from plants bearing the novel lpa1-a/lpa2-b allele combination may have much higher levels of Zn and Fe available for uptake since there is very little chelating phytic acid present.

Potential of this Novel Genetic Material to Have Germination Defects

We anticipate that soybean lines containing the lpa1-a/lpa2-b combination of alleles may have some degree of germination and/or field emergence defects when grown in environments with elevated abiotic stress (high temperature during seed development or germination after cold treatment), as this trait has previously shown for CX1834-derived materials (Anderson and Fehr, 2008; Maupin and Rainey, 2011; Oltmans et al., 2005). Rigorous analyses of germination defects require substantial amounts of seed produced and tested in multiple environments. To evaluate any large effect issues with seedling emergence, we were only able to do a small scale field study with F2 seeds. All genotypic classes for both populations from seeds produced in a growth chamber were able to germinate, emerge, and produce seed at the Bradford Research and Extension Center near Columbia, MO, during the summer of 2010, and we observed no significant segregation distortion at F2 or F3 for the cross of M766 × TN09-239 (Supplemental Table S1). Lines with all combinations of mutant lpa1/lpa2 alleles are capable of germination and field emergence. We did, however, observe segregation distortion in the cross between M766 × Williams 82, with an over-representation of wild-type samples occurring for LPA2 (Supplemental Table S1), similar to studies previously reported for low phytic acid wheat (Triticum aestivum L.) lines (Guttieri et al., 2004). This underlines the need for molecular assays for this double recessive trait to have sufficient lines to combine the low phytic acid trait with yield.

Definitive quantification of potential germination defects will require future backcrossing, as M766 is an unimproved EMS mutant line, and targeting induced local lesions in genomes (TILLING) studies have found that EMS mutant lines can have as many as one mutation per 140 kb (Cooper et al., 2008). This implies that M766 will likely contribute a significant mutation load (i.e., “yield drag”). Once sufficiently backcrossed material is developed, we can properly evaluate the extent of any germination defects. The development of an allelic series of combinations of the lpa1/lpa2 alleles from M766 will likely assist in these efforts to quantify the effects of these alleles on germination efficiency.

Conclusions

To summarize, we have developed a soybean line in which phytic acid P is reduced to a minute 2.6% of the total seed.
P using nontransgenic methods. Such material is capable of germination and field emergence although backcrossing will likely be required to remove other EMS-induced mutations from the M766 donor. This work enables the development of cultivars with even lower levels of the antinutritional compound phytic acid than is currently possible with conventional soybean germplasm. Such efforts can be accelerated through use of molecular markers we developed, and the fortuitous discovery that the exact same codon was affected in two independent mutants (Gillman et al., 2009) means that the same marker assays used for selection of M153 and/or CX1834 derived alleles can be used to select for the novel allelic combination.

**Supplemental Information Available**

Supplemental material is included with this manuscript.

Supplemental Figure S1. Cartoon depiction of \( lpa1-b \) intron single nucleotide polymorphism (SNP) (T > A 7 bp upstream of exon 10) and mRNA consequences of the presence of the intron mutation. A) Cartoon depiction of the \( Lpa1 \) or \( lpa1-b \) locus. Exons are indicated by dark boxes and introns represented by lines. The predicted transcriptional start site is indicated by arrow and the placement of the intron mutation is indicated by a red arrow. Primers used for reverse transcription polymerase chain reaction (RT-PCR) are indicated by black arrows located above exon 9 and exon 10. B) Sequence traces from RT-PCR products amplified using gene specific primers located in exon 9 and exon 10.

Supplemental Figure S2. Size fractionation of reverse transcription polymerase chain reaction (RT-PCR) products between exon 9 and 10 to detect putative splicing defect in lines bearing \( lpa1-b \).

Supplemental Table S1. Phosphorus phenotypic data for all nine possible \( F_2 \) genotypic classes as determined by genotyping assays and phenotyping on individual \( F_2 \) seeds from crosses between M766 × TN09-239 or M766 × W82.

Supplemental Table S2. Seed phosphorus phenotypic data for \( F_{2:1} \) genotypic selections from crosses between M766 × TN09-239 or M766 × W82.

Supplemental Table S3. Ionomic analysis of soybean seeds for \( F_{2:23} \) genotypic selections from crosses between M766 × TN09-239 or M766 × W82.

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**References**


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