

## Ectopic expression of a soybean phytase in developing seeds of *Glycine max* to improve phosphorus availability

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### Abstract

A transgenic approach was used to alter soybean seed phytate content by expressing a soybean phytase gene (*GmPhy*) during seed development to degrade accumulating phytic acid (IP<sub>6</sub>). An expression vector containing the soybean phytase cDNA controlled by the seed-specific  $\beta$ -conglycinin promoter ( $\alpha'$ -subunit) was used to transform embryogenic soybean cultures. Plants from four independent transgenic lines were analyzed for transgene integration and seed IP<sub>6</sub> levels. The reduction in IP<sub>6</sub> levels in transgenic seeds compared to control 'Jack' soybeans ranged from 12.6 to 24.8% as determined by HPLC. A low copy transformant was propagated to the T<sub>4</sub> generation and examined in more detail for phytase expression and enzyme activity during seed development. Expression of phytase mRNA and phytase activity increased during seed development, consistent with the use of an embryo-specific promoter. Ectopic phytase expression during seed development offers potential as an effective strategy for reducing phytate content in soybean seed.

**Abbreviations:** HPLC, high performance liquid chromatography; IP<sub>6</sub>, *myo*-inositol hexakisphosphate, phytic acid; P, phosphorus

### Introduction

Phosphorus (P) is stored in plant seeds as phytate (also known as phytic acid, *myo*-inositol hexakisphosphate or IP<sub>6</sub>) and is degraded by the enzymatic activity of phytase during germination (Reddy *et al.*, 1989). Similar to other agriculturally important crops, soybean contains 60–80% of total seed P in the form of phytate (Raboy *et al.*, 1984). Soybeans are processed to produce meal, which is commonly used in animal feed because of its high protein content. Seed phytate in animal diets is not readily digested by non-ruminant animals and is excreted in manure (Reddy *et al.*, 1989; Ravindran *et al.*, 1995a). Depending on meal treatment, a significant portion of phytate may remain undigested even in ruminant animals

(Konishi *et al.*, 1999; Park *et al.*, 1999). Inefficient utilization of phytate P necessitates the addition of supplemental phosphate to animal rations to meet optimal growth requirements.

Another anti-nutrient property of phytate is its ability to complex with mineral cations such as potassium, magnesium and calcium to form phytin, which accumulates in protein bodies in the seed (Prattley and Stanley, 1982). Mineral chelation by phytate reduces the availability of iron and zinc, leading to potential mineral deficiencies (Reddy *et al.*, 1989).

The widespread use of soybean meal in livestock feed and the inability to utilize phytate P can lead to serious environmental consequences. Repeated applications of P-rich manure to pastures and cropland increase soil P levels and the

potential for run-off into streams and lakes. The increased P influx into bodies of water, where P is limiting, can increase aquatic growth and lead to eutrophication and oxygen depletion (Sharpley *et al.*, 1994). Reducing phytate and increasing the available P in feed would improve nutrient utilization and reduce the environmental impact of livestock production.

Several alternative methods have been used to increase P availability in animal diets. To avoid the requirement for additional inorganic phosphate, one approach has been the supplementation of feed with a microbial phytase from *Aspergillus niger* (E.C. 3.1.3.8). Benefits from supplementing poultry and swine diets with the fungal phytase included an increase in available phytate P and a reduction in P excretion (Swick and Ivey, 1992; Cromwell *et al.*, 1995; Denbow *et al.*, 1995; Ravindran *et al.*, 1995b; Yi *et al.*, 1996). Another strategy for improving phytate P availability has been the expression of a recombinant fungal phytase in plant seeds including tobacco (Pen *et al.*, 1993; Verwoerd *et al.*, 1995), alfalfa (Koegel *et al.*, 1998; Austin-Phillips *et al.*, 1999), canola (Ponstein *et al.*, 2002), and soybean (Li *et al.*, 1997; Denbow *et al.*, 1998). Feeding trials have been conducted with tobacco and soybean expressing the fungal phytase based on the premise that seeds containing the recombinant enzyme could be combined with standard diets to hydrolyze feed phytate, increase the nutritional availability, and reduce excreted P. Although these goals were achieved (Pen *et al.*, 1993; Denbow *et al.*, 1998), this approach does not alleviate the cost and labor incurred by feed supplementation.

Development of soybeans with low seed phytate levels would increase P availability while also eliminating the need for phytase supplementation. Two possible strategies for altering phytate levels include blocking the phytate biosynthetic pathway or degrading phytate in developing seeds. Altering steps in phytate biosynthesis has the potential disadvantage of affecting many other cellular processes involving inositol phosphates. For this study, we approached the reduction of phytate by expressing a phytase transgene during seed development to modify the final composition of harvested seed. This involved the introduction of a previously described soybean phytase (Hegeman and Grabau, 2001) under the control of a developmentally regulated promoter. This approach

differed from previous studies that used plants as bioreactors to produce active enzyme for supplementation purposes. By expressing the phytase transgene under control of an embryo specific promoter, seed phytate accumulation is altered during development and enzyme activity is not required after harvest of mature seed or during animal feeding. Low phytate soybean meal would improve P availability and reduce P content in animal manure.

## Materials and methods

### *Phytase expression vector and genetic transformation*

The soybean phytase sequence (*GmPhy*, Accession no. AF272346) and a hygromycin resistance cassette were cloned into separate expression vectors and co-precipitated on tungsten particles prior to bombardment. The 5.6 kb soybean phytase expression vector (pPHY) consisted of the soybean phytase cDNA (Hegeman and Grabau, 2001) under regulatory control of the seed specific  $\beta$ -conglycinin  $\alpha'$ -subunit promoter and terminator (Chen *et al.*, 1986). The expression of a hygromycin resistance gene was controlled by the cauliflower mosaic virus 35S promoter with the nopaline synthase terminator (4.8 kb p*Hytru*). The hygromycin resistance gene in p*Hytru* was modified from p*Hygr* (Finer *et al.*, 1992) to eliminate an out-of-frame upstream ATG.

Transgenic soybean plants were produced via particle bombardment of embryogenic suspension cultures by a modification of a previously described procedure (Finer and McMullen, 1991). Embryogenic cultures of soybean cv. 'Jack' were maintained on semi-solid medium containing MS salts, B5-vitamins, 3% sucrose, 5 mM asparagine, 0.2% Gelrite, 20 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), pH 5.7 (Santarém and Finer, 1999). To establish liquid embryogenic suspension cultures, embryos were transferred to medium containing MS salts (minus nitrates), 10 mM  $\text{NH}_4\text{NO}_3$ , 30 mM  $\text{KNO}_3$ , B5-vitamins, 3% sucrose, 5 mM asparagine, 5 mg/l 2,4-D, pH 5.7 (Finer and McMullen, 1991). Suspension cultures were maintained with a bi-weekly subculture to fresh medium and were bombarded four days after subculture. Embryogenic soybean tissues were

selected in liquid culture for growth in the presence of 50 mg/l hygromycin for 6 to 8 weeks. Plants were regenerated from hygromycin-resistant cultures according to previously published protocols (Finer and McMullen, 1991).

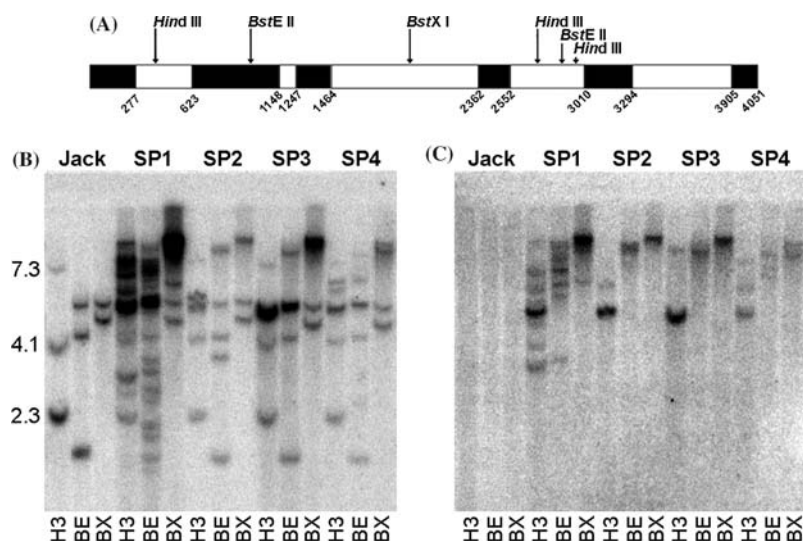
Soybean plants were grown in Scott's Metro-mix 510 in the greenhouse under a 16/8 h light/dark photoperiod with supplemental light supplied by high pressure sodium vapor lights. Day/night temperatures were constant at 28 °C. Plants were fertilized once after the emergence of the fourth trifoliolate leaf with Miracle-Gro at 0.25 × the manufacturer's recommended rate.

#### *Southern and Northern blotting*

Genomic DNA was extracted from young soybean leaves using the method of Dellaporta *et al.* (1983). Integration of the phytase transgene was analyzed by digesting genomic DNA (~10 µg) with *Hind*III, *Bst*EII, or *Bst*XI. A restriction map of genomic DNA from the soybean phytase coding region is shown in Figure 1A. Three *Hind*III sites in the phytase genomic sequence are all located within introns. Two *Bst*EII sites are located within the phytase genomic sequence, one in an exon and one in an intron. There is a single *Bst*XI site

located within an intron. The expression plasmid pPHY contains a single *Hind*III site outside the cDNA coding sequence, and a single *Bst*EII within the phytase cDNA at nucleotide position 641, and lacks any *Bst*XI sites. DNA was separated by 1.0% agarose gel electrophoresis and blotted on to nylon membranes (Sambrook *et al.*, 1989). Membranes were probed with the full length phytase coding sequence (1644 bp PCR product) or hygromycin resistance gene (1 kb *Bam*HI fragment from pHygr; Finer *et al.*, 1992) labeled using a random primer labeling kit (Stratagene, La Jolla, CA, USA) and [ $\alpha$ -<sup>32</sup>P]-dCTP (PerkinElmer, Boston, MA). Primers for amplification of the phytase PCR amplicon were 5'CGGCGGATCCGATGGCGTCAATTACTTTTTCT3' and 5'CGGCAAGCTTTTATATCGAAGCAATGCAATC3'. Autoradiographs were visualized with a Storm Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Total RNA was extracted from developing soybean seeds ranging in size from 5 to 10 mm in length using TRI-Reagent (Molecular Research Center, Cincinnati, OH). Samples (~10 µg) of total RNA from each seed size were separated by formaldehyde gel electrophoresis and transferred to nylon membranes (Sambrook *et al.*, 1989).



**Figure 1.** Southern blot analysis of soybean genomic DNA to detect the phytase transgene. (A) Soybean phytase gene structure showing the genomic sequence of the phytase coding region, location of exons (black boxes), introns (white boxes) and restriction enzyme sites. (B) Genomic DNA samples from control 'Jack' and transgenic soybean plants were digested with *Hind*III (H3), *Bst*EII (BE) and *Bst*XI (BX), subjected to agarose gel electrophoresis and Southern blot analysis. Blots were hybridized with the full length (1644 bp) phytase probe. Sizes of the endogenous *Hind*III phytase fragments are shown (7.3, 4.1 and 2.3 kb). (C) Blot was re-probed with the full length hygromycin resistance gene coding region probe (1026 bp).

Membranes were probed with a 410 bp amplicon (nucleotides 78–487) from the soybean phytase cDNA. The forward and reverse primers used for amplifying the 410 bp fragment were 5'TCACTGCCATATTCCGTCAA3' and 5'GTGTGCTTGGTTCCAATCCT3'. RNA membranes were also probed for  $\beta$ -conglycinin mRNA using a PCR product of 413 bp (nucleotides 353–765) produced from soybean genomic DNA. The forward and reverse primers used for amplifying the 413 bp fragment were 5'AGCAGAAGGAGGAACACGAA3' and 5'GTTGAACTCCAAAATGCGGT3'. Hybridization probes were labeled as described above for Southern blots.

#### *Phytase activity assay*

Protein extracts were prepared from developing seeds (5–10 mm) for assays of phytase activity. Seed samples were frozen in liquid nitrogen and ground with a mortar and pestle. Total protein was extracted using a sodium acetate buffer (50 mM NaOAc, pH 4.5, 20 mM CaCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) in a 3:1 ratio of buffer volume to sample fresh weight. The extraction solution was cleared by centrifugation and the supernatant was dialyzed overnight against 50 mM NaOAc, pH 5.5. The dialyzed samples were centrifuged to remove any precipitated protein. Total protein concentration of the supernatant was determined by using the Coomassie Plus Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA).

Phytase assays were performed by incubating 100  $\mu$ l aliquots of the protein extracts with 100  $\mu$ l aliquots of 1.0 mM sodium phytate (Sigma, St. Louis, MO, USA) in 50 mM NaOAc, pH 5.5 at 55 °C for 15 min. The addition of 400  $\mu$ l of developing reagent (50% (v/v) acetone, 2.5 mM ammonium molybdate, and 1.25 N sulfuric acid) stopped phytase activity (Heinonen and Lahti, 1981). The reaction was briefly centrifuged and samples were analyzed for P by measuring absorbance at 405 nm to determine the amount of inorganic P liberated. Phosphorus concentrations were calculated from a standard curve determined using known concentrations of potassium phosphate. Enzyme assays were replicated for four transgenic and three control plants at each of six developmental sizes (5–10 mm, in 1 mm increments).

#### *HPLC analysis of IP<sub>6</sub>*

Seed IP<sub>6</sub> content was determined for individual seeds from 'Jack' control plants ( $n = 28$ ) and each of the transgenic lines SP1 ( $n = 9$ ), SP2 ( $n = 9$ ), SP3 ( $n = 18$ ) and SP4 ( $n = 9$ ). Single seeds were pulverized to a fine powder using a Wig-L-Bug (Crescent Dental, Illinois, USA) and 100 mg of sample was added to a 2.0-ml microfuge tube containing 1.5 ml of 0.4 N HCl. After incubating the solution overnight on a rotary shaker, the samples were centrifuged at 20 000  $\times g$  for 30 min. The supernatant was filtered through a 0.45  $\mu$ m nylon filter and frozen for future analysis of inositol phosphates. Frozen seed extracts were thawed at room temperature and centrifuged ( $\sim 20\ 000 \times g$  30 min) prior to HPLC analysis by the method of Dorsch *et al.* (2003). IP<sub>6</sub> was separated from other inositol phosphates by loading 50  $\mu$ l of the seed extract on to an IonPac AS7 anion-exchange column with an Ion-Pac AG7 guard column (Dionex, Sunnyvale, CA, USA) that was previously equilibrated with 10 mM 1-methylpiperazine (pH 4.0). Inositol phosphates were eluted from the column with a linear 45 min gradient from 100% 10 mM 1-methylpiperazine to 100% 0.5 M NaCl in 10 mM 1-methylpiperazine (pH 4.0) at a flow rate of 1.0 ml/min. The column eluent was mixed with a colorimetric reagent containing 50 mM 1-methylpiperazine, 0.015% FeCl<sub>3</sub>, 0.15% sulfosalicylic acid, pH 4.0 (Wade and Morgan, 1955) at a rate of 1.0 ml/min using a mixing tee before detecting the presence of IP<sub>6</sub> at 465 nm. The amount of IP<sub>6</sub> in a sample was calculated from a standard curve determined from the peak area of 50, 100, and 200  $\mu$ g standards of sodium phytate and was reported as mg IP<sub>6</sub> per gram of seed weight.

#### *Analysis of total P, available P, and phytate in SP3 seeds*

Average values for total seed P were determined from three individual seed digests of ten SP3 transgenic plants and from three individual seed digests of four control 'Jack' plants. Single seeds were wet-ashed by heating the sample in a crucible with 3.0 ml concentrated H<sub>2</sub>SO<sub>4</sub> on a hotplate until the seed dissolved, followed by addition of 30% H<sub>2</sub>O<sub>2</sub> ( $\sim 3$  ml) in 0.5 ml aliquots until the solution was clear. The resulting solubilized

sample was decanted to a 15-ml conical tube and brought to a 10 ml volume with diH<sub>2</sub>O. P values were determined by the method of Chen *et al.* (1956).

Homozygous SP3 transgenic plants were also analyzed for available P and phytate using a modification of the method of Earley and DeTurk (1944). Ten seeds from each of ten transgenic plants and four 'Jack' control plants were weighed (~1 g), placed in 10 ml of a solution containing 0.4 N HCl and 0.7 M Na<sub>2</sub>SO<sub>4</sub>, and homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). Available P was defined as acid soluble inorganic phosphate. Extracts were shaken overnight at room temperature and cleared by centrifugation. The supernatants were filtered through miracloth and a 0.45- $\mu$ m nylon filter and 1.0 ml of the sample was analyzed for available P by the method of Chen *et al.* (1956) and reported as microgram P per gram seed weight. Phytate was precipitated from a 2.0 ml aliquot of the solution by adding an equal volume of 15 mM FeCl<sub>3</sub>. The Fe-phytate precipitate was collected by centrifugation, washed twice with 0.2 N HCl, and wet ashed as described above for total P determinations. The conversion from phytate P to phytate was calculated by multiplying phytate P by 3.55, the ratio of the molecular weight of phytate to the molecular weight of phytate P.

## Results

### *Introduction of the phytase transgene into soybean*

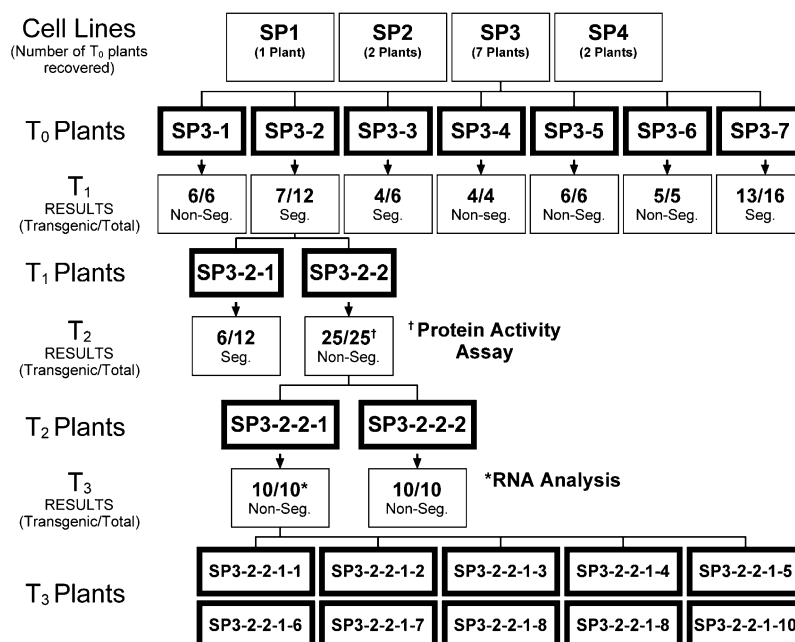
Transformation of embryogenic cultures by particle bombardment with a soybean phytase resulted in the establishment of several transgenic soybean lines, four of which were successfully regenerated to yield fertile transgenic plants (referred to as SP1-4). Genomic Southern blots were performed to test for integration of the transgene by hybridization with a phytase probe (Figure 1B). Soybean was previously shown to contain a single copy of the phytase gene (Hegeman and Grabau, 2001). Southern analysis of *Hind*III digested genomic DNA from control soybean plants (non-transformed 'Jack') using the full-length phytase cDNA probe showed three endogenous phytase bands of 7.3, 4.1 and 2.3 kb, which can be attributed to *Hind*III sites located

within the introns (Figure 1A). The large number of hybridizing *Hind*III fragments in genomic DNA digests of line SP1 indicated the presence of multiple copies, while lines SP2, SP3 and SP4 contained fewer copies of the transgene. It was estimated that line SP3 contained three copies of the transgene based on the relative intensities of the endogenous and transgenic bands. Hybridization of *Bst*EII-digested genomic DNA from the transformants also revealed three bands representing the endogenous phytase copy and showed additional large fragments that confirmed integration of the construct into the genome. *Bst*XI digestion showed two endogenous phytase bands in 'Jack' controls and additional fragments representing the integrated transgene in transformants. Reprobing the blot with the hygromycin probe showed the absence of the selectable marker in control DNA from non-transformed 'Jack' cultivar and integration of the selectable marker in the transformants (Figure 1C). The selectable marker appears to be inserted into many of the same genomic fragments as the phytase transgene as is common in biolistic transformation (Powlowski and Somers, 1998).

After the first generation, transgenic plants were indistinguishable from control 'Jack' soybeans in growth characteristics. Mean seed weights for the four transgenic lines ranged from 79 to 133% of control 'Jack' soybeans. Three of the four transgenic lines showed germination rates >97%. Germination of one of the lines (SP4) was somewhat lower (78%), but this compared favorably to control 'Jack' soybeans grown at the same time (germination rate of 68%). Phytase activity was assayed in germinating T<sub>2</sub> seeds to test for possible silencing effects on endogenous phytase expression. All four transgenic lines demonstrated mean phytase activity levels equivalent to or higher than control 'Jack' seeds in protein extracts from cotyledons at 4 to 16 days after imbibition (data not shown).

### *Heritability of the phytase transgene*

Based on the low insert number, line SP3 was examined further for heritability and genetic stability of the phytase transgene (Figure 2). Seven fertile T<sub>0</sub> plants (SP3-1 through SP3-7) were recovered from the initial SP3 transformed culture line, and 55 T<sub>1</sub> progeny were examined to determine inheritance patterns. T<sub>1</sub> progeny from three



*Figure 2.* Pedigree of transformed soybean plants and segregation results for SP3. Soybeans transformed with the phytase transgene are indicated along with the designation of individuals advanced to the next generation. Bold boxes represent individual plants propagated to another generation. Non-bolded boxes indicate the population of plants produced from an individual plant. Twelve T<sub>0</sub> plants were recovered from four stably-transformed embryogenic cultures. For the SP3 line, three T<sub>1</sub> plants showed segregation (seg.), SP3-2, SP3-3, SP3-7, and four were homozygous (non-seg.) for the transgene. Two T<sub>1</sub> plants (SP3-2-1 & SP3-2-2) were chosen for further propagation. One of those plants, SP3-2-1, segregated in the T<sub>2</sub> generation and the other, SP3-2-2, produced a homozygous T<sub>2</sub> population. Two T<sub>2</sub> plants, SP3-2-2-1 & SP3-2-2-2, both produced homozygous progeny for the presence of the phytase transgene. Populations were analyzed for protein activity and RNA expression are indicated by † and \*, respectively.

T<sub>0</sub> plants (SP3-2, SP3-3, and SP3-7) included individuals that lacked the phytase transgene by hybridization, indicating segregation for the insert. T<sub>1</sub> progeny from the remaining four T<sub>0</sub> plants (SP3-1, SP3-4, SP3-5, SP3-6) all contained the transgene and did not segregate. Seeds from two T<sub>1</sub> plants, SP3-2-1 (segregating) and SP3-2-2 (non-segregating), were grown further for analysis in the next generation. T<sub>2</sub> progeny from SP3-2-1 continued to segregate for the transgene (6 of 12 plants tested were positive). All 25 T<sub>2</sub> plants tested from SP3-2-2 contained the phytase transgene, indicating they were derived from a homozygous individual. Two of the 25 T<sub>2</sub> plants (SP3-2-2-1 and SP3-2-2-2) were tested in the next generation and yielded only phytase-containing progeny.

#### *Phytase expression in developing seed*

Phytase enzyme activity and RNA were examined in developing seeds from the homozygous T<sub>2</sub> SP3-2-2 and T<sub>3</sub> SP3-2-2-1 plants, respectively. Total

RNA was extracted from immature seeds of homozygous T<sub>2</sub> plants (T<sub>3</sub> seeds) at different developmental stages. Northern blotting results obtained using a 410 bp probe from the 5' end of the phytase coding sequence showed the absence of phytase mRNA expression in the control 'Jack' seeds (Figure 3A). In transgenic plants, phytase mRNA was detectable in immature seeds by 6 mm and expression increased as the seeds developed. Phytase transgene expression patterns paralleled the pattern of  $\beta$ -conglycinin gene expression as seen by re-probing the RNA blot with a  $\beta$ -conglycinin fragment (Figure 3B).

To measure phytase enzyme activity in developing seeds, protein extracts were prepared from control and transgenic seeds throughout seed development (from 5 to 10 mm). Phytase activity was quantified as the amount of phosphorus liberated from the sodium phytate substrate. Phytase activity was elevated above background levels in developing seeds from transgenic plants by 6 mm and activity continued to increase up to a seed size

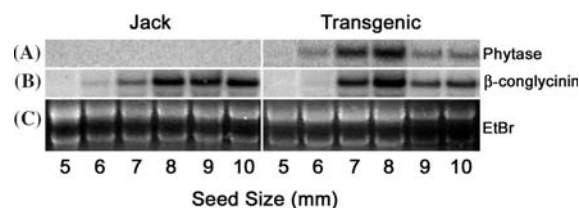


Figure 3. Northern blot analysis of phytase expression in control and transgenic soybean plants. Total RNA was extracted from developing seeds (5–10 mm) and analyzed by hybridization with (A) the 410 bp phytase probe, and (B) the 413 bp  $\beta$ -conglycinin probe. (C) RNA quality and loading was assessed by ethidium bromide staining.

of 10 mm (Figure 4). Phytase activity in control 'Jack' seeds remained low throughout development ( $0.1 \mu\text{g P}/\mu\text{g protein per hour}$ ), while the phytase activity increased 10-fold above control seeds in 10 mm transgenic seeds. The timing observed for the increase in phytase enzyme activity (Figure 4) corresponded well with expression of phytase mRNA in immature seeds by 6 mm (Figure 3A).

#### HPLC analysis of specific IP<sub>6</sub> content in T<sub>2</sub> seeds

To quantify specific IP<sub>6</sub> levels, we analyzed seed extracts from four transgenic lines and the control 'Jack' cultivar by HPLC (Figure 5). Mean IP<sub>6</sub> values ranged from 22 to 25 mg per gram seed

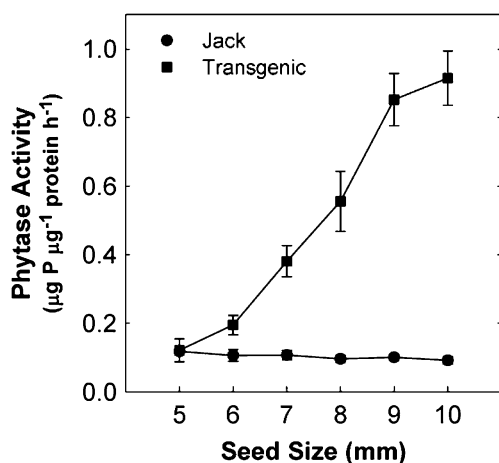


Figure 4. Phytase activity in developing seeds. Enzyme assays were performed on protein extracts prepared from immature seeds (5–10 mm) from control (●) and transgenic (■) soybean plants. Phytase activity was measured as the amount of phosphate released from sodium phytate per unit of total protein. Error bars represent standard error of the mean.

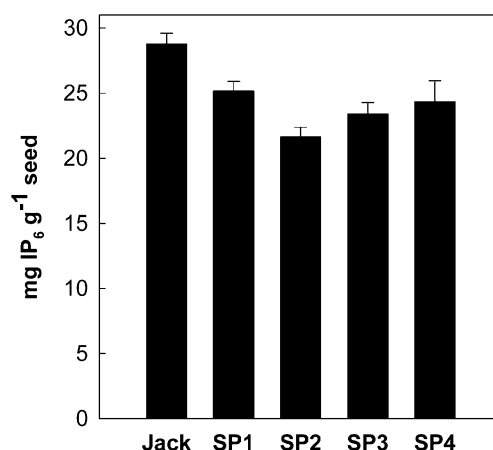


Figure 5. Seed IP<sub>6</sub> content in control and transgenic soybeans. IP<sub>6</sub> was measured by HPLC in mature T<sub>2</sub> seed of control ( $n = 28$ ), and transgenic plants, SP1 ( $n = 9$ ), SP2 ( $n = 9$ ), SP3 ( $n = 18$ ), SP4 ( $n = 9$ ). Error bars represent standard error of the mean.

weight for the transformants and 29 mg IP<sub>6</sub> per gram for control 'Jack' soybeans. This represented an average reduction in seed IP<sub>6</sub> content of 12.6, 24.8, 18.7, and 15.4% for the four lines SP1-4, respectively. Although we analyzed multiple seeds from multiple plants to generate mean values, the analysis of T<sub>2</sub> rather than T<sub>4</sub> generation includes the possibility that segregants lacking the transgene were present among the seeds tested. Nevertheless, a reduction in seed phytate content was observed for all four transgenic lines.

#### Analysis of phytate and P in mature SP3 seed

Samples of mature T<sub>4</sub> seed from homozygous SP3 plants were assayed by acid solubilization and ferric chloride precipitation as an additional method for estimating available P and seed phytate content. By this assay method, phytate content showed a reduction of ~8% in SP3 seeds compared to control seeds (Figure 6). Mean phytate P content in control plants was  $5237 \mu\text{g P}$  per gram of seed or the equivalent of 18.59 mg of phytate per gram of seed. Transgenic SP3 phytate P content was  $4809 \mu\text{g P}$  per gram of seed or 17.07 mg of phytate per gram of seed. There was no significant difference in total P content between control ( $7032 \pm 295 \mu\text{g P}$  per gram of seed) and transgenic seed ( $6828 \pm 147 \mu\text{g P}$  per gram of seed;  $P > 0.1$ ).

Available phosphorus content in control soybean seed was  $111 \mu\text{g P}$  per gram of seed compared

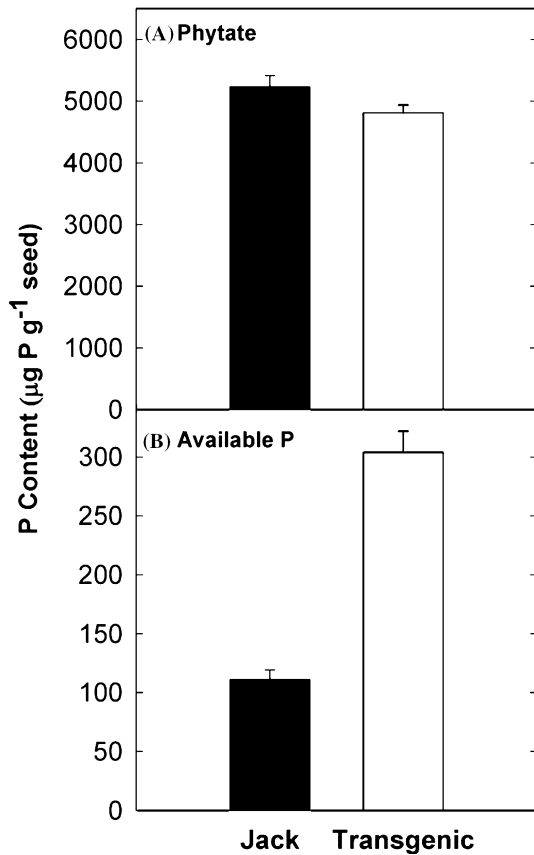


Figure 6. Seed phytate and available phosphorus content in control 'Jack' and transgenic SP3 soybeans. Phytate (A) and available phosphorus (B) were measured in mature T<sub>4</sub> seed of control ( $n = 4$ ) and transgenic ( $n = 10$ ) plants. Triplicate samples of 10 seeds each were analyzed for each T<sub>3</sub> plant tested. Error bars represent standard error of the mean.

to 304  $\mu\text{g P}$  per gram of seed from transgenic plants indicating that expression of phytase during seed development resulted in a 2.7 fold increase in available P. Detectable levels of total P and phytate P by this method in control plants were similar in range to those reported previously for field grown soybean (Raboy *et al.*, 1984).

The values for IP<sub>6</sub> content as determined by HPLC for all soybeans (transgenic and control) were higher than the levels detectable by ferric chloride precipitation. These results suggest that while detection of relative levels of phytate is reproducible using the precipitation protocol, recovery appears to be inefficient. HPLC not only allows detection of the total amount of phytic acid but also differentiates among different species of inositol phosphates, making it the method of choice for accurate and specific identification.

## Discussion

Fertile soybean plants containing a phytase transgene were recovered from four different transformed embryogenic culture lines. Phytase RNA and enzyme activity were detected in developing soybean seeds from regenerated plants. Phytase expression closely paralleled the  $\beta$ -conglycinin expression as expected from the use of the seed-specific  $\beta$ -conglycinin promoter in the transgene construct. The use of acid extraction and ferric chloride precipitation protocols showed an average seed phytate reduction of 8% and a nearly three-fold increase in P availability, without a change in total P content of mature soybean SP3 seed. However, a more precise evaluation of individual inositol phosphate species for all four transformants showed a reduction of specific IP<sub>6</sub> levels from 12.6 to 24.8%.

The range of phytate reduction previously reported for soybean mutants generated by ethyl methanesulfonate treatment was 29–55%, with an accompanying increase in available P of 3- to 6-fold (Wilcox *et al.*, 2000). In the present study, a similar 3-fold increase in available P was observed in SP3 transgenic plants. Soybean mutants generated for reduced raffinose and stachyose sugars yielded plants that also exhibited a significant reduction in phytate and increased available P (Hitz *et al.*, 2002). One mutant contained an amino acid substitution in *myo*-inositol 1-phosphate synthase, leading to impaired enzyme activity and the reduction of down-stream products, including phytate and raffinose oligosaccharides.

While promising as a demonstration of feasibility, our first set of phytase transformants did not yield the 60–80% reductions in phytate content reported in other studies. Possible explanations for the limited reduction in phytate content may include sub-optimal timing of phytase expression, incorrect sub-cellular protein localization, or possible inhibition of enzyme activity.

Seed specific phytase transgene expression was controlled by the soybean  $\beta$ -conglycinin promoter, which is expressed later in seed development than the onset of phytate accumulation. Phytate accumulation begins early in soybean embryogenesis and continues linearly throughout seed development to seed maturity (Raboy and Dickinson, 1987). In contrast, the  $\beta$ -conglycinin promoter is activated during the mid-to-late stages of seed

development (Chen *et al.*, 1989). Transgenic phytase expression may lack sufficient synchronization with phytate biosynthesis, thus effectively reducing the total phytate available for enzymatic degradation.

To address the timing of expression, phytase could be introduced under control of an alternative promoter. The soybean gene for *myo*-inositol-3-P synthase (MIPS) is expressed very early in seed development (Hegeman *et al.*, 2001). MIPS catalyzes the initial step in phytic acid biosynthesis, the conversion from glucose-6-phosphate to *myo*-inositol-3-phosphate. Use of the MIPS promoter would time phytase expression to coincide with phytic acid synthesis.

Inhibition of soybean phytase activity occurred when phytate, ATP, or polyphosphate concentrations exceeded 5.0 mM (Hegeman and Grabau, 2001). Additionally, phosphate concentrations of 18  $\mu$ M were reported to strongly inhibit phytase activity (Gibson and Ullah, 1988). Abundant accumulation of phytate in developing seeds could be inhibitory to phytase. Even the 17.07 mg of phytate per gram of seed for SP3 estimated by the ferric chloride precipitation method, which results in approximately 26 mM phytate, would be inhibitory to phytase unless compartmentalized during development. In addition, available phosphate concentrations in control plants were estimated to exceed 3.0 mM, much higher than the 18  $\mu$ M previously reported to inhibit phytase activity.

Phytate biosynthesis is postulated to take place in the cytoplasm and phytate is subsequently transported and sequestered into protein bodies (Greenwood and Bewley, 1984). Localization of the recombinant phytase in developing seeds is unknown and may not have been effective in the degradation of accumulated phytate if not localized with phytate into protein bodies. Phytase activities were measured in developing seeds verifying that active protein was produced during seed development. Unlike the fungal phytase that is secreted, the transgenic phytase may not be transported out of the cell but retained in the cytosol where optimum conditions for phytate degradation may be sub-optimal. Transgenic soybean phytase is an acid phosphatase that has a pH optimum of 4.5 and its activity declines as the pH approaches 7.0 (Hegeman and Grabau, 2001). Any sub-optimal cytosolic localization of the enzyme could be resolved by targeting phytase to vacuolar compartments. An

*Escherichia coli* phytase that was targeted to vacuoles with a soybean lectin signal sequence proved to be an effective method for reducing phytate in *Arabidopsis* seed (Coello *et al.*, 2001). Targeting the soybean phytase to protein bodies may increase the effectiveness of transgenic phytase if access to the substrate is a limiting factor.

Our results suggest that expressing phytase during seed development may offer an effective strategy for improving phosphorus availability in seeds. In these experiments, phytate reductions were obtained without regard to optimal timing of expression or protein localization. Altering phytase expression or sub-cellular localization of the enzyme to the site of phytate accumulation early in seed development may result in an optimized system for reducing seed phytate. Further reductions in phytate content should be possible without significant deleterious effects on viability or growth. The ability to reduce the amount of phytate in seed will improve nutrient availability for animal feed and reduce the environmental impact of livestock production.

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