


## Isolation of soybean mutants with high and low inorganic phosphorus

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**Abstract** In soybean (*Glycine max* (L.) Merr.) seeds, phosphorus (P) is primarily stored in the form of phytate, which is generally indigestible by monogastric animals such as human, pig, poultry, and fish. Thus, this study was conducted to isolate soybean mutants with high available P. Inorganic P content was assessed in a total of 1,266 ethyl methanesulfonate (EMS) M<sub>4</sub> lines from the Pungsannamul cultivar. Among the tested lines, four EMS lines (PE379, PE432, PE2205, and PE2503) showed higher mean inorganic P (1.21–1.56 g kg<sup>-1</sup>) than did the Pungsannamul cultivar (0.90 g kg<sup>-1</sup>). Additionally, six EMS lines (PE718, PE828, PE1466, PE1552, PE3378, and PE3386) showed lower mean inorganic P (0.38–0.60 g kg<sup>-1</sup>). The high inorganic P mutants isolated in this study will be further investigated for phytate and total P levels. Moreover, the high and low inorganic P lines will be utilized in a future study of the biochemical pathway of phytate.

**Keywords** *Glycine max* · Inorganic phosphorus · Phytate · Soybean

### Introduction

Plant seeds accumulate phosphorus (P) primarily in the form of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate; Gillman et al. 2009). The phytic acid is found chelated with cationic minerals, thereby reducing availability of Ca, Mg, Fe, and Zn in the form of phytate (Brown and Solomons 1991; Mendoza et al. 1998; Raboy 2002; Bilyeu et al. 2008). Phytate is detected in most economically important crops such as soybean, Jatropha, wheat, barley, maize, groundnut, rapeseed, sunflower, niger, and poppy (Eklund 1975; Platt and Clydesdale 1987; Fredlund et al. 1997; Wilcox et al. 2000; Mendoza et al. 2001; Makkar and Becker 2009; Hande et al. 2013). However, monogastric animals such as human, pig, poultry, and fish have limited ability to digest and utilize phytate (Brinch-Pedersen et al. 2002). The undigested and excreted phytate, in turn, causes water pollution and eutrophication in the environment (Gillman et al. 2009; Hande et al., 2013). Most phosphorus content in soybean (*Glycine max* (L.) Merr.) seeds is in the form of phytate (65–85%), leading to a deficiency in available P (Bilyeu et al. 2008). Therefore, soybean meal is usually enhanced with supplemental mineral P and/or phytase enzyme to increase the availability of P (Adeola et al. 1995; Almeida et al. 2013).

Research on development of soybean mutants with low phytate has been conducted after improvement of a simple and rapid assay to evaluate inorganic P (Wilcox et al. 2000; Hitz et al. 2002). Wilcox et al. (2000) developed two independent soybean mutants, M153 and M766, by mutagenesis using ethyl methanesulfonate (EMS). Both EMS lines showed an increase in inorganic P and decrease in phytate. The M153 line had the highest levels of inorganic P and was extensively used in development of several independent breeding lines such as the CX1834 series (CX1834-1-2, CX1834-1-3, and CX1834-1-6; Oltmans et al. 2004; Walker et al. 2006). Similar mutants with high inorganic P and low phytate were produced in barley (Larson et al. 1998) and maize (Raboy et al. 2000).

Hitz et al. (2002) showed that the myo-inositol phosphate

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synthase 1 (*MIP51*) gene was responsible for low levels of phytate in soybean mutant line LR33. However, sequence analysis of the *MIPS* gene and genetic mapping showed that it was not responsible for low phytate levels in another soybean mutant line, CX1834 (Chappell et al. 2006; Maroof et al. 2009). In a low-phytate mutant of maize (*low phytic acid 1, lpa1*), a multidrug resistance-associated protein (MRP) ATP-binding cassette transporter-encoding gene (*MRP4*) was identified as causative for the low phytate trait (Shi et al. 2007). This *MRP4* gene was identified as most closely related to the *MRP5* gene in *Arabidopsis*, which contains an integral transmembrane domain and a cytosolic ATP-binding domain (Klein et al. 2006; Shi et al. 2007). In soybean, Gillman et al. (2009) identified two homologs of the maize *MRP4* gene and *Arabidopsis MRP5* gene: *Glyma03g32500 (lpa1)* and *Glyma19g35230 (lpa2)*. They also discovered two independent mutations in the CX1834 mutant line with two recessive alleles (a nonsense mutation in the *lpa1-a* and a missense mutation in *lpa2-a*). Analysis for inorganic P in CX1834 showed an association between homozygosity for the *lpa1-a* and *lpa2-a* mutations and increased levels of inorganic P. In addition, another novel allele with a nonsense mutation (*lpa2-b*) was identified in the M766 mutant (Gillman et al. 2009).

The objective of this study was to isolate soybean mutants with increased available inorganic P in EMS mutant lines derived from the cultivar Pungsannamul. Further, genetic and molecular analyses of high and low inorganic P (HIP and LIP, respectively)

mutant lines will be helpful in understanding the phytate biochemical pathway.

## Materials and Methods

### Plant materials

To test the inorganic P trait, a total of 1,266 M<sub>4</sub> EMS lines were produced from a single M<sub>3</sub> plant of each line derived from the Pungsannamul cultivar (Lee et al. 2015; Table 1). Cultivars Pungsannamul, Williams 82, and the high inorganic P mutant 560IT-1316 (developed from the CX1834 series, CX1834-1-2 X 560IT) were used as controls. Individual seeds of four HIP (PE379, PE432, PE2205, and PE2503) and six LIP (PE718, PE828, PE1466, PE1552, PE3378, and PE3386) mutant lines were analyzed for inorganic P level (Table 2).

### Colorimetric method for inorganic phosphate determination

Inorganic P content was quantified using the modified method described by Wilcox et al. (2000). Three to four seeds of each line (0.5 g in total) were crushed with a hammer and extracted overnight with vigorous stirring in 5 mL of extraction buffer (12.5 % [w/v] trichloroacetic acid in 25 mM MgCl<sub>2</sub>). After allowing particulates to settle, 1 mL of seed extract was centrifuged at 11,269 g for 10 min. The assay (1000 μL total volume) was prepared by combining a 60 μL aliquot of the seed extract, 690 μL

**Table 1** Inorganic P content in lines of an EMS treated population of Pungsannamul (with 0.90±0.05 g kg<sup>-1</sup> inorganic P)

Inorganic P (g kg <sup>-1</sup> )	0.40~0.50	~0.60	~0.70	~0.80	~0.90	~1.00	~1.10	~1.20	~1.30
No. of lines (Total 1,266)	6	73	303	200	368	219	83	8	4

**Table 2** Inorganic P content (g kg<sup>-1</sup>) of individual seeds from each mutant line isolated with low and high inorganic P

Seed No.	Low inorganic P (LIP)						High inorganic P (HIP)				Controls		
	PE718	PE828	PE1466	PE1552	PE3378	PE3386	PE379	PE432	PE2205	PE2503	Pungsannamul	560IT-1316	Williams 82
1	0.59	0.43	0.27	0.48	0.43	0.63	2.43	2.43	1.90	1.73	1.08	2.96	0.89
2	0.58	0.50	0.57	0.44	0.64	0.52	2.08	2.40	1.55	1.45	0.95	2.86	0.82
3	0.58	0.56	0.48	0.42	0.51	0.61	1.88	1.83	1.48	1.26	0.94	2.86	0.80
4	0.55	0.55	0.70	0.41	0.54	0.65	1.88	1.73	1.48	1.26	0.93	2.77	0.78
5	0.55	0.63	0.38	0.40	0.53	0.79	1.61	1.61	1.44	1.19	0.91	2.71	0.78
6	0.54	0.52	0.55	0.39	0.68	0.54	1.38	1.52	1.44	1.19	0.90	2.42	0.69
7	0.53	0.64	0.55	0.39	0.44	0.62	1.32	1.45	1.30	1.19	0.89	2.38	0.64
8	0.52	0.50	0.78	0.37	0.81	0.45	1.32	1.38	1.30	1.08	0.88	2.31	0.63
9	0.51	0.47	0.63	0.37	0.68	0.63	1.26	1.33	1.26	1.06	0.86	2.25	
10	0.51	0.50	0.52	0.34	0.61	0.37	1.19	1.08	1.21	1.06	0.85	2.25	
11	0.50	0.51	0.42	0.29	0.46	0.62	1.19	1.04	0.97	1.01	0.83	2.17	
12	0.49	0.53	0.44	0.28	0.54	0.79	1.03	1.00	0.97	1.00	0.77	1.99	
Mean	0.54	0.53	0.52	0.38	0.57	0.60	1.55	1.56	1.36	1.21	0.90	2.49	0.75
SD*	0.03	0.06	0.13	0.06	0.11	0.12	0.41	0.45	0.24	0.20	0.07	0.31	0.09

\*SD, standard deviation

of ddH<sub>2</sub>O, and 250 µL of colorimetric reagent (1 volume of 3 M H<sub>2</sub>SO<sub>4</sub>, 1 volume of 0.02 M ammonium molybdate tetrahydrate, 1 volume of 10 % [v/v] ascorbic acid, and 2 volumes of ddH<sub>2</sub>O). The assay mix was allowed to incubate at 40°C for 60 min, and then absorbance was read at 655 nm in a Smartspec 3000 (Bio-Rad, CA, USA). A standard curve was generated with P standards (0.05 mg/mL; Megazyme, Wicklow, Ireland). Inorganic P content was converted to g kg<sup>-1</sup> (g of P per kg of seed).

## Results and Discussion

Initial screening of inorganic P content in seeds identified four HIP (>1.20 g kg<sup>-1</sup>) and six LIP (<0.60 g kg<sup>-1</sup>) mutant lines among 1,266 M<sub>4</sub> EMS lines when compared with Pungsannamul (0.90 g kg<sup>-1</sup>; Table 1). Individual seeds of each mutant line from initial screening were further tested to verify inorganic P content and to analyze homozygosity in part (Table 2). The four HIP (PE379, PE432, PE2205, and PE2503) mutant lines showed higher average values of inorganic P (1.55, 1.56, 1.36, and 1.21 g kg<sup>-1</sup>), corresponding to 72, 73, 51, and 34 % increase over Pungsannamul. In contrast, the six LIP (PE718, PE828, PE1466, PE1552, PE3378, and PE3386) mutant lines showed lower mean values of inorganic P (0.54, 0.53, 0.52, 0.38, 0.57, and 0.60 g kg<sup>-1</sup>), corresponding to 40, 41, 42, 58, 37, and 33 % decrease compared to Pungsannamul (Table 2). Three LIP mutant lines (PE718, PE828, and PE1552) showed very low variation in inorganic P among the individual seeds for each line. This finding suggests that M<sub>4</sub> plants of these lines might be fixed to homozygosity. However, the other three LIP (PE1466, PE3378, and PE3386) and four HIP (PE379, PE432, PE2205, and PE2503) mutant lines showed greater variation in inorganic P content per seed, suggesting that these lines may be heterozygous at the locus of interest or that the relevant traits might be controlled by two loci. Therefore, next generations of these lines will be carried forward to obtain homozygosity. Comparisons of the mutant lines (HIP and LIP) with Williams 82 (0.75 g kg<sup>-1</sup>) gave similar results to those with Pungsannamul (Table 2).

Wilcox et al. (2000) identified two soybean mutants with high inorganic seed P, M153 and M766. M<sub>6</sub> progeny of M153 varied in inorganic P content from 2.34 to 4.41 g kg<sup>-1</sup> and M766 had 1.21–3.84 g kg<sup>-1</sup>, compared to the cultivar Athrow (0.74 g kg<sup>-1</sup> inorganic P). They predicted that 75 % of the total P in seeds of soybean *lpa* mutants is available P, whereas nonmutant soybean have approximately 25 % available P (Wilcox et al. 2000). Similarly, the earlier reported *lpa* mutant lines in corn, barley, and rice showed more than a 50 % reduction of phytate level (Raboy and Gerbasi 1996; Ertl et al. 1998; Larson et al. 1998; Larson et al. 2000). Several reports of seed phosphorus fractions in *lpa* mutants indicate that reduction in phytate contents are largely associated with increases in inorganic P (Raboy 2007), suggesting that HIP mutants may contain low phytate levels.

In seeds, phytate synthesis pathway is complex and non-linear (Raboy 2007). The *lpa* mutant line that we used as a control, 560IT-1316, showed an average inorganic P content of 2.49 g kg<sup>-1</sup> (Table 2). Increase of inorganic P content in 560IT-1316 was due to homozygosity for double mutation in *lpa1* and *lpa2* genes (*lpa1-a* and *lpa2-a* alleles). However, a single mutant, *lpa1* or *lpa2* showed slight increase in inorganic P level compared to wild-type control (Gillman et al. 2009). Therefore, although the HIP lines showed less inorganic P than that of 560IT-1316, we suggest that these mutants might have mutation(s) in either one of *lpa* genes or mutation at different locus that encodes another enzyme involved in phytate synthesis pathway. Thus, crosses between HIP lines and wild-type control will be carried out subsequently after the selection of homozygous lines to identify the molecular basis of these HIP mutants and understand the biosynthetic pathway of phytate.

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