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Evaluation and QTL mapping of phosphorus concentration in soybean seed

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Abstract Phosphorus (P) is an essential macronutrient required for many biological and metabolic plant functions. Phosphate is the form of P used by plants. Reducing the amount of P in the seed can further aide breeding efforts to reduce the amount of P released into the environment due to the indigestibility of the complexes formed with metal ions. Analysis of the variation of phosphorus concentration in soybean seed under non-stressed conditions revealed that phosphorus ranged from 3,948.1 to 5,695.8 μg/g total phosphorous (TP) in combined years. The averages for independent years were significantly different from one another. Quantitative trait loci (QTL) analysis of TP was performed to identify candidate gene(s) that is (are) involved in P accumulation in soybean seed. One putative QTL region was identified on chromosome 12 in the combined data that contained a phosphate transporter gene. Two additional suggestive QTL were identified on chromosomes 7 and 17 with chromosome 7 having both a phosphate transport gene and a ZIP transporter gene in the region of the QTL. There were additional genes in these regions that are involved in phosphate metabolism and transport.

Keywords Phosphorus · Phytate · QTL · Seed

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Introduction

Phosphorus (P) is an essential macronutrient for all living organisms (Poirier and Bucher 2002). In plants, it is present both as a constituent of such compounds as nucleic acids, phospholipids and ATP. It also has roles as a metabolite involved in energy transfer, in the activation of proteins and in the regulation of metabolic processes (Marschner 1995). Plants take up P from the soil in the form of orthophosphate (Pi). Growth and development can be limited by the availability of Pi in most natural ecosystems (Smith et al. 2003). The availability of Pi is often limited due to it being in the soil in complex, insoluble, inorganic, and organic forms which cannot be acquired directly by the plant (Raghothama 1999; Chiou et al. 2001).

In legumes such as soybean (*Glycine max* (L.) Merr.), phytate (myo-inositol 1,2,3,4,5,6 hexakisphosphate, Ins P_6) is the major storage form of phosphorus (Reddy 1989; Raboy 1997). Phytate makes up ~75 %

of the total phosphorus (TP) in the seed and about 1–2 % of the seed composition. This composition would make TP a good measurement criterion to establish an estimate of the phytic acid (PA) status in a population (Raboy et al. 1984; Raboy 1997; Bilyeu et al. 2008). Ins P_6 represents a major pool or bottle neck in the flux of P in the world's agricultural ecology because it covalently binds to cations such as K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}. This binding results in a reduction of bioavailability of P and the cations that are complexed with it (Schlemmer et al. 1995; Tsao et al. 1997; Raboy et al. 2001). Ultimately this presents a problem because Ins P_6 is not readily digested by monogastric animals (Maupin et al. 2011). This underutilized Ins P_6 is then excreted in manure leading to an increase of P pollution and fouling of groundwater and eutrophication of inland bodies of water (Sharpley and Withers 1994; Correll 1998; Raboy 2001).

Exploiting natural variation in a population is the hallmark of plant breeding. A survey of mineral content and their interactions can provide information for breeding efforts directed toward increasing the concentration of some minerals and decreasing others. The population used in this study, Anoka × A7, had been previously characterized to have a major QTL for Fe efficiency on chromosome 3/LG N (Lin et al. 1997). The objective of this research was to determine the extent of variation of total P in the Anoka × A7 mapping population in order to determine the efficacy of using total P as a selection factor in breeding and to map total P concentration as a QTL. Understanding the extent of variation in this population could potentially aide in the selection of genotypes that are more efficient in nutrient uptake and transport.

Materials and methods

Plant material

The plant material used for QTL mapping soybean seed total phosphorus included 92 $F_{2:4}$ lines from Anoka × A7, which was previously used to identify a major QTL for Fe efficiency (Lin et al. 1997). A7 was the Fe efficient parent developed through a recurrent selection program, and Anoka was an Fe inefficient cultivar (de Cianzio and Fehr 1980; Fehr et al. 1984).

Phenotypic evaluations

The 92 F_2-derived lines and checks were planted at Bruner Farm, near Ames, IA on non-calcareous (Clarion–Nicollet loam soil type (fine-loamy, mixed, superactive, mesic Typic Hapludoll and fine-loamy, mixed, superactive, mesic Aquic Hapludoll) soil in 2008 and 2009. The experiment was set up as a randomized complete block design (RCBD) with three replications. Each line was planted in plots of forty seeds and no thinning was performed upon emergence. The plots were 1.52 m long and separated from adjacent plots by 0.91 m. Leaf samples were taken from each line from the second fully expanded trifoliate, about 3–4 weeks after planting. Leaf tissue was then stored in liquid nitrogen before being lyophilized for DNA extraction and marker analysis. Upon maturity, seed from each plot was harvested in bulk for analysis. Total phosphorus was determined using the slightly modified procedures of Gao et al. (2007) and Zheng et al. (2009). Briefly, 5 g of seed from each line were ground using a Foss Cyclotec mill (1093 Sample Mill, Foss, Eden Prairie, MN) equipped with a 1-mm screen. A 0.5 g subsample of soybean powder was taken from each genotype and vortexed with 10 ml of 2.4 % HCl in 14-ml falcon tubes until a milky-white solution could be observed. Tubes were stacked in tube racks, the racks placed on their sides, and tubes and racks were secured using laboratory tape. The samples were digested overnight ($\sim$16 h) and shaken at 220 rpm on a bench-top shaker (Daigger orbital shaker, Vernon Hills, Ill.) at room temperature. To obtain a crude extract, tubes were then centrifuged at 2,900×g at 10 °C for 15 min (Beckman Coulter, Avanti-J26XPI), and 500 µl of the supernatant of each was pipetted into a 2 ml microcentrifuge tube. Additionally, 500 µl of 20 % NaCl solution was added to the top of each sample. This crude extract was precipitated at room temperature for one to 2 h and then centrifuged at 16,100×g for 15 min on a Labnet International, Inc. Spectrafuge 24D microcentrifuge to obtain a supernatant. An aliquot of 400 µl of each supernatant was then pipetted into a 14-ml falcon tube pre-filled with 9.6 ml of double-distilled water (ddH_2O) for a dilution of 25 times. Calibration standards containing 0.00, 1.12, 2.24, 3.36, 5.6, 7.84, 8.96, and 11.2 ppm P from phytic acid sodium salt hydrate from rice (Sigma, St. Louis, MO.) were prepared using the same concentration of NaCl and HCl as the samples. Phosphorus concentrations were measured using inductive coupled plasma-optical

emission spectroscopy (ICP-OES) (Spectro Ciros CCD; Spectro Analytical Instruments) in the Iowa State Agronomy Soil and Plant Testing Facility. The samples were submitted as a 10 ml sample with a 400 µl aliquot of the supernatant in 9.6 ml of ddH_2O along with the calibration standards. Data were returned in total phosphorus (TP) ppm and converted to µg/g TP by multiplying by the dilution 500.

Statistical analysis

For this experiment, all effects were considered random. Data were analyzed using the JMP statistical package to determine ANOVA (Anonymous 2009). Broad sense heritability (h^2) for TP was estimated on an entry mean basis using expected mean squares from the combined ANOVA (Fehr 1987) as follows:

$$h^2 = \frac{F_g^2}{\left(\frac{F_e^2}{rxe} + \frac{F_{ge}^2}{e} + F_g^2\right)}$$

where F_g^2= genetic variance, F_{ge}^2 = genotype × environment interaction variance, F_e^2 = experimental error variance, r = number of replications, and e = number of environments.

Construction of the genetic linkage maps

The parental lines Anoka and A7 were surveyed for polymorphism with 916 SSR markers available from the SoyBase website (http://soybase.agron.iastate.edu). The most likely orders, and recombination rates among markers were estimated with Mapmaker 3.0 (Lander et al. 1987). Linkage groups were determined with the "group" command using a LOD of 4.0 and maximum Haldane distance of 50 centiMorgans (cM). Map order was determined using the "three point" command followed by "order", "framework", and "place." Previous markers that formed linkage groups for this population (Lin et al. 1997) were integrated into the SSR map. Additionally, 303 BARCSOYSSR markers (Song et al. 2010) were surveyed for polymorphism to increase marker density on chromosomes with larger marker intervals.

QTL mapping

Linkage maps were imported into MapQTL6 (van Ooijen 2009), and QTL positions and effects for soybean seed total phosphorus concentration for independent years and over combined years were determined using interval mapping (IM) and multiple-QTL mapping (MQM) (Lander and Botstein 1989; Jansen 1993; Jansen 1994). The significance threshold logarithm of the odds (LOD) scores for detection of the QTL was calculated based on 1,000 permutations at $P \leq 0.05$ (Churchill and Doerge 1994). As described by Liang et al. (2010), the IM method was used to determine locations of putative QTL for the total P. Subsequently, MQM was performed to eliminate inference from background markers. In order to reduce residual variance, background markers closest to the LOD peak were selected as cofactors. The mapping step size was 1.0 cM with the maximum number of neighboring markers five, and maximum number of iterations set to 200.

Results

Construction of genetic linkage maps

The current map was integrated with the original genetic map of the Anoka × A7 population generated by Lin et al. (1997). The original map contained of 82 RFLP, 14 SSRs, and one morphological marker. Informative marker data was obtained for an additional 146 SSRs and assigned to 20 linkage groups. One hundred six SSR markers completed the linkage map along with the 12 previously unmapped informative BARCSOYSSRs (Song et al. 2010). Using Haldane's mapping function and summing over all linkage groups, there was a total of 2,722.09 cM flanked by linked markers. As reported by (King et al. 2012; in press), the average length of the linkage groups was 136.10 cM ranging from 44.71 cM on chromosome 10 to 201.41 cM on chromosome 18. The average length of the marker intervals was 18.15 cM. The average number of markers per chromosome was 7.5, with a range from 3 to 14.

Total P determination

Using ICP-OES, we determined TP for all lines comprising the Anoka × A7 mapping population. Segregation for TP was observed (Table 1; Fig. 1a–c). The means significantly varied between years, with 2009 having a higher average mean than in 2008

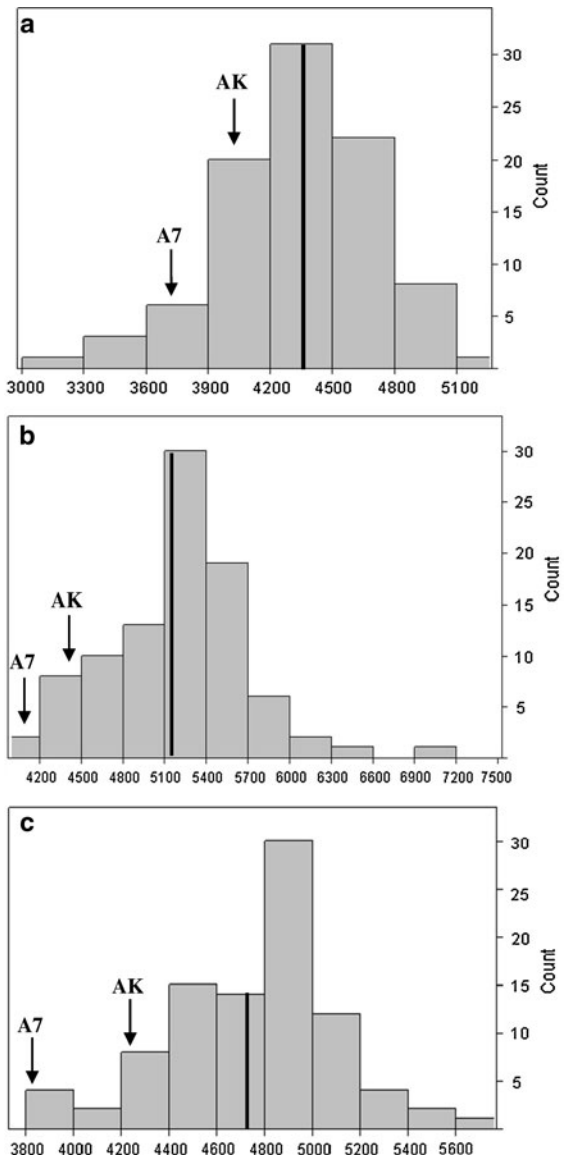

Fig. 1 Total P Distribution in the Anoka (AK) × A7 population (**a** 2008; **b** 2009; **c** combined), *vertical line* represents the population mean

(Table 2). For overall average, the mean was 4,759.4 μg/g TP. The average of the parents A7 and Anoka was 3,886.8 and 4,228.8 μg/g TP, respectively. The distribution of the combined data was slightly negatively skewed and had low kurtosis indicating a normal distribution and that TP was inherited quantitatively. The ANOVA indicated a significant year and rep within year effect with most of the variation being observed in the year effect indicating that most of variation observed is due to environmental effects. This significant year effect could be attributed to the rainy conditions observed in 2009 and hail damage received eight weeks after planting in 2008. The genotype effect was not significant at the $P = 0.05$ level, nor was the G × E interaction, indicating that one year of testing would be sufficient for evaluation of genotypes with superior genetic potential (Fehr 1987) (Table 2). The heritability estimate for TP was 0.41, indicating that about 60 % of the variation is due to non genetic factors.

Total P QTL analysis

QTL detection for loci controlling TP accumulation was determined for 2008 and 2009 and the average across years. In 2008, three suggestive QTL were detected on chromosomes 10, 18, and 20 (Table 3). Suggestive QTL were those QTL possessing peaks in LOD scores that did not exceed the genome wide threshold, but on the chromosome level were at or exceeded significant $P < 0.05$ (Liu et al. 2007; Tiwari et al. 2009; Willems et al. 2010). The LOD scores of these QTL ranged from 2.6 to 2.9 and the variation explained was 11.9–19.4 %. The positive allele for the QTL on chromosomes 10 and 18 were attributed to the A7 parent, whereas the positive allele on chromosome 20 was attributed to Anoka. In 2009, there were no QTL detected that were significant at the genome wise or chromosome wise thresholds.

With the TP averages combined over years, one significant and two suggestive QTL were detected. The major QTL was on chromosome 12 in the marker interval S12_0711–S12_1103 and had a LOD of 3.9 (Fig. 2). This QTL had a R^2 of 29.9 %, and its position was at 118.9 cM. The two suggestive QTL were on chromosomes 7 and 17. Markers *S12_0711* and S12_1103 are previously unmapped BARCSOYSSR markers used to increase marker density on chromosome 12 (Song et al. 2010). On chromosome 7, the marker interval was pk_70T-Satt308, and the QTL had LOD score of 2.4 with a R^2 of 18.4 %. The QTL on chromosome 17 was in the marker interval pa_257-Sat_277. The LOD score of this QTL was 2.3 and represented 16.3 % of the variation.

Discussion

In modern breeding programs, QTL mapping has become an increasingly important first step to marker-

Table 1 Mean (standard deviation), kurtosis, skewness, and range, in 2008 and 2009 and combined over years for the Anoka × A7 mapping population

	Population mean	Anoka	A7	Kurtosis	Skewness	Range
2008 total P (μg/g)	4,334.2 ± 392.8	4,049.6	3,712.8	0.6	−0.5	3,179.3 ~ 5,204.2
2009 total P (μg/g)	5,184.7 ± 505.9	4,407.6	4,059.6	1.9	0.5	4,134.5 ~ 7,176.8
Total P (μg/g)	4,759.4 ± 343.5	4,228.6	3,886.2	0.3	−0.2	3,948.1 ~ 5,695.8

Missing data were imputed using PROC GLIMMIX in SAS

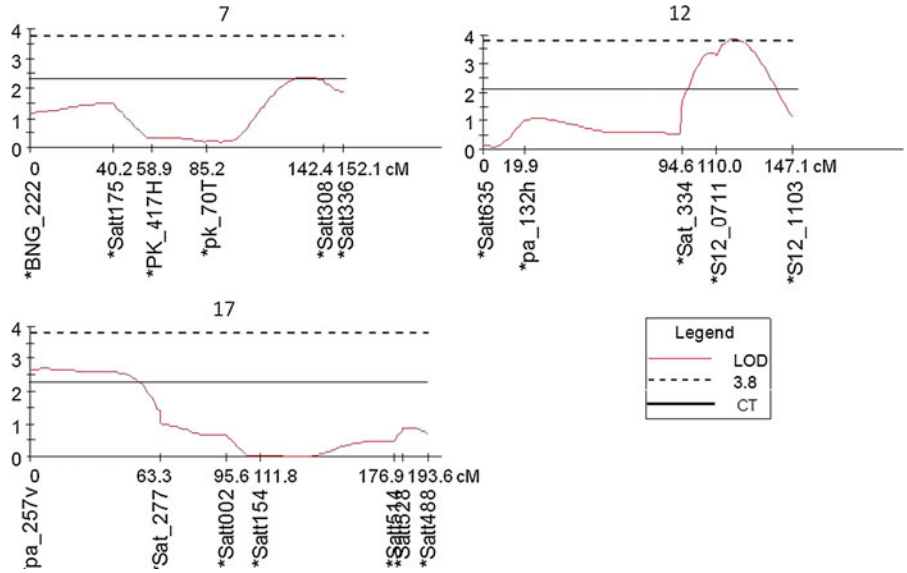

Fig. 2 TP QTL mapping in combined data with the significant QTL on Chromosome 12. *Solid line* represents the chromosome-wide threshold and the *dashed line* represents the genome-wide threshold levels of significance $P = 0.05$

Table 2 Total P ANOVA table for 2008 and 2009 with P values from the F-test

Source	DF	MS	F value	Prob > F
Year	1	99811670	181	<0.0001
Replication/year	4	7629599	13.8	<0.0001
Line	91	707752	1.28	0.0586
Year × line	91	522986	0.95	0.6144

$h^2 = 0.41$ for the data combined over years

assisted selection and gene discovery (Price 2006). In this study, TP was evaluated in soybean seed powder using ICP-OES to determine the variation within the population, and the trait was mapped as a QTL. Total P ranged between 3,948.1 and 5695.8 μg/g with an average of 4,759.4 μg/g in the combined data. TP was not significant within this population, which was similar to a finding of Oltmans et al. (2005). However, there is still the possibility that there are significant differences in levels of PA (Raboy et al. 1984). Variation in TP is influenced by genetic and non-genetic factors. Consequently, the PA is influenced by the supply of TP to the developing seed. Identifying these genetic factors gives a better understanding of P status and its importance to P homeostasis in the normal function of major metabolic pathways (Raboy et al. 2001).

In this study, one significant QTL for TP was mapped in the combined data. Additionally, five suggestive QTL for TP in 2008 and in the combined data were identified. In the combined data, the additive effects for the suggestive and significant QTL show that the positive allele was attributed to Anoka, the Fe inefficient parent. Additionally, the parental averages of TP supported this finding. This could potentially indicate that elevated TP could impact Fe inefficiency through the supply of TP absorbed and loaded in the seed. The effect of the elevated TP then could reduce the availability of seed Fe to the plant because of the binding effect associated with the PA form of P. The identification of only one significant QTL for TP was similar to that of other research. In *Arabidopsis* for

Table 3 Summary of quantitative trait loci (QTL) detected for total phosphorus (TP) concentrations in the Anoka × A7 population

	Chr/LG	Marker interval	Position (cM)	LOD	LOD threshold	R^2	QTL genotypic class means			
							A1A1	A1A2	A2A2	Additive effects
Trait										
TP-2008	10/O	Satt477–Satt123	40.3	2.7	1.9	12.3	4,466.7	4,179.3	4,467.3	−0.3
	18/G	pk_493H–pa378H	167.7	2.6	2.5	11.9	4,281.6	4,404.6	4,652.4	−185.4
	20/I	S20_1142–pa_644H	132.7	2.9	2.3	19.4	4,667.3	4,487.6	4,193.8	236.8
Combined TP										
	7/M	pk_70T–Satt308	133.7	2.4	2.3	18.4	4,957.7	4,992.0	4,649.1	154.3
	12/H	S12_0711–S12_1103	118.9	3.9	2.2	29.9	4,995.5	4,551.0	4,723.0	136.2
	17/D2	pa_257–Sat_277	7.0	2.7	2.3	16.3	5,018.9	4,677.7	4,682.4	168.3

instance, only three QTL were identified for P content (Vreugdenhil et al. 2004). Furthermore, two different studies in *Phaseolus vulgaris* L. identified a similar number of QTL for TP concentration. In one study under medium and high P fertilizer applications, one QTL for each treatment was identified (Blair et al. 2009). In the other, several QTL were identified in differing years, however no more than three were identified per year per high or low P treatment with the R^2 ranging from 11 to 40 % (Cichy et al. 2009). Differing levels of P have also been used to identify QTL in the total plant, roots, shoots, and leaves (Li et al. 2005; Liang et al. 2010). Li et al. (2005), were able to detect two QTL for low P conditions in both the roots and leaves, also showing the difficulty in detecting QTL consistently.

Once QTL regions have been identified, the next step is to identify the gene(s) or causal mechanism(s) responsible for the observed phenotype (Paran and Zamir 2003). Furthermore, Li et al. (2005), stated that the genes controlling traits for P deficiency tolerance have not been located in soybean genome. Consequently, the evaluation of mineral accumulation in soybean seed for QTL mapping may offer insight into genes encoding, for example, transporters, chelators or chelator biosynthesis enzymes (Vreugdenhil et al. 2004). The genomic locations of molecular markers on the soybean genome sequence assembly (Gmax_109) were determined to obtain the intervals of the marker positions associated with the identified QTL (Schmutz et al. 2010; http://www.phytozome.net). This allowed for the identification of phosphate related genes. In this study two suggestive QTL were identified on chromosomes 10 and 20 (Fig. 2). The QTL region on chromosome 20 encompasses the Pi transporter gene (*Glyma20g34610*) reported recently by Wu et al. (2011) (Supplementary Table 1). Additionally, one more gene in the QTL interval is associated with phosphate transport, Glyma20g34620. These low-affinity transporter genes belong to the phosphate: H+ symporter (PHS) transporter of the major facilitator super family (Pao et al. 1998). The QTL interval on chromosome 10 is just outside of the location of the Pi transporter gene reported by Wu et al. (2011). This 'off-set' positioning could potentially be due to the paucity of polymorphic markers on this chromosome. Chromosome 10 had only three polymorphic markers that only spanned 44.7 cM. Extensive screening of public SSRs failed to identify additional markers which could potentially map the QTL more precisely. Because the QTL reported in this study were identified in a non-stressed environment without any P treatments, it may be possible that these QTL represent genic regions not only involved in phosphate transport but also seed phosphate loading. The non-stress environment could further explain the lack of a strong QTL effect observed in this work. These two chromosomes are known to be highly homeologous and possessing many duplicate loci and would explain the high sequence similarity of the coding and protein sequences that was observed (Wu et al. 2011; http://www.soybase.org).

The QTL detected for TP over combined years occurred in a manner similar to that reported by Lin et al. (1997) using the same population to study iron stress. The QTL were noted in separate years and during different growth stages. Here, the significant

and two suggestive QTLs were not detected in independent years, but were detected in combined data. The extent of the environmental influence on QTL detected potential impacted QTL across environments, which can be explained by the heritability estimate of 0.41. The QTL regions in this study, however, do contain genes that are involved in phosphorus transport and utilization. The significant QTL mapped to chromosome 12. The interval for the markers *S12_0711–S12_1103* contains several genes that are involved in mineral transport or phosphate metabolism. Genes Glyma12g16410, Glyma12g30-070, and Glyma12g30100, are all a part of the ABC transporter family, which is involved in the transport of molecules through membranes (Supplementary Table 1, (Schulz and Kolukisaoglu 2006)). Other genes in this QTL region that could have a role in TP accumulation, synthesis, and transport are Glyma12g22680, Glyma12g23920, and Glyma12g29790. Glyma12g22680 is a member of the *Inositol monophosphatase family*. The enzyme activity of this gene is essential for the de novo synthesis of *myo*-Inositol (Ins) and for the recycling of Ins into Ins (1,4,5)P_3. This enzyme is a potential regulatory point for all pathways that utilize free Ins (Fu et al. 2008). Glyma12g23920 and Glyma12g29790 are both members of the *Triose-phosphate Transporter family*, which is in the P storage and homeostasis pathways and mediates the transport of Pi between the cytosol and the stroma of the chloroplast (Flügge et al. 1989, 1999; Raboy et al. 2001).

Several genes in the suggestive QTL regions play a role in P metabolism. The most important was Glyma07g34870, which is a part of the major facilitator super family, and has a KOG annotation as an *Inorganic phosphate transporter* like that of those transporters on chromosome 10 and 20 (Tatusov et al. 2003). This gene, on chromosome 7, would have a strong potential to be involved in P transport and storage and has previously gone undetected in other studies. Also in the QTL marker interval is Glyma07g34030, a *Phosphatidylinositol-4-phosphate 5-Kinase* (PIP5K), which plays an essential role in coordinating plant growth, especially in response to environmental factors (Lou et al. 2007). It also catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to form phosphatidylinositol-4-phosphate (PIP) (Divecha and Irvine 1995). Glyma07g32190 is a solute carrier of *Glucose-6-phosphate/phosphate* which is the source of *Ins* before being converted to D-*Ins* 3-phosphate, the precursor of all remaining *Ins*-containing compounds (Loewus and Loewus 1983; Raboy et al. 2001). Finally, there was a *ZIP Zinc transporter* (Glyma07g34930) in the QTL interval, which transports iron as well. Along with the Pi transporter and this gene in the interval, this could be a potential region of interest for the beginning of mineral chelation.

Similar candidate genes identified in the other QTL intervals in this study were also identified in the QTL interval on chromosome 17. These included two *triose-phosphate transporter family* genes, which also were *Glucose-6-phosphate/phosphate transporter*, two *AUX/IAA* genes, eight *ABC transporter* genes, one *Ins 5-phosphatase*, and one *PIP5K* gene. Even though, the QTL on chromosome 17 was only suggestive, the several genes in the interval are related to P metabolism and homeostasis, and contributed small effects to this QTL being detected. As with chromosome 10, the marker interval for chromosome 17 spanned ~60.0 cM with the peak occurring at 7.0 cM. Liang et al. (2010) were able to identify QTL for phosphorus content in the root, shoot, and the entire plant within this same interval under low phosphorus treatment. The interval for the authors' QTL was Sat_296-Satt458 at the position 6.42–24.52 cM on the www.soybase.org physical map. The marker interval for the TP QTL in this study was at 10.35–11.14 cM. The QTL here, along with those in the Liang et al. (2010) had QTL with LOD scores less than 2.95. This region could potentially be important for P with QTL being detected in a non-stressed environment as well as that of the low P study of Liang et al. (2010). An increase in marker density on this chromosome would help to confirm this QTL as well as to narrow the interval and list of genes actually involved in P uptake and utilization.

In summary, the objectives of this study were to determine the natural variation of total phosphorus concentration for use as selection criteria for breeding and determine the plausibility of mapping the trait as a quantitative trait locus. The variation observed amongst genotypes was not significant but allowed the mapping of QTL for TP concentration in seeds. We mapped one putative QTL as well as several suggestive QTL. One of these suggestive QTL intervals had genes previously identified to be phosphate transporters, and additional QTL newly reported on

chromosome 7. The lack of higher marker density on chromosome 10 did not allow the detection of the other phosphate transporter as it was just outside the marker interval. Additionally, it was determined that a ZIP Fe/Zn transporter gene was in a QTL interval, which in previous work (King et al. 2012; in press) identified suggestive QTL for both Fe and Zn accumulation and could potentially be a site of metal chelation. There is still work to be done in order to validate these TP QTL regions as well as the genes involved. Furthermore, this research did not rely on the population being grown in a stressed environment, but rather determined that there is minor natural variation with minor gene effects observed. A research program designed with different rates of available P could potentially elucidate the genetic effects and differentiate which genes are involved in P uptake, transport and homeostasis in this population.

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