

Extracellular Secretion of Phytase from Transgenic Wheat Roots Allows Utilization of Phytate for Enhanced Phosphorus Uptake

Samreen Mohsin¹ · Asma Maqbool¹ · Mehwish Ashraf¹ · Kauser Abdulla Malik¹

© Springer Science+Business Media, LLC 2017

Abstract A significant portion of organic phosphorus comprises of phytates which are not available to wheat for uptake. Hence for enabling wheat to utilize organic phosphorus in form of phytate, transgenic wheat expressing phytase from *Aspergillus japonicus* under barley root-specific promoter was developed. Transgenic events were initially screened via selection media containing BASTA, followed by PCR and BASTA leaf paint assay after hardening. Out of 138 successfully regenerated T₀ events, only 12 had complete constructs and thus further analyzed. Positive T1 transgenic plants, grown in sand, exhibited 0.08–1.77, 0.02–0.67 and 0.44–2.14 fold increase in phytase activity in root extracts, intact roots and external root solution, respectively, after 4 weeks of phosphorus stress. Based on these results, T2 generation of four best transgenic events was further analyzed which showed up to 1.32, 56.89, and 15.40 fold increase in phytase activity in root extracts, intact roots and external root solution, respectively, while in case of real-time PCR, maximum fold increase of 19.8 in gene expression was observed. Transgenic lines showed 0.01–1.18 fold increase in phosphorus efficiency along with higher phosphorus content when supplied phytate or inorganic phosphorus than control plants. Thus, this transgenic wheat may aid in reducing fertilizer utilization and enhancing wheat yield.

Keywords Wheat · Root-specific expression · Phytase · *Aspergillus japonicus* · Phosphorus · Phytate

Introduction

With escalating population, a major challenge of today's world is food security. Phosphorus (P), being involved in growth and development, is an essential macronutrient for plants, and thus can play a critical part toward overcoming this challenge by increasing food productivity [1–3]. Organic forms of phosphorus constitute between 50 and 80% of phosphorus pool depending upon the type of soil [4–8]. Phytate, the most abundant organic form, is relatively unavailable, since it strongly adsorbs to soil, becoming immobile and immune to degradation by soil microbes. This contrasts with inorganic phosphorus that is readily utilized by plants [9–12].

With the advent of green evolution, use of fertilizers increased over the years [13]. However, this is not a sustainable solution for two reasons: (1) natural phosphorus reserves used for manufacturing chemical phosphorus will deplete soon (2) only 10–20% of fertilizers are utilized by plants while the remaining is converted into complex inorganic or organic forms, essentially unavailable for plants [14, 15]. Thus, efforts have been made to not only have a better understanding of the mechanisms involved in phosphorus uptake and utilization by plants but also to develop plants with enhanced phosphorus use efficiency and reduced fertilizer consumption [15].

Some of the adaptations of plants to counter phosphorus deficiency include variation in root structure and architecture, greater number of Pi transporters, mycorrhizal symbiosis, secretion of organic acids and phosphatases [11, 16–21]. Conventional and marker-assisted selection

Electronic supplementary material The online version of this article (doi:10.1007/s12033-017-0020-0) contains supplementary material, which is available to authorized users.

✉ Asma Maqbool
asmamaqbool@fccollege.edu.pk

¹ Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan

methods of exploiting genetic differences in phosphorus use efficiency for developing better wheat varieties have shown little success [3]. A better and more promising approach is improving phosphorus efficiency via genetic modifications.

Various plants have been genetically modified for enhanced phosphorus use efficiency. It has been documented that over-expression of proton-pyrophosphatase gene *AVP1/AVPID* in tomato resulted in better root structure, biomass and phosphorus acquisition [22]. Over-expression of β -expansin gene *GmEXPB2* for improving phosphorus acquisition via better root architecture has also been reported in soybean and *Arabidopsis* [23, 24]. Enhanced secretion of phosphatases, especially phytase for converting organic phosphorus into inorganic phosphorus is an attractive approach as well. Transgenic soybean, *Trifolium subterraneum L.* and barley with over-expression of *AtPAP15* gene from *Arabidopsis*, *MtPHY1* and *MtPAP1* genes from *Medicago truncatula* and purple acid phosphatase *HvPAPhy_a* from barley, respectively, have been developed to utilize phytate as phosphorus source [25–27]. However, owing to higher affinity for phytate, various microbial genes have been used for developing transgenic plants to combat phosphorus deficient conditions [28]. Some of the genes used include beta-propeller phytase from *Bacillus subtilis* (*PHY_US417*), *PHYA* isolated from *Aspergillus ficuum* and *Aspergillus niger*. These genes have been used to develop transgenic *Arabidopsis*, tobacco, alfalfa, cotton and wheat with enhanced ability to use phytate as phosphorus source [8, 25, 29–33].

Wheat, having high nutritional value, is a staple crop for many countries of the world [34, 35]. Over the years, better wheat varieties have been developed in order to fulfill its demand but there is still a need to increase its yield. Phosphorus is a major limiting factor in obtaining higher wheat yield. In order to reduce phosphorus deficiency, wheat has the tendency to produce phytase, an enzyme that degrades phytate [17, 36]. Under phosphorus stress conditions, phytase activity has been reported to increase but it is still insufficient to fulfill wheat's phosphorus requirements [37–39]. Thus, exploiting phytase-producing ability of wheat by genetic modifications to utilize the phytate reserves of soil is an appealing approach of improving phosphorus efficiency of wheat. Therefore, the objective of this research was to develop and screen transgenic wheat with higher phosphorus efficiency via having root-specific enhanced expression of phytase.

Methodology

Vector Construction and Wheat Transformation

Binary vector PSB219 (obtained from Leibniz Institute of Plant Genetics and Crop Plant Research, Germany) was

used to make the construct plasmid. A 467 amino acid long *PHYA* gene from *Aspergillus japonicus* was modified at 5'-end to have a 21 amino acid long *Calotropis procera*'s ER signal peptide (accession number EF434783). The gene was placed under root-specific control of *Pht1* promoter from barley along with *nos* terminator (Fig. 1).

For source material, Faisalabad-2008 variety was obtained from Ayub Agricultural Research Institute (AARI), Faisalabad and grown under natural conditions. Calli were prepared from 15 days post-anthesis immature zygotic embryos and transformed following slightly modified protocol of Jones et al. [40] using AGL1 strain of *Agrobacterium tumefaciens*.

Screening of Transformed T₀ Plants

Putative regenerated transgenic plants were primarily selected by shifting them on Murashige and Skoog medium containing kinetin (1 mg/l), BASTA[®] (2 mg/l) and time-ntin (160 mg/l) for 2 weeks followed by another round of selection using 3 mg/l of BASTA[®] in the regeneration media for another 4 weeks. After regeneration, plants were transferred and acclimatized in peat moss: vermiculite: perlite (2:1:1) pots under laboratory conditions and then transferred to greenhouse [41]. DNA was extracted from leaves of T₀ plants using CTAB protocol [42] for screening via PCR. Gene junction primers shown in Table 1 were used. Positive transgenic events were further screened via BASTA leaf paint assay by painting leaf samples with 0.005% BASTA[®] herbicide and scoring their color change from green to yellow after a week [43].

Analysis of Phytase Activity of T1 Generation

For screening of transgenic events, 10 plants per positive T₀ event were grown in sterile sand pots under controlled conditions for a month, providing them 10 ml of full-strength Hoagland's solution for first 3 weeks and with 15 ml per week afterward following the protocol described by Ma et al. [26] with slight modifications [44]. Positive T1 plants were screened via BASTA leaf paint assay and PCR using gene junction primers mentioned in Table 1. Positive T1 transgenic plants were shifted to P-stress conditions for a month by providing 1 mM phytate (Phytic acid sodium salt-Sigma P8810-100G) as P source in Hoagland's solution equivalent to the concentration of inorganic phosphorus in full-strength of Hoagland's solution. After stress period, phytase activity in root extracts, external root solution and intact roots was determined following slightly modified protocols described by Hayes et al. [45], Richardson et al. [17] and Mohsin et al. [39].

Briefly for determining phytase activity in root extracts, 0.5 g of clean root samples was grinded in 5 ml of MES/CA

Fig. 1 Simplified C219-B construct containing *Pht1* promoter from barley, ER signal peptide from *Calotropis procera*, *PhyA* phytase gene from *Aspergillus japonicus* and nos terminator with XHO1 and EcoR1 restriction sites at the ends

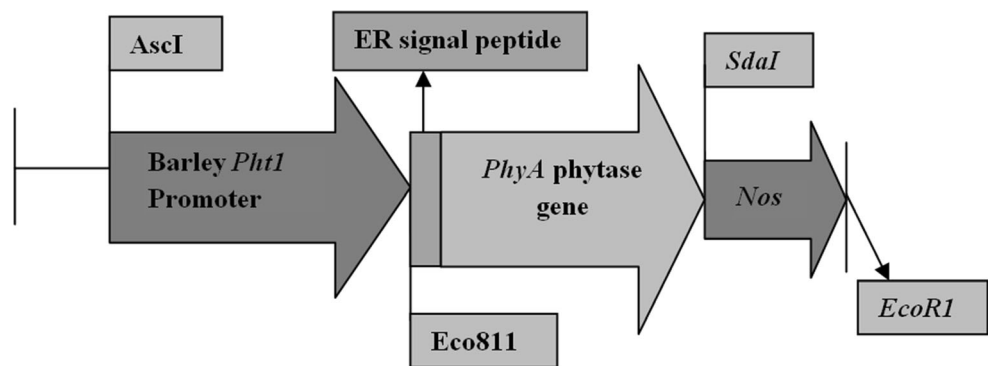


Table 1 Gene junction primers used for confirmational analysis of T_0 plants

Primer name	Primer sequence
CBphyR1	5'-GTTTCGGAAAAGCATTGG-3'
CBPF1	5'-CAAGTAGCGAGGAAATGT-3'
Phy nosF1	5'-ACTCTACAACGGGACTAA-3'
Phy nosR1	5'-GCGGGACTCTAATCATAA-3'

buffer (15 mM MES, 1 mM EDTA, 0.5 mM CaCl_2 , pH 5.5) and then incubated at 37 °C for an hour at 250 rpm. Samples were centrifuged at 4 °C, and then 250 μl of sample was transferred to an Eppendorf. After addition of 250 μl of MES/Ca buffer containing 2 mM phytate, samples were incubated at 37 °C for 30 min. Reaction was terminated by adding 500 μl of 10% trichloroacetic acid (TCA) solution. Absorbance of sample was measured at 820 nm by mixing 100 μl of sample with 900 μl of molybdate-blue color reagent and incubating the sample for 15 min at 50 °C. Phytase activity was calculated in unit per gram (where 1 unit = amount of enzyme that will catalyze transformation of 1 micromole of substrate per minute under standard conditions) using the following formula:

$$\text{Phytase activity} \left(\frac{U}{g} \right) = \frac{\text{gradient of standard curve} \times \text{sample absorbance} \times \text{dilution factor}}{\text{mass of sample} \times \text{incubation time} \times \text{volume of sample used}}$$

For phytase activity in external root solution, plant roots were incubated for 4 h in MES/Ca buffer and then 450 μl of the root incubation solution was further incubated at 37 °C for 30 min with 500 μl of MES/Ca buffer containing 2 mM phytate. Reaction was terminated by addition of 500 μl of 10% TCA solution, and 100 μl of sample was then incubated with 900 μl of molybdate-blue color reagent for 15 min at 50 °C before measuring absorbance at 820 nm. Phytase activity was calculated using the same formula used for phytase activity of root extracts.

In case of phytase activity of intact roots, plant roots were incubated for 1 h in MES/Ca buffer containing 2 mM phytate, rinsed with distilled water and then incubated for 30 min at 37 °C in 0.9% sodium chloride solution. After adding 500 μl of MES/Ca buffer containing 2 mM phytate to 500 μl of the incubated sample solution, further incubation at 37 °C for 30 min was given and then reaction was stopped using 500 μl of 10% TCA solution. To 100 μl of sample, 900 μl of molybdate-blue color reagent was added and incubated for 15 min at 50 °C before measuring absorbance at 820 nm. Phytase activity was calculated using the same formula used for phytase activity of root extracts.

Analysis of Phytase Activity of T2 Generation

Transgenic events with high phytase activity in T1 generation were selected for further analysis. For T2 generation analysis, like T1 generation, 10 plants per line of the four selected transgenic events were grown in sand pots under controlled conditions, screened via BASTA leaf paint assay and PCR using same conditions as used for T1 plants, followed by phytase activity analysis after stress period following the same protocols used for T1 generation plants.

Phosphorus Content and Phosphorus Efficiency of T2 Generation

Phosphorus content of shoots was also determined in case of T2 generation [46]. Fresh weight of shoot was measured, and then samples were dried in oven at 65 °C after stress period. Weight of dried samples was also measured followed by preparation of ash of samples in a muffle furnace at 550 °C for 4–5 h. White ash formed was dissolved in 0.9 M sulfuric acid at approximately 10 mg/ml DW/ml acid. The phosphate content was then determined via molybdate-blue assay.

Phosphorus efficiency was calculated using the dry weights of transgenic plants grown under controlled conditions for a month and then split into phosphorus deficient

and sufficient conditions for another month [47]. Following formula was used to calculate phosphorus efficiency:

$$\text{Phosphorus efficiency} = \frac{\text{Shoot dry weight at low P}}{\text{Shoot dry weight at high P}} \times 100.$$

Quantitative Real-Time PCR Analysis

RNA was extracted from roots of plants under P-stress conditions using method described by Das et al. [48] followed by cDNA preparation via Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit (#K1632). Integrity of the cDNA prepared was checked via RT-PCR using actin primers (ActWhtF 5'-GCCACACTGTTCCAATCTATGA-3' and ActWhtR 5'-TGATGGAATTGTATGTCGCTTC-3') followed by real-time PCR analysis using gene-specific primers for determining the relative fold expression of phytase gene in transgenic plants as compared to control Faisalabad-2008. Actin gene was used as a reference gene for normalization while various dilutions of positive phytase plasmid were used for preparing a standard curve. Livak method [49] was used for analysis of results.

Statistical Analysis

All assays were done in triplicates. Data analyses were done using ANOVA in SPSS version 17.0 and where *F* ratios were significant ($p < 0.05$), means were compared using Dunnett test.

Results

Generation of Transgenic Wheat

True calli prepared from Faisalabad-2008 (FSD-08) variety of wheat were transformed using AGL1 strain of *Agrobacterium tumefaciens* containing phytase construct. Out of 488 inoculated calli, 242 regenerated putative transgenic plants survived on selection medium containing BASTA. On shifting to peat moss pots only 138 regenerated plants survived. BASTA leaf paint assay and PCR analysis of these plants were done for screening positive transgenic events. Out of 46 positive events only 12 had complete construct while the remaining 34 had truncated construct. T1 seeds of these 12 events were collected and used for further analysis.

Phytase Activity of T1 Plants

After phosphorus stress period in which plants were provided organic phosphorus instead of inorganic phosphorus,

root-associated and extracellular phytase activity was determined (Fig. 2). In case of phytase activity of intact roots of T1, seven out of twelve transgenic events showed significantly higher enzyme activity than non-transgenic control plants (Fig. 3). Event 5 (E-5) exhibited the highest phytase activity of intact roots, i.e., 0.0836 U/g which was 0.67-fold higher than that of control plants. Phytase activity in external root solution of T1 generation was significantly higher for only five out of twelve events as compared to control plants (Fig. 4). Highest enzyme activity was 0.0983 U/g of event-11 (E-11) as compared to 0.0313 U/g of control plants. Results of phytase activity in root extracts of T1 generation are shown in Fig. 5. Events 1, 5, 6, 8, 9, 10, 11 and 12 showed significantly higher phytase activity than control plants, E-5 having the highest enzyme activity (0.669 U/g). Events 5,9,10 and 11 showed significantly higher enzyme activity in all three assays and thus were selected for further analysis on T2 generation.

Phytase Activity Analysis of T2 Plants

For T2 analysis, ten plants per line of each of the selected four events were grown in sand pots and selected via BASTA leaf paint assay and PCR just like T1 generation. After stress period, phytase activity was determined following the protocols used for T1 generation and transgenic lines with best results shown in Figs. 6, 7 and 8. Results of phytase activity of intact roots are shown in Fig. 6. Significant results were shown by ten transgenic lines. Maximum enzyme activity (2.084 U/g) of intact roots was shown by transgenic line A of event 9 (E9-A) as compared to 0.036 U/g of control FSD-08 plants. In case of external root solution, all transgenic lines showed significant results with 0.60–15.40 fold increase in their enzyme activity as compared to control plants (Fig. 7). Maximum phytase activity observed was 0.771 U/g of line E of transgenic event 5 (E5-E) while lowest was of E9-D (0.075 U/g), whereas control plants had enzyme activity of 0.047 U/g. In case of phytase activity in root extracts, except for E10-J all transgenic lines showed significant results (Fig. 8). Highest enzyme activity was shown by line E9-A (0.661 U/g) while that of control plants was 0.285 U/g.

Phosphorus Content and Phosphorus Efficiency Analysis

Shoot phosphorus content was determined under two conditions, i.e., when phytate (P+) was supplied during stress conditions and when inorganic P (Pi) was supplied throughout the experiment. Results indicated that there was significant increase in P content of all transgenic lines in the presence of P+ as compared to that of control plant while in case of Pi, majority of transgenic lines had higher

Fig. 2 a From left to right: transgenic plants grown in absence of phosphorus, when phytate was supplied and in the presence of inorganic phosphorus, respectively. **b** Control FSD-08 plants grown in absence of phosphorus, when phytate was supplied and in the presence of inorganic phosphorus, respectively

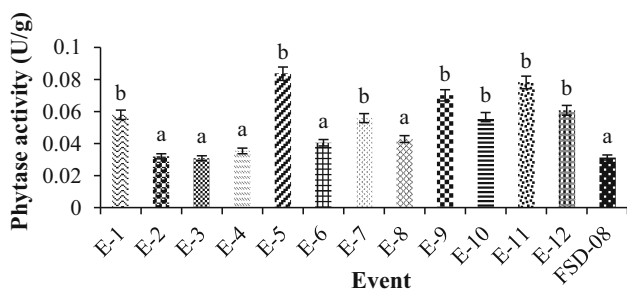
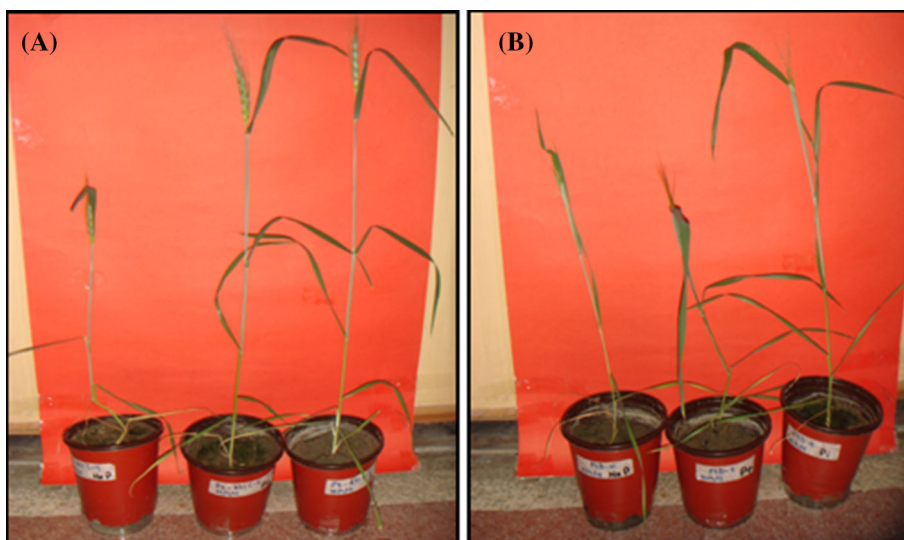


Fig. 3 Phytase activity of intact roots of T1 transgenic plants grown under P-stress conditions

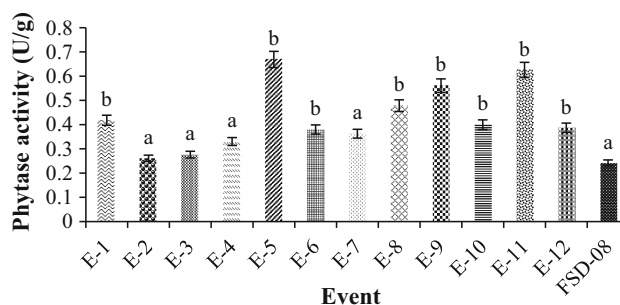


Fig. 5 Phytase activity in root extracts of T1 plants grown under P-stress conditions

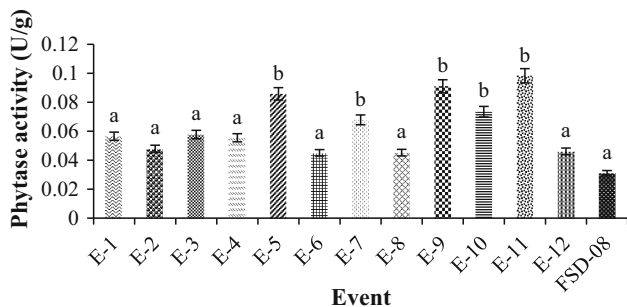


Fig. 4 Phytase activity in external root solution of T1 transgenic plants grown under P-stress conditions

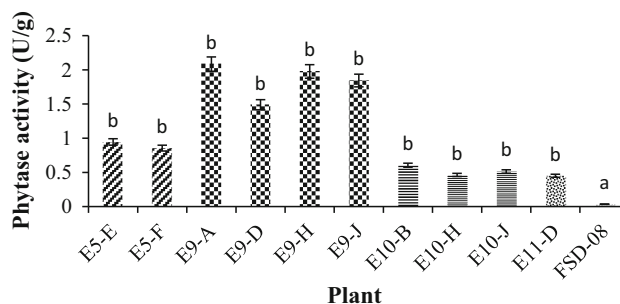


Fig. 6 Phytase activity of intact roots of T2 plants grown under P-stress conditions

P content as shown in Table 2. In both, P+ and Pi conditions E9-H showed highest P content, 220 and 236.75 μg , respectively. P content as well as shoot dry weight was higher for most of the transgenic lines when grown in the presence of phytate compared to inorganic P. In case of shoot dry matter, while majority of lines showed significantly higher results than control plants only E5-E, E5-F and E9-J showed significantly higher results than control

plants in the presence of inorganic P. Maximum shoot dry weight in the presence of phytate was 0.926 g (E5-F) and 0.712 g (E9-J) in Pi condition. Except for E5-E, E9-J and E11-D, all lines also showed significant fold increase in phosphorus efficiency. Maximum phosphorus efficiency was 199.02% which indicated a fold increase of 1.18 as compared to non-transgenic FSD-08 plants.

Real-Time PCR Analysis

Quantitative real-time PCR was used to determine phytase expression in transgenic lines. Results were analyzed via relative quantification method in which samples were normalized against wheat actin gene. Standard curve was made via different concentrations of phytase gene containing plasmid for determining quantification range. Livak

method was used to interpret the results [49]. Non-transgenic FSD-08 plants showed negligible expression while transgenic lines showed 1.58–19.8 fold increase in phytase expression (Fig. 9). E9-A had maximum fold expression while lowest was of E9-H.

Discussion

Adequate level of phosphorus is imperative for good yield of wheat. However, wheat is largely unable to utilize the large reserves of organic phosphorus present in soil naturally or due to fertilizers [50]. Microbes exist in soils that have the ability to degrade phytate and thus contribute significantly to phosphorus acquisition by plants. However, various studies show that microbes may compete with plants for nutrients and efficiency of microbial phytase is greatly affected by the distribution of microbes and the distance between microbes, phytate and plants [28, 51]. Moreover, modification of rhizosphere for phytate utilization may not be efficient due to poor ecological fitness or low secretion of metabolites or variation in inoculums [52]. To meet the world's requirement, it is essential to increase wheat's yield via genetic modification. Over the years, phytase gene from various microbes such as *Selenomonas ruminantium*, *Bacillus subtilis*, *E. coli*, yeast, *Aspergillus ficuum*, *A. niger*, *Thermomyces lanuginosus* has been used to produce transgenic plants, owing to their greater affinity for phytate, with enhanced phosphorus efficiency [26, 53]. Tobacco, soybean, *Arabidopsis*, maize, canola, wheat, potato, rice, sesame and *Medicago truncatula* have all been successfully transformed via phytase gene [53–56]. The phytase gene from *Aspergillus* species has been extensively used for making transgenic plants due to its high thermostability, broad pH tolerance and proteolytic resistance for better results

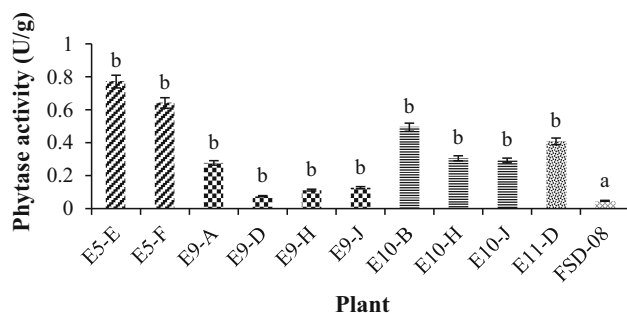


Fig. 7 Phytase activity in external root solution of T2 plants grown under P-stress conditions

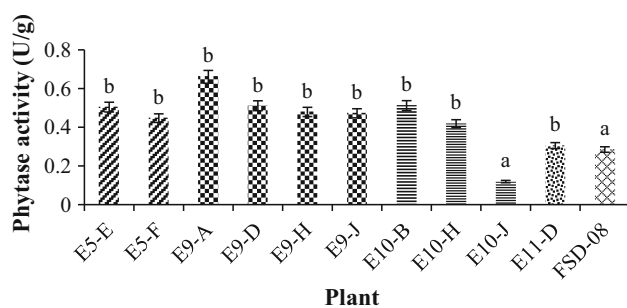


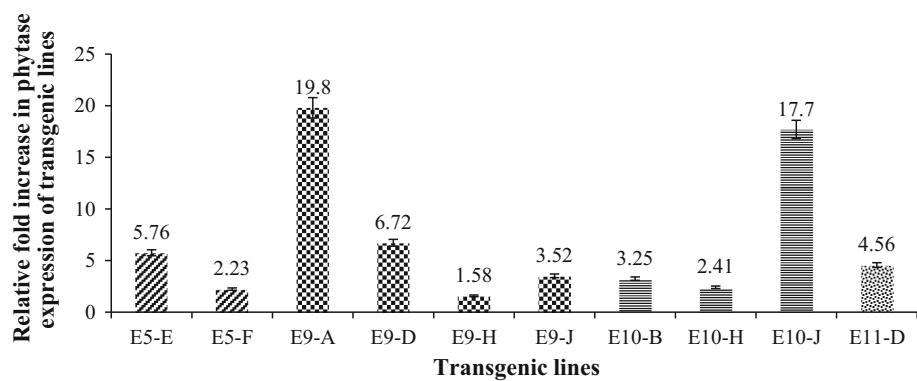
Fig. 8 Phytase activity in root extracts of T2 plants grown under P-stress conditions

Table 2 Phosphorus content, shoot dry weight and phosphorus efficiency (P.E) of T2 transgenic plants when supplied with phytate (P+) or inorganic P (Pi)

Plant	P content (P+)/ μg	P content (Pi)/ μg	Shoot dry weight (P+)/g	Shoot dry weight (Pi)/g	P.E (P+/Pi) (%)	Fold increase in P.E
E5-E	170.75 ^b	147.75 ^a	0.457 ^a	0.605 ^b	75.54 ^a	-0.17
E5-F	192.38 ^b	162.63 ^b	0.926 ^b	0.622 ^b	148.87 ^b	0.63
E9-A	210.25 ^b	198.38 ^b	0.823 ^b	0.427 ^a	192.74 ^b	1.11
E9-D	204.75 ^b	208 ^b	0.624 ^b	0.512 ^a	121.88 ^b	0.33
E9-H	220.50 ^b	236.75 ^b	0.609 ^b	0.306 ^a	199.02 ^b	1.18
E9-J	196.63 ^b	197.88 ^b	0.665 ^b	0.712 ^b	93.40 ^a	0.02
E10-B	155.75 ^b	144 ^a	0.806 ^b	0.496 ^a	162.50 ^b	0.78
E10-H	166.75 ^b	146.25 ^a	0.674 ^b	0.396 ^a	170.20 ^b	0.86
E10-J	189.13 ^b	188.75 ^b	0.279 ^a	0.251 ^a	111.16 ^b	0.22
E11-D	215.63 ^b	200.38 ^b	0.406 ^a	0.439 ^a	92.48 ^a	0.01
FSD	149.38 ^a	159.75 ^a	0.470 ^a	0.514 ^a	91.44 ^a	-

Different superscript letters show that significant difference exists when $p < 0.05$

Fig. 9 Fold increase in expression of phytase gene in T2 transgenic plants grown in sand pots



[53, 57]. Moreover, various researches have shown that extracellular expression of phytase from *Aspergillus niger* in *Arabidopsis thaliana* and *Trifolium subterraneum* resulted in better acquisition of organic phosphorus from laboratory media as compared to endogenous expression of gene, without affecting adversely any of the stored form of phosphorus in plants [29, 31]. Hence, in the present work, phytase gene from *Aspergillus japonicus* with modified ER signal was expressed under barley root-specific promoter to ensure enzyme secretion without effecting internal phosphorus of the plant [26].

Insertion of bar gene in the construct made transformed plantlets resistant to BASTA herbicide and thus allowed initial screening in MS media containing BASTA. PCR analysis using gene junction primers for gene and terminator ensured that only transformants with complete construct were selected. Ten plants per twelve confirmed transformants were then grown in sand for phytase activity analysis of T1 progeny. Phytase activity in root extracts was greatest for all twelve transgenic events followed by phytase activity in external root solution and intact roots, respectively (Fig. 3, 4, 5). Previously, Asmar [58] has also reported greater root-associated phytase activity as compared to root-released extracellular phytase activity. Only four transgenic events (E-5, E-9, E-10 and E-11) showed significantly higher enzyme activity in all three assays and thus were selected for T2 analysis. Root-associated enzyme activity was higher than the extracellular for T2 progeny as well. However, in case of T2 generation, phytase activity of intact roots was greater than that of root extracts and external root solution. Variation was seen in the phytase activity of different events and lines. This may be due to the expression level of the transgene that is affected by the position of transgene insertion or copy number [59]. Insertion of phytase gene may have varied the root morphology because of which phytase activity may have varied among different transgenic lines (Figure S1).

Shoot phosphorus content was also determined for T2 generation under two different phosphorus sources. In the

presence of phytate, as compared to control plants, all lines showed significantly higher results while in case of inorganic phosphorus majority of lines had higher P content (Table 2). Also majority of lines had higher P content in the presence of phytate as compared to inorganic phosphorus. E9-H had the highest phosphorus efficiency even though E9-A had higher enzyme activity in all three assays. This could be explained by the difference in root architecture which has shown to greatly affect nutrient uptake and interaction with rhizosphere (Figure S1) [28]. Enhanced phosphorus content due to increased phytase activity has been previously reported by Richardson et al. [29], George et al. [31] and Wang et al. [3]. These results advocate that transgenic plants were able to utilize phytate and were supported by results of shoot dry weight as well as phosphorus efficiency (Table 2). Increased shoot dry weight in the presence of phytate and enhanced phosphorus efficiency indicated that these transgenic lines have better ability to survive in soil with large phytate content which otherwise is phosphorus deficient condition for the non-transgenic wheat. The variation in phosphorus efficiency may be due to variation in phytase expression in different lines or utilization of phosphorus at cellular level.

Phytase expression analysis for T2 generation was done via quantitative real-time PCR, a highly sensitive and reliable method. Transgenic lines showed 1.58–19.8 fold increase in phytase expression compared to control plants (Fig. 9). E9-A had the highest real-time expression as well as shoot phosphorus content, phosphorus efficiency (199.02%) and phytase activity in root extracts as well as intact roots. The fold increase shown by real-time PCR results did not correlate with that of phytase activity, phosphorus content and phosphorus efficiency results of all lines. This might be because actual phytase protein being produced by plants may be less than the mRNA owing to post-translational changes like protein folding and RNA splicing.

The effect of transgenic plants and their exudates on the microbial growth of soil is not clear yet. While some studies show no effect, others show small effects on some

components of the microbial community [60–64]. Further progenies of these transgenic lines can be analyzed to determine homozygous line with stable expression in natural environment and their effect on rhizosphere or microbial community of soil. Successful development of this transgenic wheat will result in decrease in cost of production by reducing chemical fertilizer usage.

Acknowledgements Authors are grateful to Pakistan Science Foundation (PSF) (Grant No. PSF/NSLPP-FCCU (244)) for funding of this project. We would like to thank Ms. Sahrish Farooqi for her valuable input.

References

- Almeida, J. P. F., Hartwig, U. A., Frehner, M., Nosenberger, J., & Luscher, A. (2000). Evidence that P deficiency induces feedback regulation of symbiotic N₂ fixation in white clover (*Trifolium repens* L.). *Journal of Experimental Botany*, *51*, 1289–1297.
- George, T. S., & Richardson, A. E. (2008). Potential and limitations to improving crops for enhanced phosphorus utilization. In P. J. White & J. P. Hammond (Eds.), *Ecophysiology of plant-phosphorus interactions* (pp. 247–270). Berlin: Springer.
- Wang, J., Sun, J., Miao, J., Guo, J., Shi, Z., He, M., et al. (2013). A wheat phosphate starvation response regulator Ta-PHR1 is involved in phosphate signalling and increases grain yield in wheat. *Annals of Botany*, *111*(6), 1139–1153.
- Iyamuremye, F., Dick, R. P., & Baham, J. (1996). Organic amendment and phosphorus dynamics: II. Distribution of soil phosphorus fractions. *Soil Science*, *161*, 436–443.
- Turner, B. L., Paphazy, M. J., Haygarth, P. M., & Mckelvie, I. D. (2002). Inositol phosphates in the environment. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *357*, 449–469.
- Cade-Menun, U. (2005). Using phosphorus-31 nuclear magnetic resonance spectroscopy to characterize organic phosphorus in environmental samples. In D. S. Baldwin, B. L. Turner, & E. Frossard (Eds.), *Organic phosphorus in the environment* (pp. 21–44). Wallingford: CABI Publishing.
- Richardson, A. E., George, T. S., Hens, M., & Simpson, R. J. (2005). Utilization of soil organic phosphorus by higher plants. In D. S. Baldwin, B. L. Turner, & E. Frossard (Eds.), *Organic phosphorus in the environment* (pp. 161–184). Wallingford: CABI Publishing.
- Belgaroui, N., Berthomieu, P., Rouached, H., & Hanin, M. (2016). The secretion of the bacterial phytase *PHY-US417* by *Arabidopsis* roots reveals its potential for increasing phosphate acquisition and biomass production during co-growth. *Plant Biotechnology Journal*, *14*(9), 1914–1924.
- Bowman, B. T., Thomas, E. L., & Elrick, D. E. (1967). The movement of phytic acid in soil cores. *Soil Science Society of America Journal Proceedings*, *31*(4), 477–481.
- McKercher, R. B., & Anderson, G. (1989). Organic phosphate sorption by neutral and basic soils. *Communications in Soil Science and Plant Analysis*, *20*, 723–732.
- Vance, C. P., Uhde-stone, C., & Allan, D. L. (2003). Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. *New Phytologist*, *157*, 423–447.
- Gerke, J. (2015). Phytate (inositol hexaisphosphate) in soil and phosphate acquisition from inositol phosphates by higher plants. A review. *Plants*, *4*, 253–266.
- Childers, D. L., Corman, J., Edwards, M., & Elser, J. J. (2011). Sustainability challenges of phosphorus and food: Solutions from closing the human phosphorus cycle. *BioScience*, *61*(2), 117–124.
- Holford, I. C. R. (1997). Soil phosphorus: Its measurement and its uptake by plants. *Australian Journal of Soil Research*, *35*, 227–239.
- Tian, J., Wang, X., Tong, Y., Chen, X., & Liao, H. (2012). Bioengineering and management for efficient phosphorus utilization in crops and pastures. *Current Opinion in Biotechnology*, *23*, 1–6.
- Gilroy, S., & Jones, D. L. (2000). Through form to function: Root hair development and nutrient uptake. *Trends in Plant Science*, *5*, 56–60.
- Richardson, A. E., Hadobas, P. A., & Hayes, J. E. (2000). Acid phosphomonoesterase and phytase activities of wheat (*Triticum aestivum* L.) roots and utilization of organic phosphorus substrates by seedlings grown in sterile culture. *Plant, Cell and Environment*, *23*, 397–405.
- Bates, T. R., & Lynch, J. P. (2001). Root hairs confer a competitive advantage under low phosphorus availability. *Plant and Soil*, *236*, 243–250.
- Lynch, J. P., & Brown, K. M. (2001). Topsoil foraging—An architectural adaptation of plants to low phosphorus. *Plant and Soil*, *237*, 225–237.
- Shen, H., Chen, J., Wang, Z., Yang, C., Sasaki, T., Yamamoto, Y., et al. (2006). Root plasma membrane H⁺-ATPase is involved in the adaptation of soybean to phosphorus starvation. *Journal of Experimental Botany*, *57*, 1353–1362.
- Wang, X., Shen, J., & Liao, H. (2010). Acquisition or utilization, which is more critical for enhancing phosphorus efficiency in modern crops. *Plant Science*, *179*, 302–306.
- Yang, H., Zhang, X., Gaxiola, R. A., Xu, G., Peer, W. A., & Murphy, A. S. (2014). Over-expression of the *Arabidopsis* proton-pyrophosphatase AVP1 enhances transplant survival, root mass, and fruit development under limiting phosphorus conditions. *Journal of Experimental Botany*, *65*(12), 3045–3053.
- Guo, W., Zhao, J., Li, X., Qin, L., Yan, X., & Liao, H. (2011). A soybean β -expansin gene GmEXPB2 intrinsically involved in root system architecture responses to abiotic stresses. *Plant Journal*, *66*(3), 541–552.
- Zhou, J., Xie, J., Liao, H., & Wang, X. (2013). Overexpression of β -expansin gene GmEXPB2 improves phosphorus efficiency in soybean. *Physiologia Plantarum*, *150*(2), 194–204.
- Wang, X., Wang, Y., Tian, J., Lim, B. L., Yan, X., & Liao, H. (2009). Overexpressing AtPAP15 enhances phosphorus efficiency in soybean. *Plant Physiology*, *151*(1), 233–240.
- Ma, X. F., Tudor, S., Butler, T., Ge, Y., Xi, Y., Bouton, J., et al. (2012). Transgenic expression of phytase and acid phosphatase genes in alfalfa (*Medicago sativa*) leads to improved phosphate uptake in natural soil. *Molecular Breeding*, *30*, 377–391.
- Holme, I. B., Dionisio, G., Madsen, C. K., & Brinch-Pedersen, H. (2017). Barley HvPAPhy_a as transgene provides high and stable phytase activities in mature barley straw and in grains. *Plant Biotechnology Journal*, *15*, 415–422.
- Richardson, A. E., Barea, J. M., McNeill, A. M., & Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, *321*, 305–339.
- Richardson, A. E., Hadobas, P. A., & Hayes, J. E. (2001). Extracellular secretion of Aspergillus phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. *Plant Journal*, *25*(6), 641–649.
- Gaxiola, R., Li, J., Undurraga, S., Dang, L., Allen, G., Alper, S., et al. (2001). Drought and salt-tolerant plants result from over-expression of the AVP1 H⁺-pump. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 11444–11449.
- George, T. S., Richardson, A. E., Hadobas, P. A., & Simpson, R. J. (2004). Characterization of transgenic *Trifolium subterraneum*

- L. which expresses phyA and releases extracellular phytase: Growth and P nutrition in laboratory media and soil. *Plant, Cell and Environment*, 27, 1351–1361.
32. Lung, S. C., Chan, W. L., Yip, W., Wang, L., Yeung, E. C., & Lim, B. L. (2005). Secretion of beta-propellar phytase from tobacco and *Arabidopsis* roots enhances phosphorus utilization. *Plant Science*, 169, 341–349.
 33. Liu, J. F., Zhao, C. Y., Ma, J., Zhang, G. Y., Li, M. G., Yan, G. J., et al. (2011). *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum* L.) with a fungal phytase gene improves phosphorus acquisition. *Euphytica*, 181(1), 31–40.
 34. Lung, S. C., & Lim, B. L. (2006). Assimilation of phytate-phosphorus by the extracellular phytase activity of tobacco (*Nicotiana tabacum*) is affected by the availability of soluble phytate. *Plant and Soil*, 279(1–2), 187–199.
 35. Curtis, T., & Halford, N. G. (2014). Food security: The challenge of increasing wheat yield and the importance of not compromising food safety. *Annals of Applied Biology*, 164(3), 354–372.
 36. Khan, I., & Zeb, A. (2007). Nutritional composition of Pakistani wheat varieties. *Journal of Zhejiang University Science B*, 8(8), 555–559.
 37. Centeno, C., Viveros, A., Brenes, A., Canales, R., Lozano, A., & de la Cuadra, C. (2001). Effect of several germination conditions on total P, phytate P, phytase, and acid phosphatase activities and inositol phosphate esters in rye and barley. *Journal of Agricultural and Food Chemistry*, 49, 3208–3215.
 38. George, T. S., Gregory, P. J., Hocking, P., & Richardson, A. E. (2008). Variation in root-associated phosphatase activities in wheat contributes to the utilization of organic P substrates in vitro, but does not explain differences in the P-nutrition of plants when grown in soils. *Environmental and Experimental Botany*, 64(3), 239–249.
 39. Mohsin, S., Malik, K. A., & Maqbool, A. (2015). Comparison of phytase activity in roots of wheat varieties grown under different phosphorus conditions. *Research in Biotechnology*, 6(3), 31–41.
 40. Jones, H. D., Doherty, A., & Wu, H. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. *Plant Methods*, 1, 5.
 41. Abid, N., Maqbool, A., & Malik, K. A. (2014). Screening commercial wheat (*Triticum aestivum* L.) varieties for *Agrobacterium* mediated transformation ability. *Pakistan Journal of Agricultural Sciences*, 51(1), 83–89.
 42. Kang, T. J., & Yang, M. S. (2004). Rapid and reliable extraction of genomic DNA from various wild-type and transgenic plants. *BMC Biotechnology*, 4, 20.
 43. Visarada, K. B. R. S., Saikishore, N., Kuriakose, S. V., Rani, V. S., Royer, M., Rao, S. V., et al. (2008). A simple model for selection and rapid advancement of transgenic progeny in sorghum. *Plant Biotechnology Reports*, 2, 47–58.
 44. Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Circular - California Agricultural Experiment Station*, 347, 1–32.
 45. Hayes, J. E., Richardson, A. E., & Simpson, R. J. (1999). Phytase and acid phosphatase activities in extracts from roots of temperate pasture and legume seedlings. *Australian Journal of Plant Physiology*, 26, 801–809.
 46. Murphy, J., & Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, 27, 31–36.
 47. Gunes, A., Inal, A., Alpaslan, M., & Cakmak, I. (2006). Genotypic variation in phosphorus efficiency between wheat cultivars grown under greenhouse and field conditions. *Soil Science & Plant Nutrition*, 52, 470–478.
 48. Das, A., Saha, D., & Mondal, T. K. (2013). An optimized method for extraction of RNA from tea roots for functional genomics analysis. *Indian Journal of Biotechnology*, 12, 129–132.
 49. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408.
 50. Syers, J. K., Johnston, A. E., & Curtin, D. (2008). *Efficiency of soil and fertilizer phosphorus use: Reconciling changing concepts of soil phosphorus behavior with agronomic information*. Rome: Food and agriculture organization of the United Nations.
 51. Tarafdar, J. C., & Jungk, A. (1987). Phosphatase activity in the rhizosphere and its relation to the depletion of soil organic phosphorus. *Biology and Fertility of Soils*, 3(4), 199–204.
 52. Shenoy, V. V., & Kalagudi, G. M. (2005). Enhancing plant phosphorus use efficiency for sustainable cropping. *Biotechnology Advances*, 23, 501–513.
 53. Iti, G., Tantai, K., Rajput, L. P. S., & Tiwari, S. (2012). Transgenic plants expressing phytase gene of microbial origin and their prospective application as feed. *Food Technology and Biotechnology*, 50(1), 3–10.
 54. Pedersen, H. B., Hatzack, F., Stöger, E., Arcalis, E., Pontopidan, K., & Holm, P. B. (2006). Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): Deposition pattern, thermostability, and phytate hydrolysis. *Journal of Agriculture and Food Chemistry*, 54, 4624–4632.
 55. Hong, Y. F., Liu, C. Y., Cheng, K. J., Hour, A. L., Chan, M. T., Tseng, T. H., et al. (2008). The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato. *Plant Molecular Biology*, 67, 347–361.
 56. Pires, A. S., Cabral, M. G., Fevereiro, P., Stoger, E., & Abranches, R. (2008). High levels of stable phytase accumulate in the culture medium of transgenic *Medicago truncatula* cell suspension cultures. *Biotechnology Journal*, 3, 916–923.
 57. Promdonkoy, P., Tang, K., Sormlake, W., Harnpicharnchai, P., Kobayashi, R. S., Ruanglek, V., et al. (2009). Expression and characterization of *Aspergillus* thermostable phytases in *Pichia pastoris*. *FEMS Microbiology Letters*, 290(1), 18–24.
 58. Asmar, F. (1997). Variation in activity of root extracellular phytase between genotypes of barley. *Plant and Soil*, 195, 61–64.
 59. Zeller, S. L., Kalinina, O., Brunner, S., Keller, B., & Schmid, B. (2010). Transgene × environment interactions in genetically modified wheat. *PLoS ONE*, 5(7), e11405. doi:10.1371/journal.pone.0011405.
 60. Bruinsma, M., Kowalchuk, G. A., & Van-Veen, J. A. (2003). Effects of genetically modified plants on microbial communities and processes in soil. *Biology and Fertility of Soils*, 37, 329–337.
 61. Castaldini, M., Turrini, A., Sbrana, C., et al. (2005). Impact of Bt corn on rhizospheric and soil eubacterial communities and on beneficial mycorrhizal symbiosis in experimental microcosms. *Applied and Environmental Microbiology*, 71, 6719–6729.
 62. Griffiths, B. S., Caul, S., Thompson, J., Birch, A. N. E., Scrimgeour, C., Cortet, J., et al. (2006). Soil microbial and faunal community responses to Bt maize and insecticide in two soils. *Journal of Environmental Quality*, 35, 734–741.
 63. Philippot, L., Kuffner, M., Cheneby, D., Depret, G., Lauguerre, G., & Martin-Laurent, F. (2006). Genetic structure and activity of the nitrate reducers community in the rhizosphere of different cultivars of maize. *Plant and Soil*, 287, 177–186.
 64. Lupwayi, N. Z., Hanson, K. G., Harker, K. N., Clayton, G. W., Blackshaw, R. E., O'Donovan, J. T., et al. (2007). Soil Microbial biomass, functional diversity and enzyme activity in glyphosate-resistant wheat–canola rotations under low-disturbance direct seeding and conventional tillage. *Soil Biology & Biochemistry*, 39, 1418–1427.