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Transgenic expression of phytase in wheat endosperm increases bioavailability of iron and zinc in grains

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Abstract Phytate is a major constituent of wheat seeds and chelates metal ions, thus reducing their bioavailability and so the nutritional value of grains. Transgenic plants expressing heterologous phytase are expected to enhance degradation of phytic acid stored in seeds and are proposed to increase the in vitro bioavailability of mineral nutrients. Wheat transgenic plants expressing *Aspergillus japonicus* phytase gene (*phy*A) in wheat endosperm were developed till T_3 generation. The transgenic lines exhibited 18–99 % increase in phytase activity and 12–76 % reduction of phytic acid content in seeds. The minimum phytic acid content was observed in chapatti (Asian bread) as compared to flour and dough. The transcript profiling of *phy*A mRNA indicated twofold to ninefold higher expression as compared to non transgenic controls. There was no significant difference in grain nutrient composition of transgenic and non-transgenic seeds. In vitro bioavailability assay for iron and zinc in dough and chapatti of transgenic lines revealed a significant increase in iron and zinc contents. The development of nutritionally enhanced cereals is a step forward to combat nutrition deficiency for iron and zinc in malnourished human population, especially women and children.

Keywords Phytase · Phytic acid · Bioavailability · Transgenic wheat · Iron · Zinc

Introduction

One-third of the world population is at risk of iron and zinc deficiency (Alloway 2009; Lee and Okam 2011; Paul et al. 2013). Majority of the developing countries around the world have poverty levels of around 40 %. The scenario has resulted in malnutrition. The Global

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Nutrition Report (2014), the Scientific Advisory Committee on Nutrition (SACN 2010) and The National Nutrition Survey of Pakistan (2011) indicate that the deficiencies of iron, zinc and vitamin A are increasing at an alarming rate in women and children. The primary ailment as a consequence is anemia and immune deficiencies. Zinc deficiency is the fifth leading risk factor for sickness in the developing world (Maret and Sandstead 2006), which mostly causes disorders of eyesight, taste, smell and memory (Ikeda et al. 2008). Therefore, an adequate intake of these minerals is important for meeting nutritional needs to overcome the 'hidden hunger' and 'food security' (Kennedy et al. 2003).

Although sufficient quantities of iron and zinc are available in cereal grains (Raboy 2001; Borg et al. 2009; Paul et al. 2013), the bioavailable Fe and Zn in staple food crop grains are as low as 5 and 25 %, respectively (Bouis and Welch 2010). Fortification of wheat with Fe has been adopted but is limited to urban areas because majority of the population in the developing world lives in rural areas where they have their own mechanism of grinding wheat into flour (called "Chakki" in South Asia). Under such circumstances, biofortification can be a much better choice.

The greatest brunt on zinc and iron absorption in the body is mainly by phytic acid or Myo-inositol hexaphosphate (InsP6) also known as phytate. It is a powerful chelator that binds free metal ions to form mixed salts resulting in their excretion due to non-availability of relevant enzymes for its metabolism in humans and other non-ruminant animals such as poultry, swine, and fish (Raboy 2001; Erdal et al. 2002; Jin et al. 2009; Bouis and Welch 2010).

The transgenic crops are being developed for reduced content of phytic acid (Paul et al. 2013; Frontela et al. 2009) in grains. Breeding for nutritional quality is a viable, practicable, and cost-effective strategy to complement existing interventionist strategies (Bouis and Welch 2010; Clugston and Smith 2002). The problem is that some varieties with high iron and zinc are tall, traditional and low-yielding (Bouis and Welch 2010; Paul et al. 2013). However, the objective is not only to increase the content of micronutrients in the edible part of the plant but also to increase their bioavailability which can be achieved if the anti-nutrient component (phytate in this case) can be degraded in situ. Therefore, a long term solution using crop varieties with increased zinc and iron bioavailability is highly desirable.

To cope with the issue of mineral deficiency, many efforts are being made for the genetic improvement of cereal crops. These efforts focus on increasing the bioavailable iron and zinc contents of the staple grains either through conventional breeding and selection of low seed phytate content (Raboy 2001; Hotz and McClafferty 2007; White and Broadley 2009) or developing transgenic crops using fungal phytase as transgene for seed specific expression. Phytase is an enzyme that can breakdown the indigestible phytic acid in grains and oil seeds. The cereals like barley and wheat naturally deposit purple acid phytases in seeds known as PAP type a and also produce PAP type b during germination. These phytases are generally involved in seed germination by providing an initial pool of bioavailable phosphate by the degradation of phytate. The kinetic studies on the wheat phytase enzymes indicate that these phytases have an optimum activity at 45 °C (Dionisio et al. 2011). Although these phytases produce sufficient phosphate for seed germination yet they are not able to release enough bioavailable iron and zinc to meet the dietary requirements in humans (Dionisio et al. 2011). Phytase gene (*phy*A) from *Aspergillus* species has been widely reported to breakdown phytate in rice grains to enhance germination and nutritional value (Lucca et al. 2001). The *Aspergillus japonicus* phyA has 10 N-glycosylation sites (Asn-X-S/T) that indicate ER specific processing for N-glycosylation, RHGXRX and dipeptide HD motif indicative of histidine type acid phosphatase (Promdonkoy et al. 2009; Fonseca-Maldonado et al. 2014).

Several transgenic crops with enhanced levels of metallic ions, vitamin A, C and other micronutrients have been reported (Khush 2008; Gontia et al. 2012; Ali et al. 2013; Perez-Massot et al. 2013). Wheat is ranked as the 2nd most globally consumed staple food after rice. The legume crops on the other hand follow the cereals (FAO statistical pocketbook 2015). Brinch-Pedersen et al. (2000, 2003) transformed wheat with *Aspergillus phy*A under a constitutive (Ubi) promoter with and without an α-amylase signal peptide for ER specific and cytosolic expression respectively. Therefore, the transgenic protein can be stored in ER or can be directed for secretion in the apoplast. The phytic acid breakdown products by the fungal phyA were examined through the above studies. However, these studies did not address seed specific expression of *phy*A and bioavailability of metal ions in wheat flour,

dough and chapatti. The present study was thus aimed to fill the gap and determine the feasibility of wheat biofortification for increased bioavailability of iron and zinc in wheat seeds specifically, which is consumed as "chapatti" in South Asia. This study reports transformation of wheat with synthetic phytase (*A. japonicus*; *phy*A) in endosperm tissues, its expression and the bioavailability of iron and zinc. To ensure sufficient release of phytase in flour the deposition of enzyme was directed to ER using a single gene construct and also deposition in ER and Apoplast through a double gene construct. The transgenics of two elite wheat varieties (*Triticum aestivum*) were obtained. Total and bioavailable iron and zinc contents were estimated in wheat flour, dough and chapatti (cooked bread).

Materials and methods

Plant material, bacterial strains and plant transformation vectors

Seeds of two elite winter commercial wheat (*Triticum aestivum*) varieties namely Sahar 2006 and Faisalabad-2008 were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. The T_0 and T_1 plants were grown in pots having peat-moss, vermiculite and perlite in a ratio of 2:1:1 respectively in a controlled temperature room set at 25 °C. The T_2 transgenics were grown in the containment facility during the winter season.

The pBRACT 304 was obtained from Mark Smedley, John Innes Centre, UK. This vector is based on pGreen/pSoup binary vector system (Hellens et al. 2000). The phytase gene cassettes were initially developed in pBRACT 304. The phytase constructs were co-transformed with pAL154 (Ms. Caroline Sparks, Rothamsted Research, UK, kindly provided *Agrobacterium tumefaciens* AGL1 and pAL154) into *AgL1* strain of *A. tumefaciens* having C58 chromosome background. The pAL154 provides replication function in trans to pBRACT 304 and also has a 15 Kb Komari fragment having additional virulence genes (virB, virC and virG542) for efficient wheat transformation (Wu et al. 2008). The phytase cassettes were later lifted from pBRACT 304 and cloned in pSB219 (obtained from Leibniz Institute of Plant Genetics and Crop Plant Research, Germany). The expression vectors were maintained in *Escherichia coli* strain *DH10*. The phytase constructs developed in pSB219 were used for *Agrobacterium* (AgL1) mediated wheat transformation.

Gene and promoter resources

The *phyA* gene accession number EU786166 from *A. japonicus* codes for a histidine acid phosphatase (HAPs). The nucleotide sequence was retrieved from GenBank. The coded protein comprises of 467 amino acids. The first 21 amino acids represent an ER specific signal and amino acid 22–467 constitutes the functional phytase. The fungal ER signal was replaced by a 21 amino acid long plant ER signal peptide (Fig. S1) from accession number EF434783 (Expansin EXPA3). The ER retention signal KDEL (Munro and Pelham 1987) was added to the end of phytase cassette 1 (Fig. S3A). Retrieval to ER mediated by the retention signal occurs in an early golgi complex (cis-golgi) without further golgi-specific processing (Pelham 1988; Stornaiuolo et al. 2003). However, the KDEL signal was deleted from the second phytase expression cassette to direct this phytase to secretary pathway for deposition in apoplast. A synthetic gene, codon optimized for expression in wheat, was designed using GENEius (http://www.geneius.de/GENEius/Security_login.action) and got synthesized from Operon Technologies, USA.

A 435 bp truncated D-hordein promoter fragment was amplified from *Hordeum vulgare* and a 930 bp truncated glutenin promoter was amplified from *Triticum aestivum* for endosperm specific expression (Furtado et al. 2009; Sorensen et al. 1989, 1996). The D-hordein and glutenin promoters were fused to the phytase gene for developing construct 1 and 2 respectively (Fig. S2).

Phytase expression vector and wheat transformation

The immature zygotic embryos (at 8–10 day-post anthesis) of two elite wheat varieties (Faisalabad-2008 and Sahar-2006) were transformed through *Agrobacterium* mediated transformation. The binary monocot transformation vector (pSB219) was obtained from Leibniz Institute of Plant Genetics and Crop Plant Research, Germany. The GFP expression cassette in pSB219 was replaced with phytase cassette 1 in

construct 1 and phytase cassette 1 and 2 in construct 2 (Fig. S2E–S2H), which were initially developed in pBRACT304 (Fig. S2A-S2D). The pBRACT 304 was kindly provided by John Innes Centre, UK and modified in our lab by replacing Ubiquitin 2 promoter and *Gus* with a 177 bp rare cutter adopter between the *Hin*dIII and *Sac*I sites (Fig. S2b). Two independent constructs in pSB219 having selectable marker (*PAT* gene) and synthetic *PhyA* gene were used for wheat transformation. The construct 1 had single phytase expression cassette (Fig. S2G), while construct 2 had two phytase expression cassettes (Fig. S2H).

Wheat plants (*Triticum aestivum* L.) of two elite varieties, Faisalabad-2008 and Sahar-2006, were grown in the green house at 18–20 °C day/night temperature and in field under natural conditions during winter season. The embryos were obtained at 15 days post anthesis (DPA). Transformation was done according to the procedure of Jones (2005). The regeneration and selection was performed as described by Abid et al. (2014). The putative transgenic (T_0) plants were shifted to soil and further grown to maturity in the green house. The seeds were harvested and kept desiccated at 4 °C until further use.

Screening and molecular analysis of transgenic plants

Total genomic DNA from putative transgenic plants (T_0) was isolated using CTAB method (Kang and Yang 2004) and analyzed through conventional PCR to detect the presence of integrated phytase cassette/s applying the promoter-cds and cds-terminator junction primers (Table S1; Fig. S3). Total genomic DNA isolated from wild type plants and mocks (transformed with the plain vector) was used as negative control. On the other hand, the phytase vector constructs were used as positive controls.

T_1 transgenic plants were screened for *PAT* expression at the five-leaf stage by painting 0.005 % Basta solution to a marked area on the second or third leaf of the plantlet. The herbicide resistance was scored at 7–10 days post application.

Phytase activity assay and phytic acid content

Phytase activity and phytic acid content were determined in T_1 and T_2 transgenic lines respectively. Phytase activity was determined in wheat flour by following Richardson et al. (2000) with little modifications. About 500 mg of the control and transgenic seeds were pulverized, and extracted in 5 ml of MES/Ca buffer (15 mM MES, 0.5 mM $CaCl_2$ and 1 mM EDTA) by thorough vortexing followed by incubation in shaking incubator at 250 rpm (24 °C) for 1 h. The samples were then centrifuged for 10 min at 14000 rpm. About 250 µl of the supernatant was incubated at 37 °C for 30 min with 250 µl of MES/Ca buffer containing 2 mM phytic acid as substrate. The enzyme reaction was stopped by adding 500 µl of 10 % trichloroacetic acid (TCA). The liberated phosphate ions were quantified spectrophotometrically by mixing 100 µl of stopped assay mixture with 900 µl of coloring reagent (ammonium molybdate reagent) and absorbance was obtained at 820 nm after 15 min of incubation at 50 °C.

Phytic acid content was assayed in wheat flour, dough and chapatti according to Latta and Eskin (1980) with some modifications. The seeds from different plants of the same T_2 transgenic wheat line were pooled and 100 g of the seed were ground to obtain flour, dough and bread through conventional means. For phytic acid analysis, 500 mg of sample from transgenic and control wheat were extracted with 5 mL of 2.4 % HCl for 1 h at room temperature in shaking incubator. After centrifugation at 14,000 rpm, 3 ml of supernatant was passed through the SAX column (pre- activated with 5 % NaOH and extensively washed with water). The column was cleaned to remove Pi passing 8 ml of 0.1 M NaCl. The phytate was finally eluted with 5 ml of 0.7 M NaCl. To measure phytate, 3 ml of the extract was mixed with 1 ml of Wade's reagent and absorbance was measured after 10 min at 500 nm using UV-1800 (Shimadzu, Japan).

Expression studies

Total RNA was extracted from immature wheat seeds (8–10 DPA) from parent and selected transgenic wheat lines using Invitrogen Plant RNA Purification Reagent (Cat#12322-012) following the manufacturer's protocol. Total RNA ($\sim$1.2 µg) was reverse transcribed with oligo-dT using RevertAid^{TM} H minus first-strand cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's instructions. The cDNA was used for RT-PCR and subsequently qRT-PCR for determining relative expression of *PhyA* gene in the control and transgenic lines.

The template equalization was done using *Actin* gene as an internal control. ActWhtF and ActWhtR primers

were used for *Actin* amplification, while PhyCLC11F and PhyCLC11R were used for *PhyA* amplification (Table S1). The qRT-PCR was performed on the validated template for phytase gene expression studies. The reaction mixture comprised of 2 µl (500 ng/µl) cDNA, 12.5 µl 2X Maxima SYBR Green q-PCR Master Mix (Thermo Scientific), 1.5 µl (25 mM) $MgCl_2$, and 25 ng each of forward and reverse *phytase* gene primers (Table S1). Wheat *actin* gene was used as an internal control in the qRT-PCR experiment using *actin* specific primers (Table S1). The PCR profile was set to initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. The final extension was set at 72 °C for 7 min.

Iron and zinc bioavailability analysis

Bioavailability of iron and zinc was determined in wheat dough and chapatti (Asian bread) of selected T_2 lines. The total iron and zinc contents were determined by using standard AOAC methods (2005). The bioassay was carried out according to the method described by Miller et al. (1981) that involved simulated gastrointestinal digestion (using double enzyme digestion method) followed by measurement of soluble, low molecular weight iron and zinc as described in method S1.

All the estimations were made in triplicate. The data were analyzed statistically using SPSS version 17.0. One way Analysis of Variance (ANOVA) was applied at a confidence level of 95 %. The data presented here is the mean of triplicate). Comparisons of mean were performed using Tukey's test and explained with each relevant figure.

Grain nutrients composition

Protein, fat and gluten contents of the control and six selected transgenic lines were determined according to AOAC (2012). The starch content was estimated following Pearson (1991).

Results

Phytase expression vectors

Phytases from fungi have been reported to degrade phytic acid and release the bound micronutrients (Wodzinski and Ullah 1996; Singh and Satyanarayana 2015). The single and double gene phytase cassettes were analyzed by restriction analysis and DNA sequencing. The physical maps of the two cassettes are provided in Figure S2 (S2G and S2H respectively). The single phytase gene cassette in construct 1 comprised of 2605 bp that included 435 bp Hordein promoter (having 95 % identity to NCBI accession numbers X84368), ER signal from GenBank accession number EF434783, synthetic phytase gene with 3′ terminal AAGGACGAGCTCTGA sequence coding for KDEL, the stop codon and the *CaMV* terminator. The first phytase gene cassette in construct 2 had the same size and order of sequences as the phytase cassette in construct 1. The second phytase gene cassette in construct 2 comprised of 2625 bp. In this cassette, the phytase gene was without a KDEL coding sequence, while the ER specific signal was retained and the expression was controlled by 930 bp wheat glutenin promoter (having 97 % identity to NCBI accession number EU074252). A *Nos* terminator was attached downstream to the phytase gene (Fig. S3A). The cleavage prediction using SignalP4 (Peterson et al. 2011) indicated that the ER signal is removed at the same site (TLA-VPA) as the original signal peptide sequence of *A. japonicus* (GLA-VPA), thus releasing the correct amino acid sequence for proper folding in wheat tissues (Fig. S4).

Transformation and selection of transgenics

Two wheat varieties (Faisalabad-2008 and Sahar-2006) were used for *Agrobacterium*-mediated transformation. Five thousand embryos of each variety were isolated. The true calli derived from immature embryos were infected with *A. tumefaciens* harboring either of the two phytase constructs (Fig. S5). One hundred and twenty putative transgenic plants were regenerated, 13 from Sahar-2006 and 107 from Faisalabads-2008, and shifted on selection medium having 3 mg/L phosphinothricin. The 70 plants survived to maturity, 7 from Sahar-2006 and 63 from Faisalabad-2008. The plants were separated into two groups based on the type of transformed construct (Construct 1 and Construct 2). The PCR analysis was carried out on total genomic DNA from putative transgenic plants by using four sets of gene junction primers (two pairs for each cassette) and is shown in Figs. 1 and 2. The location of primers in

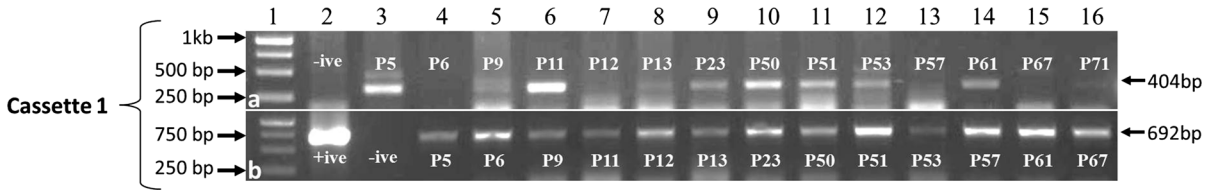

Fig. 1 PCR analysis of the genomic DNA isolated from putative transgenic wheat plants transformed with construct 1. (*a*) PCR amplification of promoter-cds junction region of the integrated phytase 1 cassette using primers HorPhyF and HorPhyR. *Lane 1*, 1 kb DNA ladder. *Lane 2*, negative control (non-transgenic). *Lanes 3–16*, PCR amplifications from total DNA obtained from the leaves of putative transgenic plants. (*b*) PCR amplification of cds-terminator junction region of the phytase cassette 1 using primer pair PhyCamvF and PhyCamvR. *Lane 1*, 1 kb DNA ladder. *Lane 2*, PCR amplification from positive control. *Lane 3*, negative control. *Lane 4–16*, PCR amplifications from putative transgenic plants chromosomal DNA. Amplification of both 404 and 692 bp fragments from putative transgenics plants was assumed to be complete integration of phytase cassette 1 in wheat genome. The primer sequences are provided in Table S1

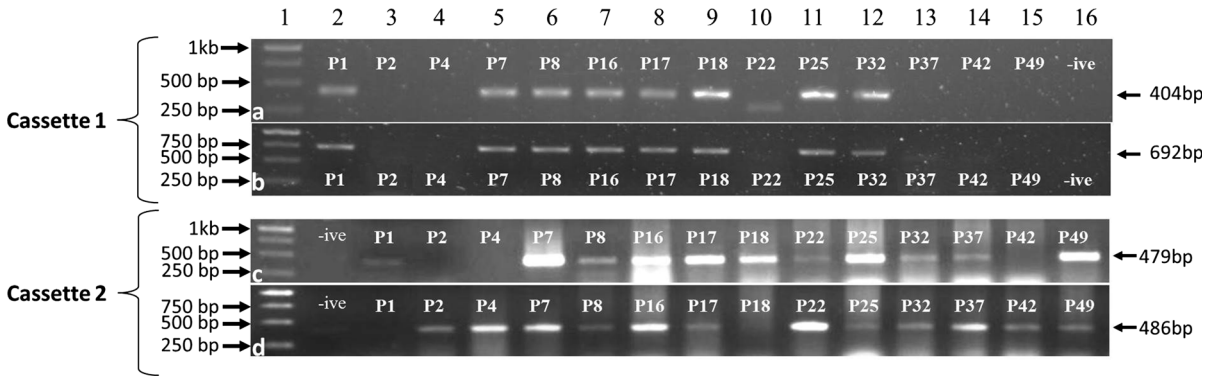

Fig. 2 PCR analysis of the total genomic DNA isolated from putative transgenic wheat plants transformed with construct 2. (*a*) PCR amplification of promoter-cds junction region of the phytase 1 cassette in construct 2 using primers HorPhyF and HorPhyR. *Lane 1*, 1 kb DNA ladder. *Lanes 2–15*, PCR amplifications from total DNA obtained from the leaves of putative transgenic plants. *Lane 16*, negative control (total DNA from non-transgenic plant). (*b*) PCR amplification of cds-terminator junction region of the phytase cassette 1 using primer pair PhyCamvF and PhyCamvR. *Lane 1*, 1 kb DNA ladder. *Lanes 2–15*, PCR amplifications from total DNA obtained from putative transgenic plants. *Lane 16*, negative control. Amplification of both 404 and 692 bp fragments from putative transgenics plants was assumed to be complete integration of phytase cassette 1. (*c*) PCR amplification of promoter-cds junction region of phytase 2 cassette in wheat transgenics transformed with construct 2. *Lane 1*, 1 kb DNA ladder. *Lane 2*, negative control. *Lane 3–16*, PCR amplifications from total genomic DNA from putative transgenic plants using GluPhyF and GluPhyR primers. (*d*) PCR amplification of cds-terminator junction region in phytase 2 cassette using primer pair PhyNosF and PhyNosR, *Lane 1*, 1 kb DNA ladder. *Lane 2*, negative control. *Lanes 3–16*, PCR amplifications from the total genomic DNA of putative transgenic plants. Amplification of 404, 692, 479 and 486 bp fragments from putative transgenics plants was assumed to be complete integration of phytase cassette 1 and 2 in wheat genome. Any lane with missing amplifications was considered for incomplete integration of phytase cassette 1 and 2 in the genomic DNA. The PCR amplifications indicate that plants P7, P8, P16, P17, P18 and P25 have both the phatase 1 and phytase 2 cassettes from construct 2. The primer sequences used for PCR amplifications are provided in Table S1

construct 1 and construct 2 is provided in Fig. S3A-S3E.

The differential primer approach provided a good idea of transgene integrity. It helped to discard truncated or rearranged transgenics at an earlier stage and selecting those with complete cassettes to undergo more detailed expression analysis. Thirty transgenic plants indicating complete transgene cassette integration were selected. These plants had 4 transgenics from Sahar-2006 and 26 from Faisalabad-2008. The overall transformation efficiency was 0.04 % for Sahar-2006 and 0.26 % for Faisalabad-2008. Basta leaf paint assay was done on T_1 plants in order to check the resistance of progeny lines to the herbicide. The sensitive versus resistance ratio of 1:1(P50) to 1:2.5(P61) was observed among the Basta painted leaves from different transgenic lines indicating heterologous expression of *PAT* gene at T_1 stage (Fig. S6).

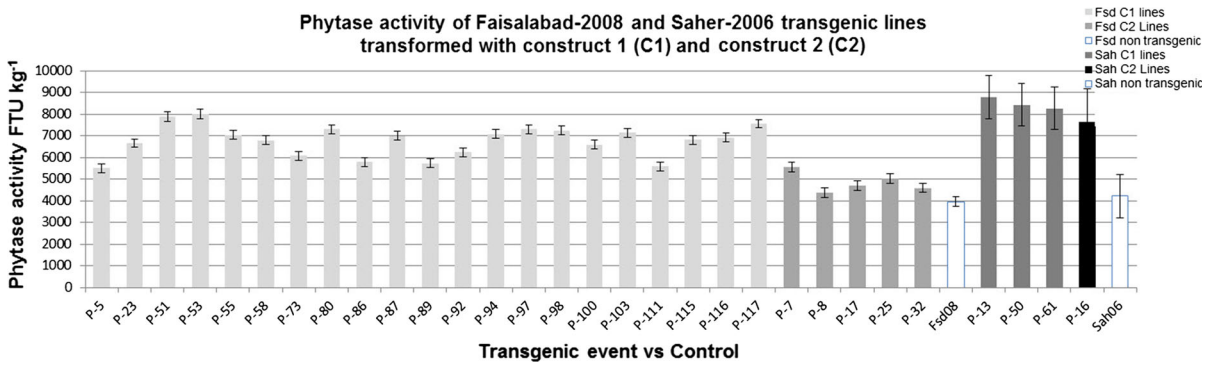

Fig. 3 Phytase activity in seeds of non-transgenic (control) and transgenic lines of Faisalabad-2008 (single phytase gene-construct 1, P5-P117; double phytase gene-construct 2, P7-P32) and Sahar-2006 (single phytase gene-construct1, P13-P61; double phytase gene-construct 2, P16). *Error bars* indicate mean ± SD of three replicates (Turkeys's Test $P < 0.05$)

Phytase activity and phytic acid content

Phytase activity was determined on pooled T_1 seeds of 30 individual putative transgenic plants selected through conventional PCR indicating complete phytase gene integration. On the basis of higher phytase activity the T_1 seeds of six T_0 transgenic plants, representing six different transgenic events were selected for further study. These lines included P7, P13, P50, P51, P53, and P61. The line P7 was transformed with construct 2, while the remaining five lines had phytase cassette integration from construct 1. The phytase activities of the pooled seeds, of the six individual selected transgenic lines, were higher than controls (Fig. 3). However, a variation in the phytase activity of all tested transgenic plants was detected. Maximum phytase activity was observed in P13 (parent of E1Sah) that was 8785.89 FTU. Amongst the six selected lines, P7 (parent of E1Fsd) showed the minimum phytase activity that was 5560.83 FTU as compared to controls Fsd08 (3965.38 FTU) and Sah06 (4221.79 FTU). Over all, an increase of 40–99 % in phytase activity was observed in the T_1 seeds of selected transgenic wheat lines (Fig. 4).

The phytic acid content was determined in T_2 seeds from the selected plants of the six picked transgenic wheat lines (Fig. 4). The individual plant selection was based on phytase transcript level in the respective plant (Fig. S7). The highest transcript level was observed in plants F10, E1, C5, D8, I9 and D7. The F10 plant was selected from the T_2 line obtained from P7 and was named E1Fsd (Fig. S7a). E1 plant was selected from the T_2 line obtained from P51 and was named E2Fsd (Fig. S7b). C5 plant was selected from the T_2 line obtained from P53 and was named E3Fsd (Fig. S7c). D8 plant was selected from the T_2 line obtained from P13 and was named E1Sah (Fig. S7d). I9 plant was selected from the T_2 line obtained from P50 and was named E2Sah (Fig. S7e). D7 plant was selected from the T_2 line obtained from P61 and was named E3Sah (Fig. S7f). The schematic diagram representing the selection of single transgenic plants mentioned above is provided in Figure S7.

The phytic acid content in the T_2 seeds obtained from E1Fsd, E2Fsd, E3Fsd, E1Sah, E2Sah and E3Sah was significantly reduced as compared to control (Fig. 4). Highest phytic acid content was observed in flour (65–100 mg/100 g) followed by dough (62–82 mg/100 g) and chapatti (58–65 mg/100 g). The phytic acid content determined in flour for the transgenic lines fell in the range of 0.69–0.99 g kg^{-1}, which is 12–41.6 % less than the phytic acid content in the flour of control lines Fsd08 (122 mg/100 g) and Sah06 (112 mg/100 g).

Expression analysis

Semi-quantitative RT-PCR was conducted to select T_2 lines based on transcript level. Total RNA was used to prepare cDNA from developing seeds (10DPA) of T_2 plants that germinated out of ten T_1 seeds planted for each selected transgenic wheat line. The RT-PCR results are given in Figure S7. The *PhyA* transcript was detected by the amplification of a 325 bp fragment using phytase specific primers given in Table S1. The wheat actin gene was used as

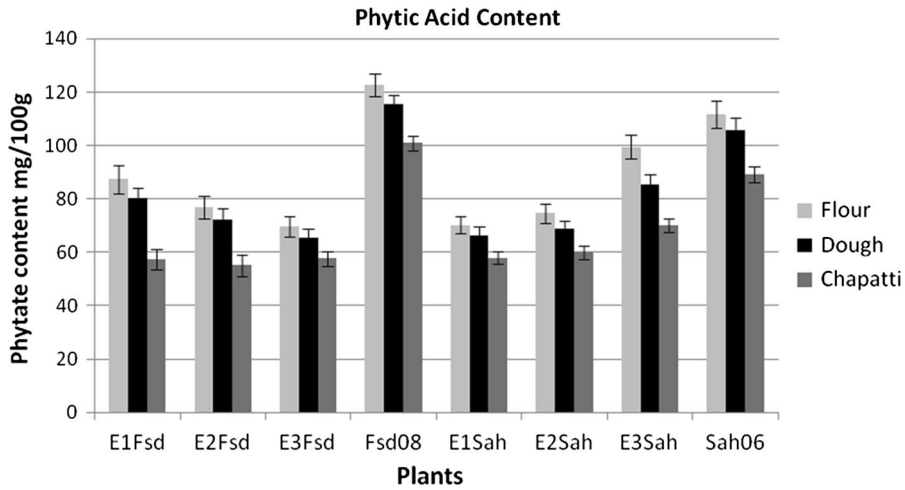

Fig. 4 Phytic acid content in flour, dough and chapatti obtained from transgenic and non-transgenic (control) lines of Fsd08 and Sah06. The lowest phytic acid content was observed in chapatti followed by dough and flour. The transgenic lines E1Fsd and E3Sah showed highest phytic acid content reduction ratio in flour, dough and chapatti. *Error bars* indicate mean ± SD of three replicates (Turkeys's Test $P < 0.05$)

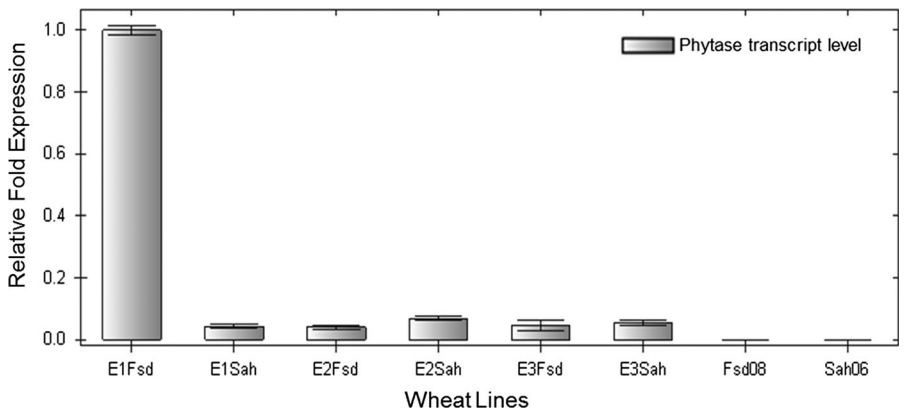

Fig. 5 Relative fold expression (ΔC_q) of phytase in endosperm samples of control and transgenic lines by Real Time PCR. Expression was observed only in transgenic wheat lines (E1.Fsd-E3.Fsd; E1.Sah-E3.Sah) whereas no expression in non-transgenic lines (Fsd08 and Sah06). Among all transgenic lines, the lowest expression (onefold) was observed in E2.Fsd and the highest expression was observed in E1.Fsd (ninefold). The rest of the transgenic lines E3.Fsd, E1.Sah, E2.Sah and E3.Sah showed 1.3 fold, 1.2 fold, 2 fold and 1.5 fold expression, respectively. *Error bars* indicate mean ± SD of three replicates

an internal control and is represented by a 369 bp fragment (Fig. S7). The T_2 lines showing highest transcript level were selected for determining phytic acid content (Fig. S7). qRT-PCR was used to determine the expression level of *PhyA* in control and selected T_2 transgenic plants. The expression profiling indicated that the transformed lines had twofold to ninefold higher transcript level verses no transcript detection in wild plants. Among all transgenic lines, E1Fsd showed about ninefold higher expression of phytase transcript (Fig. 5).

Comparative studies of bioavailable iron and zinc

The quantitative estimation of the bioavailable iron by FAAS (Flame Atomic Absorption Spectrophotometer) indicated that total bioavailable iron level in endosperm of transgenic wheat lines increased up to 38 % as compared to non-transgenic wheat. Iron and zinc bioavailability of all transgenic lines was higher than non-transgenic plants. However, the bioavailability of iron and zinc was significantly enhanced in chapatti as compared to dough (Fig. 6).

In case of dough, maximum bioavailable iron content of 22.59 % was observed in E3Sah and minimum bioavailable iron content of 19.46 % was observed in E1Fsd. Over all, an increase of 18–38 % in bioavailable iron content was estimated in dough of transgenic lines. In case of chapatti, maximum bioavailable iron content was observed in E3Sah that was 90.50 % and minimum bioavailable iron content was found in E2Sah that was 48.01 %. However, an increase of 31–218 % was detected in bioavailable iron content of chapatti cooked from different transgenic lines (Fig. 6).

On the other hand, in dough the maximum bioavailable zinc content was observed in E1Fsd that was 14.77 % and minimum was detected in E1Sah that was 11.28 %. An assorted increase of 29–99 % in bioavailable zinc content was found in dough of transgenic lines. In case of chapatti maximum bioavailable zinc content was observed in E2Sah that was 46.99 % and minimum bioavailable zinc content was noted in E3Sah that was 22.72 % (Fig. 6). Over all, an increase of 4–115 % in bioavailable zinc content was observed in chapatti prepared from different transgenic lines.

Grain nutrients composition

The grain nutrient composition for total proteins, fats, starch and gluten is provided in Fig. 7. The statistical analyses on the triplicate estimations indicate that there is no significant difference between the controls and transgenics. These results emphasize that expression of phyA in wheat endosperm does not disturb the major nutritional composition of both the wheat lines used in this study.

Discussion

The degradation of phytic acid by phytase enzyme may be achieved if sufficient amount of phytase is stored in the grains during seed development. The processing of grains or flour for cooking could then activate the enzyme and cause subsequent degradation of phytic acid, thus releasing the desired elements. A thermostable enzyme may, however, prolong the enzyme activity and can result into greater release of iron and zinc under higher temperatures. Phytases from *Aspergillus* species have been reported to have highest activity at 50 °C and retain >50 % of their activity at 90 °C for 15 min (Promdonkoy et al. 2009). A codon optimized thermostable phytase gene analogous to the *A. japonicus* nucleotide sequence accession no. EU786166 and protein sequence accession no. ACE79228 respectively, was fused with an N-terminal expansin ER signal for ER processing and N-glycosylation (Fig. S1). The phyA storage was directed to ER using C-terminal KDEL sequence (Construct 1) and secretion in apoplast for additional storage by inserting a second phyA expression cassette lacking KDEL sequence in Construct 2. ER specific storage of phytase has been widely reported (Brinch-Pedersen et al. 2000; Lucca et al. 2001; Drakakaki et al. 2005; Liu et al. 2006; Peng et al. 2006), while apoplast specific secretion has not been tested in wheat endosperm. The grinding of seeds to make flour, does not essentially break the cells completely, thus most of the enzyme might have been intracellular and could not be accessed for phytic acid degradation. The apoplast storage was considered to provide extra amount of phytase enzyme.

In rice, soybean, canola and wheat various signal peptides from proteins like barley ß-glucanase (Lucca et al. 2001), tobacco pathogenesis related proteins (Liu et al. 2006; Peng et al. 2006), α-amylase (Brinch-Pedersen et al. 2000) and murine immunoglobulin (Drakakaki et al. 2005) have been used to target the post translational modification. For this purpose, we utilized an efficient ER signal from expansin gene (Naseer et al. 2010). The endosperm specific expression of double gene cassette was controlled by a truncated barley hordein promoter for cassette 1 and wheat glutenin promoter in cassette 2 (Fig. S2).

The wheat flour goes through the process of doughing before cooking chapatti (Indian bread). The dough is usually kept at room temperature between 15 to 30 min and then used for making the bread. The wheat phytase has a temperature optimum at 45 °C (Nakano et al. 1999), while the fungal phytase becomes active at higher temperatures (50 °C and above). We assumed that a greater phytic acid degradation could be achieved in the bread making process using a thermostable phytase and depositing it in the apoplast and the ER of wheat endosperm tissues.

A population of 70 putative transgenic plants based on tolerance to Basta were regenerated and screened by genomic PCR to identify primary transgenics. The genomic PCR was carried out by using two sets of

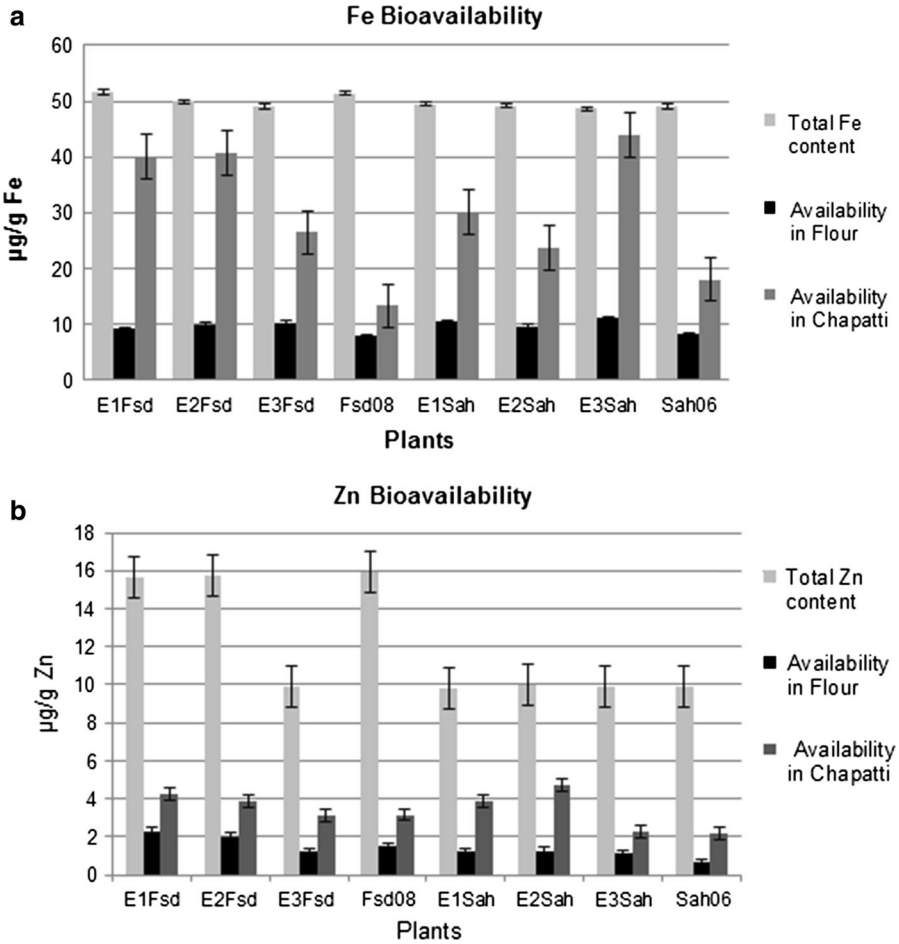

Fig. 6 Bioavailability of iron and zinc in control and transgenic lines. **a** Comparison of total iron content and bioavailable Fe in control and transgenic wheat dough and chapatti. The highest levels of bioavailable iron were observed in cooked chapatti from transgenic lines E1Fsd, E2Fsd and E3Sah. **b** Comparison of bioavailable Zn in control and transgenic wheat dough and chapatti. The highest bioavailable Zn levels were detected in the cooked chapatti from transgenic lines E1Fsd, E2Fsd, E1Sah and E2Sah. *Error bars* indicate mean ± SD of three replicates (Turkeys's Test $P < 0.05$)

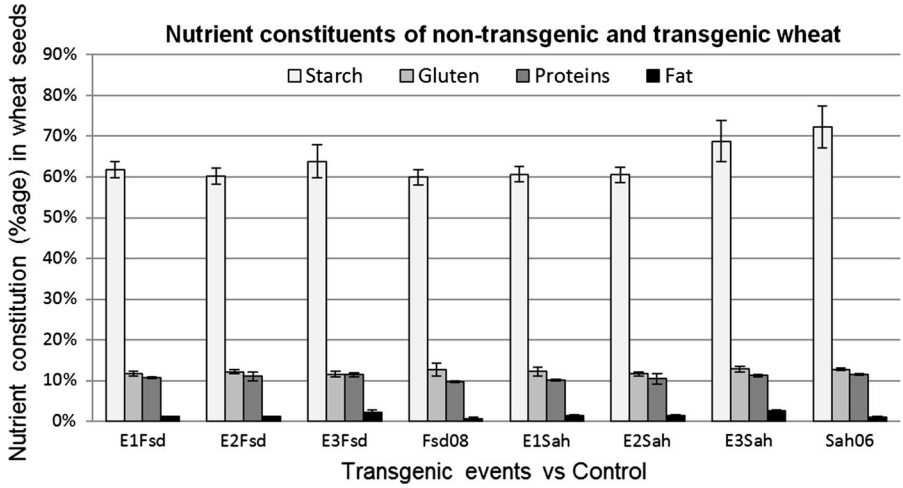

Fig. 7 Nutrient constituents in control and transgenic lines. No significant difference in starch, gluten and protein contents was observed between non-transgenic and transgenic lines. The higher fat content was observed in Sah06 as compared to the Fsd08 control lines and a similar pattern was detected in the respective transgenic lines. *Error bars* indicate mean ± SD of three replicates (Turkeys's Test $P < 0.05$)

junction primers (promoter-cds and cds-terminator junction) (Table S1; Fig. S3). The double primer verification of a transgene may be a better approach to detect the integration of complete gene cassette. The PCR on genomic DNA yielded 30 transgenics (each representing an independent transgenic event) that indicated complete expression cassette integration in wheat genome (Figs. 1, 2). The T_1 progenies generated from the 30 transgenic events were propagated. About 10 plants from each T_2 line were selected for expression profiling of phytase transcripts in the endosperm tissues through RT-PCR. Variability in transcript level was observed within transgenic lines and also the lines from the same transgenic event. This variability may be due to the variations in gene integrations, host response or gene stability (Butaye et al. 2005). We assume that it may also be due to heterologous gene expression as T_2 plants might not represent the true homozygous lines.

Phytase expression profiling of the six selected transgenic lines was performed by Real Time PCR. Expression of phytase gene was observed in all the transgenic lines. The primers designed on the synthetic sequence did not indicate identity with the known wheat phytases, both in the non-redundant and EST databases. The primers were validated for >95 % PCR efficiency using Cyber green chemistry. Relative fold expression between the six transgenics was determined by ΔΔ CT method. Five of the transgenic lines (E1Sah, E2Fsd, E2Sah, E3Fsd, E3Sah) indicated relatively equal expression levels, while the transgenic line E1Fsd indicated a ninefold higher expression of phytase transcripts in several repetitions (Fig. 5). This higher expression could not be correlated with the phytase activity or reduction in phytic acid content. However, this transgenic line had almost similar level of phytase activity and phytic acid degradation as most of the other selected lines.

Six transgenic events from the two transformed varieties (Sahar-2006 and Faisalabad-2008) were selected based on the clarity and persistence in RT-PCR amplifications. The phytase activity of the T_1 generation seeds indicated a maximum of 99 % increase (Fig. 3). The phytase activity in transgenics selected from wheat variety Sahar-2006 was higher than those of Faisalabad-2008, which might be a varietal response. On the other hand, the transgenics transformed with double gene cassette showed a significantly lower phytase activity as compared to single gene integrations directed for apoplast specific deposition of phytase. It is assumed that the double gene expression might have caused a competition between two transcripts directed to ER or deposition of phytase in ER is a better choice than release in apoplast.

Rice and maize have been the key cereals for the expression of fungal phytases. In previous studies, 130 fold increase of phytase activity in transgenic rice plants (Lucca et al. 2001) and 50 fold increase in phytase activity of transgenic maize seeds was observed (Chen et al. 2008). These cereals naturally deposit negligible amount of phytase in their seeds, while wheat has been reported to accumulate significant amounts of phytase in the mature seeds (Brinch-Pedersen et al. 2000). However, fungal phytase expression in wheat has been reported to enhance fourfold enzyme activity (Brinch-Pedersen et al. 2000). Our results indicate that transgenic phytase activity is comparatively lower in the best selected wheat transgenics as compared to that reported for rice and maize. This difference could be due to host response or heterologous gene expression.

To increase endosperm specific phytase level, we introduced a synthetic version of *A. japonicus* phytase gene under the control of D-hordein promoter and double phytase genes under the control of D-hordein and glutenin promoters respectively. Endosperm specific expression of multivitamins and phytase has been reported earlier in corn (Chen et al. 2008; Naqvi et al. 2009) and canola (Peng et al. 2006) respectively using similar strategies. Our analysis of phytase activity and assays on bioavailable iron and zinc levels indicated that phytase expression in wheat seeds resulted in comparatively lower enhancement of phytase activity and the nutrients as compared to the values reported for rice and maize but were significantly higher to improve the nutritional value of transgenic wheat. Our results correlate with Gao et al. (2007) who introduced a linear gene cassette (35S-phytase gene-nos) having T-DNA borders into soybean genome through the pollen tube pathway and obtained 2.5-fold increase in transgene expression in soybean.

An increase in phytase activity could be correlated with the reduction in phytic acid content (Shen et al. 2008). A direct but relatively weaker correlation was observed in this study. To determine phytase activity, phytic acid content and bioavailability we pooled the

seeds for each event. It is expected that due to segregation, the samples present heterologous phyA expression. Thus we couldn't expect high level of correlation.

These studies demonstrated that the transgenic lines with higher phytase activity had lower phytic acid content. The phytic acid content determined for the transgenic lines fell in the range of 69.59–99.44 mg/g, which is 12–76 % less than the phytic acid content of non-transgenic lines (Fig. 4). Drakakaki et al. (2005) and Peng et al. (2006) reported 30 %–50 % reduction of phytate in transgenic maize and canola plants. Reduction of phytic acid is positively correlated with increased iron absorption (Frontela et al. 2009; Akhtar et al. 2012). In the present study, the selected transgenic lines (E1Sah, E2Sah, E3Sah, E1Fsd, E2Fsd, E3Fsd) with lower phytate content were used for determining iron and zinc bioavailability. We carried out quantitative profiling of the bioavailable iron and zinc in dough and chapatti of selected transgenic and non-transgenic lines by using FAAS (Flame Atomic Absorption Spectrophotometer). Bioavailable iron and zinc were higher in chapatti (218 and 115 %, respectively) as compared to dough (38; 99 %; Fig. 6), which indicated that fungal phytase worked more efficiently at higher temperature.

Nutritional impact of transgenic wheat with low phytate

Daily intake of wheat in Pakistani population is ~342 g/person/day (Pakistan Agricultural Research Council 2013). The ranges of iron and zinc contents (dry weight basis) in wheat grains of the most popular elite wheat varieties in Punjab, Pakistan were reported as 3.41–5.55 mg/100 g for iron and 0.71–3.00 mg/100 g for zinc (Akhtar et al. 2012). In this study, total iron content is 5.14 ± 0.02 mg/100 g for Faisalabad-2008 and 4.91 ± 0.05 mg/100 g for Sahar-2006. The total zinc content is estimated to be 1.59 ± 0.01 and 0.99 ± 0.01 mg/100 g in Faisalabad-2008 and Sahar-2006 respectively. The percent bioavailability of iron and zinc in transgenic wheat plants was found to be much higher than parent varieties (Fig. 6). The bioavailable iron in transgenic wheat achieved 52.14 % of recommended daily intake (RDI) which is 14.28 % higher than non-transgenic wheat. Similarly, the zinc bioavailability is 10.66 % of RDI, 4 % higher than normal. The nutrient composition analysis indicated non-significant differences in control and transgenic wheat lines. Therefore, the nutritional values of the phyA transgenic seeds remain the same as the elite cultivars. The transgenic wheat varieties can be utilized for good uptake of the micro nutrients like iron and zinc. However, achieving the desired bioavailable iron and zinc levels in wheat would require sustainable developments in our breeding programs and continuous efforts in development and screening of transgenics.

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