Transgenic Res
DOI 10.1007/s11248-007-9138-3

ORIGINAL PAPER

Transgenic maize plants expressing a fungal phytase gene

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在文中，作者为了研究不同转基因玉米颗粒中肌醇六磷酸酶的表达水平，采用Geno2000同时研磨大量的玉米种子。这样既提高了试验的效率，又确保了数据的准确性和重现性。

Received: 19 December 2006 / Accepted: 24 August 2007

Abstract Maize seeds are the major ingredient of commercial pig and poultry feed. Phosphorus in maize seeds exists predominately in the form of phytate. Phytate phosphorus is not available to monogastric animals and phosphate supplementation is required for optimal animal growth. Undigested phytate in animal manure is considered a major source of phosphorus pollution to the environment from agricultural production. Microbial phytase produced by fermentation as a feed additive is widely used to manage the nutritional and environmental problems caused by phytate, but the approach is associated with production costs for the enzyme and requirement of special cares in feed processing and diet formulation. An alternative approach would be to produce plant seeds that contain high phytase activities. We have over-expressed *Aspergillus niger phy*A2 gene in maize seeds using a construct driven by the maize embryo-specific globulin-1 promoter. Low-copy-number transgenic lines with simple integration patterns were identified. Western-blot analysis showed that the maize-expressed phytase protein was smaller than that expressed in yeast, apparently due to different glycosylation. Phytase activity in transgenic maize seeds reached approximately 2,200 units per kg seed, about a 50-fold increase compared to non-transgenic maize seeds. The phytase expression was stable across four generations. The transgenic seeds germinated normally. Our results show that the phytase expression lines can be used for development of new maize hybrids to improve phosphorus availability and reduce the impact of animal production on the environment.

Keywords Maize · Phytate · Fungal phytase · *phy*A2 · Transgenic plant

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Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate; $InsP_6$) is synthesized during seed development and accumulated in mature seeds. It exists *in planta* as phytate, a mixed cation salt of phytic acid, or a complex of phytate and proteins, also known as phytin. Phytate serves as the principal storage of phosphorus,

myo-inositol and a variety of mineral cations for seed germination and seedling growth. Phytate represents approximately 75–80% of the total phosphorus in plant seeds (Cosgrove 1966; Austin et al. 1994). Cereal grains and oilseed meals are major ingredients of animal feed. The amount of phosphorus in cereal grains and oilseed meals would meet the requirement of optimal growth of animals if all the phosphorus from phytate were available to animals. However, monogastric animals (e.g., pigs, poultry, and fish) can barely utilize phytate phosphorus because they have very low levels of phytase activity in the digestive tract (Ravindran et al. 1995; Schroder et al. 1996; Wodzinski and Ullah 1996; Brinch-Pedersen et al. 2002; Vohra and Satyanarayana 2003). Therefore, inorganic phosphate has to be added to animal rations to achieve optimal growth. Consequently, a large amount of undigested phytate phosphorus is excreted in animal waste. The high concentration of phytate in the animal manure contributes significantly to algal blooms and eutrophication of surface waters. Furthermore, phytate is an anti-nutritional factor. It combines in vivo with nutritionally important cations, such as Fe^{3+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, and K^{+}, preventing absorption of the minerals by animals (Asada et al. 1969; Urbano et al. 2000).

Phytases ($InsP_6$ phosphohydrolase) are a special class of phosphatases which catalyze the sequential hydrolysis of phytic acid to produce less phosphorylated *myo*-inositol derivatives and inorganic phosphate (Wyss et al. 1999a). Phytases are found in all types of organisms. A number of phytase genes have been isolated and characterized from plants (Gibson and Ullah 1988; Hegeman and Grabau 2001), bacteria (Kerovuo et al. 1998; Greiner et al. 1993; Yao et al. 2001) and fungi (Wodzinski and Ullah 1996; Wyss et al. 1999b; Yao et al. 1998). *Aspergillus niger phy*A genes have been ectopically expressed in *A. niger*, *A. awamori*, *A. terreus*, *A. ficuum*, and *A. fumigatus* (Lei and Stahl 2001; Mitchell et al. 1997; Mullaney et al. 2000; Vohra and Satyanarayana 2003). Microbial phytases are widely used as a feed additive to increase phosphorus availability of plant-based feed to animals and reduce phosphorus excretion in manure (Verwoerd et al. 1995; Brinch-Pedersen et al. 2002).

The *phy*A2 gene from *A. niger* line 963 encodes 467 amino acids with a signal peptide at the N terminus (Yao et al. 1998). The gene shares 91.6% amino acid sequence identity with the *phyA* gene from *A. ficuum* (Ullah 1988). The yeast-expressed recombinant *phy*A2 enzyme that lacks the signal peptide has a number of unique characteristics, making it an ideal feed additive. The recombinant *phy*A2 enzyme has high specific activity (100 U/mg protein) on phytic acid with two optimum pH of 1.6–2.0 and 5.5–5.9. The specific activity at pH 1.8 is 77% of the activity displayed at pH 5.8. At pH 3.0, the average pH in the animal digestive tract, *phy*A2 retains 40% of its activity. The widely used *phy*A enzyme, however, exhibits the pH optima of pH 2.5 and 5.9. The specific activity at pH 2.5 is 50% of the activity at pH 5.9. Only 25% of the activity remains at pH 3.0. The *phyA*2 gene has been expressed in *Pichia pastoris* for the large-scale fermentation (Yao et al 1998). The *phy*A2 phytase enzyme is marketed as a commercial feed additive in China.

To reduce phytase production costs, new prokaryotic and eukaryotic expression systems were explored. Phytase genes from various *Aspergillus* species have been expressed in transgenic tobacco seeds and leaves (Reddy et al. 1982; Pen et al. 1993; Verwoerd et al. 1995; Ullah et al. 1999), transformed soybean cell-suspension cultures (Li et al. 1997), transgenic soybean and alfalfa (Denbow et al. 1998; Ullah et al. 2002), and transgenic wheat, rice, and canola seeds (Brinch-Pedersen et al. 2000; Zhang et al. 2000; Lucca et al. 2001; Ponstein et al. 2002; Hong et al. 2004). The plant-produced phytase has a lower molecular mass than the enzyme produced in fungi due to a difference in glycosylation (Pen et al. 1993; Li et al. 1997). Poultry-feeding studies demonstrated that the plant-produced phytase can substitute for the enzyme produced from microbial fermentation (Pen et al. 1993; Denbow et al. 1998; Zhang et al. 2000).

The objective of this research was to develop maize lines that accumulate significant quantities of the active phytase in the seed to eliminate the need for phosphorus supplementation of monogastric animal feed. The *phy*A2 gene was ectopically expressed in transgenic maize plants. Analyses on phytase activity, the stability of *phy*A2 expression and the integration pattern of the transgene indicate that the *phy*A2 transgenic lines can be incorporated in breeding programs to develop new maize hybrids.

Materials and methods

Plant materials

Maize plants of Hi-II (Armstrong et al. 1991) grown in the field and greenhouse were used as a source of immature embryos (1.0–2.0 mm long). Immature ears were dehulled, sterilized with 5% (v/v) NaOCl for 30 min and washed extensively with sterile distilled water. The isolated immature embryos were placed on N6 1-100-25 medium (Armstrong et al. 1991) containing 0.2% (w/v) phytagel (Sigma, St. Louis, MO, USA) for callus induction.

Plasmid construction

The phytase over-expression constructs used for maize transformation consists of the maize embryo-specific globulin-1 (Glb) promoter, the *A. niger phyA*2 gene and the Glb terminator. The construct pSPHP3303T-Phy contains a synthetic barley α-amylase signal peptide sequence (5′-GGATCCATG GGGAAGAACGGCAGCCTGTGCTGCTTCTCTC TG CTGCTGCTGCTGCTTCTCGCCGGGTTGGC GTCCGGC-3′) immediately after the start codon of the *phy*A2 gene for secretion of the expressed phytase into the intercellular space. The construct pSPHP3303Phy does not have the signal peptide and the phytase would remain in cytosol. To make plasmid pSPHP3303Phy, pSP72 (Promega, USA) was digested with *Xho*I, resulting sticky ends were blunted with T4 DNA polymerase and ligated to generate pSP72-1. Maize Glb promoter was excised with *Hind*III and *Bam*HI from PHP3303 and subcloned into pSP72-1 to generate pSPHP3303-1. Maize Glb terminator was excised with *Nde*I and *Sac*I and subcloned into pGEM5zf (+) (Promega, USA) to generate pSPHP3303-2. The terminator then was excised with *Sac*I and *Eco*RV from pSPH3303-2 and subcloned into pSPHP3303-1 to generate pSPHP3303. The *phy*A2 gene was amplified from vector Te-phy-2 using forward primer (5′-GGATCCATGTCCAAGTCCTGCGATAC-3′) and reverse primer (5′-GTTAACCTATTATCAACTA-AAGCACTCTCCCC-3′). The PCR product was cloned into pGEM5zf (+) to generate pGEM5zfPhy. The phytase gene then was excised from pGEM5zf-Phy with *Bam*HI and *Hpa*I and subcloned into pSPHP3303 to produce pSPHP3303Phy. pSPHP3303T-Phy was constructed by inserting the signal peptide sequence of barley α-amylase gene immediately behind the start codon of the *phy*A2 gene. The chimeric gene cassettes were sequenced to confirm their correctness.

The plasmid PHP17042BAR carrying the maize histone H2B (H2B) promoter (Joanin et al. 1992), the maize Ubiquitin 5′-UTR intron-1, the *bar* gene and the potato protease II (PINII) terminator (An et al. 1989) was used as the transformation selectable marker. To make PHP17042BAR, pAHC25 (Christensen and Quail 1996) was digested with *Pst*I and the *bar* gene fragment was subcloned into pGEM5zf (+) to generate pGEM5zfBar. The *bar* gene was excised from pGEM5zfBar by *Bam*HI and *Eco*RV digestion and subcloned into PHP17042 which contains the maize H2B promoter to produce PHP17042BAR.

Maize transformation

The chimeric phytase expression cassette was excised with restriction enzyme *Nde*I from plasmid pSPHP3303Phy and pSPHP3303T-Phy for transformation. The *bar* gene expression cassette was excised with *Hind*III and *Sac*I from plasmid PHP17042BAR. Maize transformation was carried out with tungsten microprojectiles carrying the phytase and *bar* gene according to the method described by Tomes (1995).

Production of transgenic plants

The putatively transformed calli were recovered on medium containing Bialaphos. Seedlings were regenerated from the putatively transformed calli and transplanted into greenhouse. Regenerated transgenic plants confirmed by herbicide leaf painting were grown in greenhouse and pollinated with the inbred line Huangzaosi or Mo17 to produce T1 seeds. Progenies were self-pollinated to produce Tn.

Leaf-painting assay

Basta (Bayer, AG) was mixed with lanolin to obtain a paste with 1% of the active ingredient for leaf painting. The paste preparation was applied to a

marked area on the third or fourth leaf of maize seedlings at the five-leaf stage. The herbicide resistance was scored at 5–7 days after herbicide applications. Transgenic plants are resistant to Basta due to the presence of the *bar* gene and the wild-type segregants are susceptible.

Determination of phytase activity, phytic acid, and Pi contents

Single or pooled kernels were ground with a Geno/Grinder2000 (Sepx CertiPrep, USA). Phytase activity, phytic acid, and Pi contents were measured.

To determine phytase activity, 100-mg samples were placed into an eppendorf tube and 1-ml extraction buffer (50 mM NaAc, 1 mM $CaCl_2$, pH 5.5) was added. The tubes were shaken on a shaker at room temperature for 1 h. The tubes were then centrifuged at 3,000*g* for 10 min. Supernatants were transferred into fresh tubes and used for phytase activity measurement with the method described by Wyss et al. (1999a). In brief, 100 µl of the supernatant was mixed with 900 µl of 5 mM phytic acid, and incubated at 37°C for 30 min. The reaction was stopped by adding 1 ml of 15% (v/v) aqueous trichloroacetic acid (TCA). To set up controls, TCA was added to the supernatant first, followed by the phytic acid substrate, and incubated in the same conditions. The released Pi was quantified colormetrically using 0.6 M H_2SO_4–2% ascorbic acid–0.5% ammonium molybdate. Standard solutions of potassium phosphate were used as a reference. One unit (U) of phytase activity was defined as the amount of activity that liberates 1 µmol of phosphate per min at 37°C.

Phytic acid was assayed according to Latta and Eskin (1980) and Vaintraub and Lapteva (1988). Thirty-milligram samples were extracted with 1 ml of 0.4 M HCl–15% TCA at room temperature for 3 h. After centrifugation at 3,000*g* for 15 min, 25 µl of the supernatant was mixed with 275 µl of 36.3 mM NaOH and 100 µl of 0.03% (w/v) $FeCl_3 \cdot 6H_2O$–0.3% sulfosalicylic acid. The mixture was centrifuged at 2,000*g* for 10 min. Then, transfer 200 µl supernatant into a new 96-well plate. The absorbance at 500 nm was measured using a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices, USA). The same extract was used to determine the Pi content according to Wyss et al. (1999a).

DNA gel blot analysis

Genomic DNA was isolated from maize leaves as described (Tomes 1995). Ten microgram of DNA was digested with *Bam*HI, *Eco*RV, or *Sca*I, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane (Bio-Rad, USA). The membrane was hybridized with ^{32}P-labeled probes and washed according to the manufacturer's instructions. The phytase probe was prepared with a 500-bp fragment of the *phy*A2 gene and the *bar* probe contains the entire *bar* gene sequence. For *bar* gene blot analysis, the membrane previously hybridized with the *phy*A2 probe was stripped and re-hybridized with the *bar* probe.

Western blot analysis

Proteins were extracted from seed meals. Ten-milligram samples were placed into microfuge tubes with screw cap and extracted with 200 µl of extraction buffer containing 60 mM Tris, pH 6.8, 100 mM DTT, and 2% (w/v) SDS. The tubes were incubated at 100°C for 4 min, vortexed briefly, heated at 100°C for another 4 min and centrifuged at 13,000*g* for 10 min. Supernatants were transferred into fresh tubes for SDS-PAGE and Western blotting analysis. After separation on SDS-PAGE gel, proteins were transferred to a NC membrane (Bio-Rad, USA).

The blot was probed with a polyclonal antibody raised in rabbits against the yeast-expressed *phy*A2 protein. The *phy*A2 gene was cloned into a yeast expression vector to make plasmid pPIC9 and expressed in *Pichia pastoris* GS115 in an extracellular manner. The *phy*A2 protein was purified with a standard procedure (Yao et al. 1998). Two rabbits were injected with purified *phy*A2 protein three times. The antibody specificity was verified by agar-gel double immunodiffusion and the titer was 1/32. The antibody was diluted by 2,000× for Western blot analysis. The goat anti-rabbit IgG labeled with alkaline phosphatase was used as the second antibody.

Deglycosylation of purified *phy*A2 protein was carried out by using endoglycosidase H (New England Biolabs, USA) according to manufacturer's instructions.

Results

Production of transgenic plants

The construct pSPHP3303Phy and pSPHP3303T-Phy was used for maize transformation. A total of 40 independent transgenic events were obtained; 33 were from pSPHP3303Phy and 7 from pSPHP3303T-Phy. All events produced T1 seeds. To determine *phyA*2 gene expression, five T1 seeds from each event were randomly selected to assay phytase activity. The non-transgenic maize seeds had phytase activity ranging from 5 to 40 U per kg seed (U/kg) (data not shown). Many transgenic events had phytase activity higher than 40 U/kg, indicating *phyA*2 transgene expression. Because T1 seeds were produced by crossing the T0 plant with a non-transgenic line, the transgenic and wild-type seeds were segregating in the ear. Therefore, the phytase activity determined by using five randomly selected T1 seeds under-estimates the expression level of the transgene. Based on the phytase activity in T1 seeds, selected events were planted in greenhouse and self-pollinated to produce T2 seeds. Up to four ears from each event were assayed for phytase activity. The phytase activity determined by using five pooled kernels from each ear is presented in Table 1. The expression levels of the *phyA*2 gene in T1 and T2 seeds are comparable. The event B21-4-2 showed the highest expression level with phytase activity ranging from 1,136.6–1,227.4 U/kg. The four ears from the event B15-18-3 had similar levels of *phyA2* expression and the average phytase activity was 748.9 U/kg. The phytase activity in the event B23-3-1 varied among the four ears assayed. Two of them had phytase activity 607.4 and 872.9 U/kg (Table 1). The expression level in other ear was low with phytase activity of 121.5 U/kg. The ear with phytase activity of 18.9 U/kg apparently is a wild-type segregant.

The T1 seeds of B21-4-2 germinated with a low frequency and resultant T1 plants were weak. B23-3-1 and B15-18-3 produced vigorous seedlings and the seedling grew normally. The two events were propagated to produce T4 and T5 seeds. Seed germination frequency and seedling vigor of T4 in the field and T5 in greenhouse are presented in Table 2. All lines derived from these two events had higher seed germination frequency in greenhouse than in the field. The seed germination frequency ranged from 75 to 88% in the field and 80–92.5% in greenhouse which were similar to the wild-type segregant control.

Table 1 Phytase activity in T2 seeds of maize transgenic events

Event	Phytase activity (U/kg)			
	Ear 1	Ear 2	Ear 3	Ear 4
pSPHP3303Phy				
B13-10-1	79.2	69.1	NT	NT
B13-17-1	43.2	40.5	45.9	42.3
B15-14-1	59.8	45.5	45.5	NT
B15-15-1	57.1	NT	NT	NT
B15-17-11	50.0	29.4	NT	NT
B15-17-2	64.2	31.2	86.5	106.2
B15-17-3	53.5	65.1	38.4	34.8
B15-18-2	40.1	26.8	8.0	44.6
B15-18-3	595.7	822.5	873.8	703.7
B15-19-1	18.0	39.6	79.2	NT
B17-4-3	59.4	52.2	53.1	NT
B17-7-1	77.4	43.2	20.7	NT
B19-5-1	36.9	10.8	3.6	4.5
B28-15-1	19.8	39.6	21.6	3.6
pSPHP3303T-Phy				
B14-5-5	61.2	45.9	90.0	55.8
B14-9-1	19.6	NT	NT	NT
B14-10-1	45.5	NT	NT	NT
B21-4-2	1136.6	1227.4	NT	NT
B21-12-2	14.4	8.1	NT	NT
B23-3-1	18.9	872.9	607.4	121.5
B23-6-1	10.4	3.8	5.7	3.8

Up to four ears from each event were analyzed. Five kernels from each ear were randomly selected, pooled, and phytase activity assayed. One unit of phytase activity was defined as the amount of activity that liberates 1 μmol of phosphate per min at 37°C. U/kg, phytase units per kilogram of seed. NT, not determined

Segregation analysis

T2 seeds of the event B15-18-3 and B23-3-1 were planted in the field to determine transgene

Table 2 Seed germination frequency and seedling vigor of transgenic plants and wild-type segregant controls at T4 and T5 generation

Event	Lines	T4			T5		
		No. of seeds	Germination[a] (%)	Vigor[b]	No. of seeds	Germination (%)	Vigor
B15-18-3	1	60	75	Good	20	80	Good
	2	40	80	Good		NT	
	3	80	75	Good		NT	
B23-3-1	1	60	75	Good	35	92.5	Good
	2	40	88	Good	40	85	Good
Wild-type segregant[c]	1	40	75	Good	40	80	Good

[a] Germination was scored 15 days after planting; NT, not determined

[b] Seedling vigor was scored on a scale of 1–5: 1 = poor; 3 = good; 5 = outstanding

[c] The wild-type segregant was derived from the event B23-3-1

segregation patterns. Leaf painting was carried out at the five-leaf stage. Out of 65 seedlings obtained from the event B15-18-3, 48 were Basta resistant and 17 susceptible. The ratio of transgenic and wild-type seedlings fits 3 to 1 segregation (χ^2 test, $P = 0.830$), indicating the *bar* gene is integrated at a single locus. A total of 443 seedlings were analyzed for the event B23-3-1. Three hundred and twenty-two plants were Basta resistant and 121 susceptible. The segregation ratio of 3:1 (χ^2 test, $P = 0.261$) suggested this event also has only one integration site for the *bar* gene.

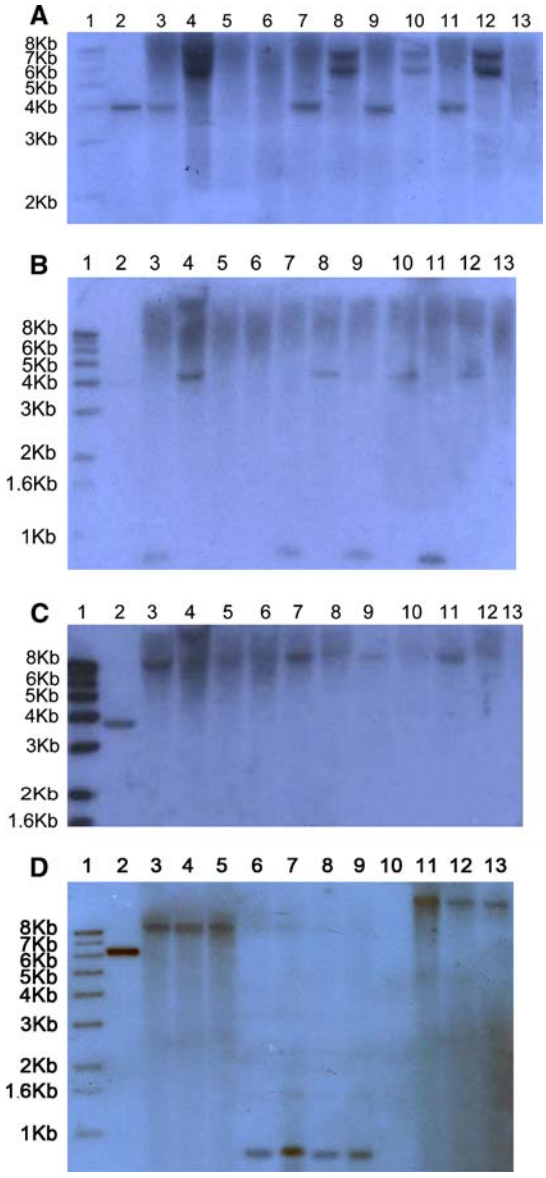
To determine if the selectable marker and the *phy*A2 gene co-segregate, 20 T2 seedlings of the event B23-3-1 were leaf-painted with Basta and leaf samples were taken for PCR analysis of the *phy*A2 gene. Four plants were Basta sensitive and the *phy*A2 gene was not presented in these plants. Sixteen Basta resistant plants were PCR positive for the *phy*A2 gene. These results indicated that the selectable marker *bar* gene and the *phy*A2 gene are located in the same locus.

Determination of transgene integration

Southern blotting was conducted to estimate the copy number of the transgene in the event B23-3-1, using four Basta resistant and *phy*A2 PCR positive T2 plants, one wild-type segregant that was Basta susceptible and *phy*A2 PCR negative, and one non-transgenic control plant. Genomic DNA was digested with *Eco*RV, *Bam*HI, or *Sca*I to determine integration patterns of the *phy*A2 gene and the *bar* gene. There is only one *Bam*HI restriction site located between the Glb promoter and the *phy*A2 gene in the construct pSPHP3303Phy. *Sca*I cut the chimeric phytase transgene twice. There was no *Eco*RV site in the expression cassette. A 500-bp probe derived from the *Phy*A2 coding region revealed a hybridizing band in the *Eco*RV digested DNA of transgenic plants (Fig. 1A). The *Bam*HI digestion produced two bands (Fig. 1A), indicating two copies of the transgene in the event because *Bam*HI does not cut the *PhyA*2 coding region. The *Sca*I digestion released an internal fragment of 750 bp from the *phy*A2 expression cassette, as expected (Fig. 1B). The transgenic *phy*A2 gene was not presented in the wild-type segregant and the non-transgenic control plant. The hybridization patterns can be best explained by the presence of two copies of the *phy*A2 transgene in the event.

Southern hybridization patterns of the *bar* gene also were simple in the event B23-3-1. The *Sca*I digested DNA showed an 8-kb band. However, no band was detected in the *Eco*RV digested samples. Neither *Sca*I nor *Eco*RV cut the *bar* gene. The *Eco*RV fragment that contains the *bar* gene possibly is too large to be resolved on the 0.8% (w/v) agarose gel. Apparently a piece of DNA with an *Eco*RV restriction site is presented between the *phy*A2 gene and the *bar* gene because the *phy*A2 probe hybridized with a 4-kb *Eco*RV fragment and this fragment did not hybridize with the *bar* gene probe (Fig. 1B and C).

Genomic DNA also was extracted from leaves of T4 and T5 plants of the event B23-3-1 and digested with *Xba*I, *Pst*I (these two enzymes do not cut the phytase gene expression cassette) or *Sca*I (Fig. 1D).



◀ **Fig. 1** Southern-blot analysis of the phytase transgenic plant of event B23-3-1. (**A**) The *Eco*RV- and *Bam*HI-digested geneomic DNA hybridized with the phytase probe. Lane 1, DNA molecular weight markers; lane 2, the insert of the phytase gene from the transformation vector as a positive control; lanes 3, 5, 7, 9, and 11, *Eco*RV digestion; lanes 4, 6, 8, 10, and 12, *Bam*HI digestion. The DNA in lanes 5 and 6 was isolated from a wild-type segregant plant which is Basta susceptible and phytase PCR negative. Other four pairs are the individual transgenic T2 plants resistant to Basta and PCR-positive for phytase. Lane 13, *Eco*RV-digested DNA from non-transgenic maize plant. (**B**) The *Sca*I- and *Eco*RV-digested genomic DNA hybridized with the phytase probe. Lane 1, DNA molecular weight markers; lane 2, the insert of the phytase gene and the *bar* selectable marker gene from the transformation vector as positive controls; lanes 3, 5, 7, 9, and 11, *Sca*I digestion; lanes 4, 6, 8, 10, and 12, *Eco*RV digestion, the same digestion as in the panel (**A**). The DNA in lanes 5 and 6 was isolated from a wild-type segregant plant which is Basta susceptible and phytase PCR negative. Other four pairs are the individual transgenic T2 plants resistant to Basta and PCR-positive for phytase. Lane 13, *Eco*RV-digested DNA from non-transgenic maize plant. (**C**) The *Sca*I- and *Eco*RV-digested genomic DNA hybridized with the *bar* probe. The same blot used for the panel (**B**) was stripped and re-hybridized with the *bar* probe. (D) The *Xba*I-, *Sca*I-, and *Pst*I-digested genomic DNA from T4 and T5 plants hybridized with the phytase probe. Lane 1, DNA molecular weight markers; lane 2, the insert of the phytase gene from the transformation vector as a positive control; lanes 3, 4, and 5, *Xba*I digestion; and lanes 6, 7, 8, and 9, *Sca*I digestion; lanes 11, 12, and 13, *Pst*I digestion; lane 10, *Sca*I-digested, non-transgenic maize plant DNA. The DNA in lanes 3, 4, 6, 7, 11, and 12 were isolated from T5 plants. The DNA in lanes 5, 8, 9, and 13 were isolated from T4 plants

The result showed that the phytase gene was stably integrated into maize genome.

Immunodetection of transgenic phytase proteins

To determine protein expression levels of the transgenic phytase in seeds, proteins were extracted from mature T2 seeds of the event B15-18-3 and B23-3-1. The transgenic phytase protein band was not resolved on SDS-PAGE (Fig. 2A). The *phy*A2 protein expressed and purified from yeast is glycosylated and its molecule weight is approximately 75 kD. De-glycosylated protein has a molecular weight of about 55 kD, as predicted from its amino acid sequence. The dominant band about 30 kD in lane 4 is probably the endoglycosidase H used for deglycosylation of the yeast-expressed *phy*A2 protein (Fig. 2A). The phytase antibody recognizes the yeast-expressed *phy*A2 in both glycosylated and de-glycosylated form (Lanes 3 and 4 in Fig. 2B) and revealed a band of approximately 60 kD in transgenic seed samples (Fig. 2B). The band was not detected in the non-transgenic control seed. The maize expressed phytase is smaller than the yeast expressed protein, but larger than the de-glycosylated *phy*A2 protein (Fig. 2B), indicating the ectopically expressed *phy*A2 protein is glycosylated differently in maize and yeast.

Phytase activity, phytic acid, and Pi in transgenic seeds

Homozygous T4 seeds were assayed for phytase activity. The event B15-18-3 had phytase activity of

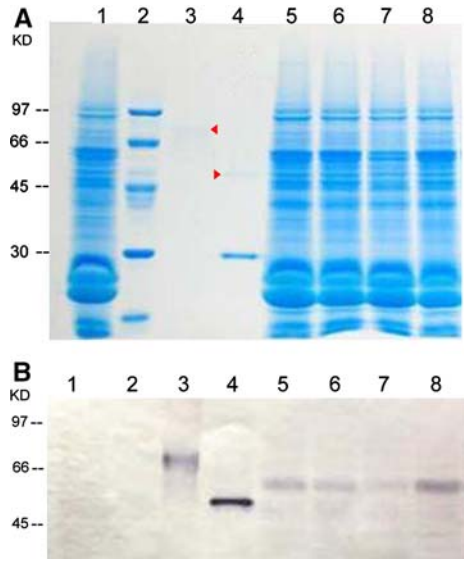


Fig. 2 Phyase expression in transgenic mature maize seeds. (**A**) SDS-PAGE gel. (**B**) Western blot analysis. The blot was probed with an antibody against yeast-expressed phytase. Lane 1, non-transgenic seed control; lane 2, protein molecular weight markers; lane 3, yeast-expressed and purified phytase (arrowhead); lane 4, deglycosylated phytase that was expressed in yeast (arrowhead); lanes 5 and 6, transgenic seed No. 1 and No. 2 from the event B15-18-03; lanes 7 and 8, transgenic seed No.1 and No. 2 from the events B23-03-01

approximately 2,000 U/kg seed. The average activity for the event B23-3-1 was 2,200 U/kg. The phytic acid content in wild-type control seeds was about 3.30 mg per gram seed (mg/g), while the phytic acid content was reduced to 2.39–2.66 mg/g in transgenic seeds. The Pi contents were about 0.12 and 0.41–0.56 mg/g in the wild-type and transgenic seeds, respectively. The phytic acid content in transgenic seeds was reduced by approximately 23% compared to wild-type control seeds and the Pi was increased about 3-fold (Table 3).

Discussion

Maize grains are the major ingredient of animal feed. About 65% of the maize seed produced in China is used as feed. If maize seeds contain enough phytase, the supplementation of microbial phytase additive will not be required. This will reduce feed costs and simplify feed processing and diet formulation. In addition, the phytase produced in maize seeds are convenient for storage and transportation. In this study, we demonstrated that transgenic maize seeds can express the fungal phytase *phyA*2 gene in embryos without affecting seed germination. The phytase activity in transgenic seeds reached about 2,200 U/kg seed. A similar level of phytase expression also was obtained when the *A. niger phy*A gene was expressed in maize endosperm under the control of the rice glutelin-1 promoter (Drakakaki et al. 2005). Previous feeding trials have shown that the effectiveness of phytase as a feed additive was associated with the amount that is included in the diet and responses in swine was maximized at approximately 750–1,000 units per kg of diets (Jongbloed et al. 1996; Lei et al. 1994). Because maize grains typically constitute at least 50% of the diet, the

Table 3 Phytase activity, phytic acid, and inorganic phosphate contents in transgenic maize T4 seeds

Event	Ear[a]	No. of seeds	Phytase activity[b] (U/kg)	Phytic acid-P[c] (mg/g)	Phosphate-P[c] (mg/g)
B15-18-3	1	20	1915.3 ± 500.2	2.59 ± 0.23	0.41 ± 0.06
	2	20	1790.8 ± 367.4	2.54 ± 0.12	0.44 ± 0.04
	3	20	2219.8 ± 382.2	2.39 ± 0.09	0.46 ± 0.05
B23-3-1	1	20	1955.8 ± 413.0	2.58 ± 0.08	0.52 ± 0.10
	2	20	2502.3 ± 377.9	2.66 ± 0.19	0.56 ± 0.08
Wild-type segregant[d]	1	20	38.3 ± 11.7	3.30 ± 0.09	0.12 ± 0.01

[a] T4 seeds were produced from T0 plants that were crossed with inbred line Huangzaosi, followed by three generations of self-pollination

[b] Phytase activity was determined using individual seeds. One unit of phytase activity was defined as the amount of activity that liberates 1 μmol of phosphate per min at 37°C. U/kg: phytase units per kilogram of seed

[c] Phytic acid and inorganic phosphate is expressed as mg elemental P in phytic acid or inorganic phosphate per grams of seed

[d] The wild-type segregant was derived from the event B23-3-1

transgenic seeds containing about 2,000 U/kg phytase can substitute for the phytase additive and achieve the same effect.

The two lead events from this study have a single transgene integration site. The event B23-3-1 contains two copies of the transgene. This event is suitable for commercial product development. In this study, we observed a low frequency of the transgenic events with high expression levels when the fungal *phyA*2 gene is expressed in cytosol of maize embryo cells (construct pSPHP3303Phy). Out of 33 events analyzed, only one has the phytase activity high enough to make a product. However, two high-expression events were identified from seven transgenic events produced from the construct pSPHP3303T-Phy. The only difference between the two constructs is the signal peptide. The signal peptide targets the expressed protein to the intercellular space and is frequently used for high-level expressions of recombinant enzymes in transgenic seeds (Hood et al. 2003; Streatfield et al. 2003; Van Droogenbroeck et al. 2007). Richardson et al. (2001) reported a dramatic increase of the phytase expression in *Arabidopsis* roots when a signal peptide is fused to the N terminus of the *A. niger phy*A protein. The signal peptide in pSPH3303T-Phy possibly is the reason for the increase in the frequency of the high-expression events. However, the event number in this study is small and the effect of a signal peptide on *phy*A2 expression levels in maize embryos needs further investigation. To further increase phytase expression, we have tested constructs that target the phytase to vacuoles and obtained events with the phytase activity higher than 20,000 U/kg (unpublished data). Other approaches to increase the phytase activity in seeds include using stronger promoters and expressing the gene in endosperm.

The maize expressed *phy*A2 protein is smaller than that expressed in fungi, as revealed on Western blots. Molecule weight shift of ectopically expressed phytases also has been observed in other expression systems (Pen et al. 1993; Brinch-Pedersen et al. 2000). This is likely due to different glycosylation of the phytase protein in different hosts. Despite the different glycosylation patterns, the plant expressed *A. niger phy*A and *E. coli* appA conserve the catalytic properties of the native enzyme (Coello et al. 2001; Ullah et al. 2002). More importantly, inclusion of the transgenic tobacco seeds expressing *phy*A in animal diets improved the phosphorus availability and broiler growth rate (Pen et al. 1993). The effect of different glycosylation on heat tolerance of the maize-expressed *phy*A2 remains to be determined.

Expression of the fungal *phy*A2 gene in maize embryo reduces seed phytate and increases Pi contents. The phytate reduction and Pi increase are unlikely artifacts introduced during assay because TCA in the extraction buffer inactivates the phytase and prevents phytate from hydrolysis during sample preparation. Transgenic soybean over-expressing phytase also showed phytate reduction and Pi increase in seeds (Chiera et al. 2004). A higher level of phytate reduction and Pi increase was observed in transgenic *Arabidopsis* seeds when the *E. coli* appA phytase was targeted to vacuoles (Coello et al. 2001). However, the germination of the *Arabidopsis* transgenic seed was impaired. Phytate plays an important role in seed germination, maturation, and initiation of dormancy and in control of inorganic phosphate levels in both developing seeds and seedlings. The transgenic seeds of the event B23-3-1 and B15-18-3 germinated normally (Table 2), and there was no significant difference in plant growth and seed development between the transgenic and wild-type segregants. For commercial product development, it is critical to obtain transgenic plants that have high phytase activity and their agronomic characteristics are not affected. The transgenic events obtained in this study can be used for developing new maize hybrids that help phosphorus management in animal production.

Acknowledgments We thank Mr. Li Denghai, Prof. Zhang Shihuang and Dr. Li Mingshun for their help. We acknowledge Key National Project of China Crop Genetic Resources and Gene Improvement for providing greenhouse and other facilities. This work was partially supported by the National Basic Research and development Program of China (973 Program) (No. 2005CB120905) and a research grant from Pioneer Hi-Bred International, a DuPont Company.

References

An G, Mitra A, Choi HK, Costa MA, An K, Thornburg RW, Ryan CA (1989) Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1:115–122

Armstrong CL, Green CE, Philips RL (1991) Development and availability of germplasm with high type II culture formation response. Maize Genet Coop News Lett 65:92–93

Asada K, Tanaka K, Kasai Z (1969) Formation of phytic acid in cereal grains. Ann NY Acad Sci 165:801–814

Austin S, Bingham ET, Koegel RG, Mathews DE, Shahan MN, Straub RJ (1994) An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. In: Bajpai RK, Prokop A (eds) Recombinant DNA Technology II. New York Academy of Sciences, New York, pp 234–244

Brinch-Pedersen H, Olesen A, Rasmussen SK, Holm PB (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Mol Breed 6:195–206

Brinch-Pedersen H, Sorensen LD, Holm PB (2002) Engineering crop plants: getting a handle on phosphate. Trends Plant Sci 7:118–125

Chiera JM, Finer JJ, Grabau EA (2004) Ectopic expression of a soybean phytase in developing seeds of Glycine max to improve phosphorus availability. Plant Mol Biol 56: 895–904

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Coello P, Maughan JP, Mendoza A, Philip R, Bollinger DW, Veum TL, Vodkin LO, Polacco JC (2001) Generation of low phytic acid Arabidopsis seeds expressing an *E. coli* phytase during embryo development. Seed Sci Res 11:285–291

Cosgrove DJ (1966) The chemistry and biochemistry of inositol polyphosphates. Rev Pure Appl Chem 16:209–215

Denbow DM, Grabau EA, Lacy GH, Kornegay ET, Russell DR, Umbeck PF (1998) Soybeans transformed with a fungal phytase gene improve phosphorus availability for broilers. Poult Sci 77(6):878–881

Drakakaki G, Marcel S, Glahn RP, Lund EK, Pariagh S, Fisher R, Christou P, Stoger E (2005) Endosperm specific co-expression of recombinant soybean ferritin and Aspergillus phytase in maize results in significant increases in the levels of bioavailable iron. Plant Mol Biol 59:869–880

Gibson DM, Ullah AH (1988) Purification and characterization of phytase from cotyledons of germinating soybean seeds. Arch Biochem Biophys 260:503–513

Greiner R, Konietzny U, Jany K (1993) Purification and characterisation of two phytases from *Escherichia coli*. Arch Biochem Biophys 303:107–113

Hegeman CE, Grabau EA (2001) A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. Plant Physiol 126:1598–1608

Hong CY, Cheng KJ, Tseng TH, Wang CS, Liu LF, Yu SM (2004) Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. Transgenic Res 13:29–39

Hood EE, Bailey MR, Beifuss K, Magallanes-Lundback M, Horn ME, Callaway E, Drees C, Delaney DE, Clough R, Howard JA (2003) Criteria for high-level expression of a fungal laccase gene in transgenic maize. Plant Biotechnol J 1:129–140

Joanin P, Gigot C, Philipps G (1992) Nucleotide sequence and expression of two cDNA coding for two histone H2B variants of maize. Plant Mol Biol 20:581–588

Jongbloed AW, Kemme PA, Mroz Z (1996) Phytase in swine rations: impact on nutrition and environment. In: BASF Technical Symposium. January 29, 1996, Des Moines, IA, BASF, Mount Olive, NJ, pp 44–69

Kerovuo J, Lauraeus M, Nurminen P, Kalkkinen N, Apajalahti J (1998) Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64:2079–2085

Latta M, Eskin M (1980) A simple and rapid colorimetric method for phytate determination. J Agric Food Chem 28:1313–1315

Lei XG, Ku PK, Miller ER, Yokoyama MT, Ullrey DE (1994) Calcium level affects the efficacy of supplemental microbial phytase in corn-soybean meal diets of weanling pigs. J Anim Sci 72:139–143

Lei XG, Stahl CH (2001) Biotechnological development of effective phytases for mineral nutrition and environmental protection. Appl Microbiol Biotechnol 57:474–481

Li J, Hegeman CE, Hanlon RW, Lacy GH, Denbow MD, Grabau EA (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol 114:1103–1111

Lucca P, Hurrell R, Potrykus I (2001) Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. Theor Appl Genet 102:392–397

Mitchell DB, Vogel K, Weimann B, Pasamontes L, van Loon APGM (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology 143:245–252

Mullaney EJ, Daly CB, Ullah AH (2000) Advances in phytase research. Adv Appl Microbiol 47:157–199

Pen J, Verwoerd TC, van Paridin PA, Beukeder RF, van der Elzen PJM, Geerse K et al (1993) Phytase-containing transgenic seed as a novel feed additive for improved phosphorus utilization. Bio/Technol 11:811–814

Ponstein AS, Bade JB, Verwoerd TC, Molendijk L, Storms J, Beudeker RF et al (2002) Stable expression of Phytase (phyA) in canola (*Brassica napus*) seeds: towards a commercial product. Mol Breed 10:31–44

Ravindran V, Bryden WL, Kornegay ET (1995) Phytates: occurrence, bioavailability and implications in poultry nutrition. Poult Avain Bio Rev 6:125–143

Reddy NR, Sathe SK, Salunkhe DK (1982) Phytates in legumes and cereals. Adv Food Res 28:1–92

Richardson AE, Hadobas PA, Hayes JE (2001) Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. Plant J 25:641–649

Schroder B, Breves G, Rodehutscord M (1996) Mechanisms of intestinal phosphorus absorption and availability of dietary phosphorus in pigs. Dtsch Tierarztl Wochenschr 103:209–214

Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, Howard JA (2003) Corn as a production system for human and animal vaccines. Vaccine 21:812–815

Tomes DT (1995) Direct DNA transfer into plant cell via microprojectile bombardment. In: Gamborg OL, Philipps GC (eds) Plant cell tissue and organ culture: fundamental methods. Springer-Verlag Publisher, Berlin, pp 197–213

Ullah AH (1988) *Aspergillus ficuum* phytase: partial primary structure, substrate selective, and kinetic characterization. Prep Biochem 18:459–461

Ullah AH, Sethumadhavan K, Mullaney EJ, Ziegelhoffer T, Austin-Phillips S (1999) Characterization of recombinant fungal phytase (phyA) expressed in tobacco leaves. Biochem Biophys Res Commun 264:201–206

Ullah AH, Sethumadhavan K, Mullaney EJ, Ziegelhoffer T, Austin-Phillips S (2002) Cloned and expressed fungal phyA gene in alfalfa produces a stable phytase. Biochem Biophys Res Commun 290:1343–1348

Urbano G, Lopez-Jurado M, Aranda P, Vidal-Valverde C, Tenorio E, Porres J (2000) The role of phytic acid in legumes: antinutrient or beneficial function? J Physiol Biochem 56:283–294

Vaintraub IA, Lapteva NA (1988) Colorimetric determination of phytate in unpurified extracts of seeds and the products of their processing. Anal Biochem 175:227–230

Van Droogenbroeck B, Cao J, Stadlmann J, Altmann F, Colanesi S, Hillmer S, Robinson DG, Van Lerberge E, Terryn N, Van Montagu M, Liang M, Depicker A, De Jaeger G (2007) Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. Proc Natl Acad Sci USA 104:1430–1435

Verwoerd TC, van Paridon PA, van Ooyen AJ, van Lent JW, Hoekema A, Pen J (1995) Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. Plant Physiol 109:1199–1205

Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 23(1):29–60

Wodzinski RJ, Ullah AHJ (1996) Phytases. Adv Appl Microbiol 42:263–302

Wyss M, Brugger R, Kronenberger A, Remy R, Fimbel R, Oesterhelt G, Lehmann M, van Loon AP (1999a) Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance. Appl Environ Microbiol 65(2):359–366

Wyss M, Pasamontes L, Friedlein A, Remy R, Tessier M, Kronenberger A, Middendorf A, Lehmann M, Schnoebelen L, Rothlisberger U, Kusznir E, Wahl G, Muller F, Lahm HW, Vogel K, van Loon AP (1999b) Biophysical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65:367–373

Yao B, Yuan TZ, Wang YH. Cao SS, Wang YR, Shi XY, Fan YL (2001) Cloning of neutral phytase gene nphy from *Bacillus subtilis* and its expression in *Escherichia coli*. Sheng Wu Gong Cheng Xue Bao 17:11–15

Yao B, Zhang CY, Wang JH, Fan YL (1998) Recombinant *Pichia pastoris* overexpressing bioactive phytase. Sci China C 41:330–336

Zhang ZB, Kornegay ET, Radcliffe JS, Denbow DM, Veit HP, Larsen CT (2000) Comparison of genetically engineered *Aspergillus* and canola in weanling pig diets. J Anim Sci 78:2868–2878