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minireview

Transgenic Plants Expressing Phytase Gene of Microbial Origin and Their Prospective Application as Feed

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Summary

Phytate is the primary storage form of phosphate in plants. Phytases from microbial sources are supplemented to feedstuff of monogastric animals to increase the uptake of phytate phosphorus. The use of microbial phytase is associated with high production cost and also requires special care in feed processing. Expression of phytase in transgenic plants is an alternative approach to the production of phytase for commercial use (organic farming) as well as for animal feed. This review summarizes the current knowledge of transgenic plants overexpressing *phy* gene and their potential application in animal feed. The need for alterations in the *phy* gene for enhanced expression, accumulation and activity of phytase in transgenic plants is also discussed.

Key words: transgenic plants, phytase, *phy* gene, animal feed

Introduction

Plant seeds are major source of proteins and other nutrient elements in animal feed. The majority of the phosphorus in the seeds of higher plants is stored as *myo*-inositol-1,2,3,4,5,6-hexakisphosphate, otherwise known as phytic acid or phytate. Plant seeds that have high phytate content are used as animal feed ingredients such as oilseed meals, cereal grains and legumes (*1*). The amount of phosphorus in cereal grains and oilseed meals meets the requirement for optimal growth of animals if all the phosphorus from phytate is available. Phytase is an enzyme that catalyses the breakdown of phytate into inorganic phosphorus and *myo*-inositol. Since monogastric animals like poultry, pigs and fish have very low or no phytase activities in their digestive tracts, they are unable to efficiently utilize phosphorus from the feed present in the form of phytate (*2,3*). In addition, phytate is considered as an antinutritional factor because under acidic conditions it has strongly negative charge and chelates cations such as iron, zinc, magnesium, manganese, copper and calcium (*4*). At low pH, phytate binds to amine groups of proteins, resulting in phytate-protein complex, which is generally insoluble and more resistant to hydrolysis by digestive enzymes (*5*). Binding of minerals and proteins to phytate impacts its nutritional quality by decreasing bioavailability of essential nutrients (*6*). Supplementation of inorganic phosphorus to feed can solve this nutritional problem (*7*). As a result, additional phosphorus is often provided to the feed and unutilized organic phosphorus (phytate) leaches and persists in animal manures. Such manures if applied routinely to the fields, cause serious pollution and ecological problems known as eutrophication (*8*). If inorganic phosphate is substituted by phytase and provided to monogastric animals, both phosphorus in phytate and bound metals become available to animals and the phosphorus pollution problem can also be resolved to some extent.

Microbial phytase produced by fermentation is used as a feed additive to manage the nutritional and environmental problems caused by phytate. However, this approach is associated with high production costs for the enzyme and requirement of special care in feed processing and diet formulation, which limits its extensive commercial use. Many strategies have been developed for improving phosphate and mineral availability in

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feed and food. These comprise the pretreatment of grains to activate endogenous phytase, mutation of enzymes involved in phytate biosynthesis for reduction of seed phytate levels, genetic engineering of livestock for secretion of phytase in the saliva, and transformation of crop plants for production of microbial phytase (*2*). The approach to produce transgenic crops having high phytase expression is becoming a prerequisite to improve the bioavailability of phosphorus in food/feed instead of direct supplementation of microbial phytase to animal feed. As a cost-effective option, transgenic plants have been evaluated as bioreactors for the production of recombinant phytases to meet the industrial demand (*9,10*). Enzymes can be expressed, secreted, folded and post-translationally modified in plants to produce functional recombinant proteins at high level (*11,12*). This review summarizes the current knowledge of various transgenic plants overexpressing microbial *phy* gene and their potential application in animal feed.

Source Organisms for *phy* Gene

Both prokaryotic and eukaryotic microbes have been used as a source of *phy* gene for transformation of crop plants. Some bacterial *phy* genes of *phyC* from *Bacillus subtilis* (*13*), *SrPf6* from *Selenomonas ruminantium* (*14*), and *appA* and *appA2* from *E. coli* (*15*) have been used to develop transgenic plants. Yeast is again an excellent source of *phy* gene for genetic transformation studies. The *phy* gene from yeast (*Schwanniomyces occidentalis*) has been used to develop transgenic rice plants (*4*). This is the first and only report about the use of *phy* gene from yeast in genetic transformation. A long list of phytase-producing fungi includes *Aspergillus ficuum* (*16*), *A. niger* (*17,18*), *Emericella nidulans*, *Talaromyces thermophilus* (*19*), *A. fumigatus* (*20*), *Thermomyces lanuginosus* (*21*), *Peniophora lycii*, *Agrocybe pediades*, *Ceriporia* sp. and *Trametes pubescens* (*22*). *A. niger* IARI 363 and *A. ficuum* IARI 1461 have shown to produce thermostable phytase using waste carbon source (*23,24*). Fungal *phyA* gene from *A. niger* and *A. fumigatus* has been used to develop transgenic plants such as tobacco, maize, soybean, rice, wheat, canola and alfalfa. The *phy* gene from fungi has been extensively employed in transformation studies since they exhibit stability in a wide range of pH and temperature.

Expression of Phytase Gene in Plants

Phytase gene from diverse microbial origin has been overexpressed in various plants such as tobacco (*9,25–31*), canola (*32,33*), soybean (*10,34–38*), wheat (*6,39,40*), rice (*4,41–43*), alfalfa (*44*), sesame (*45*), *Arabidopsis* (*28,46*), maize (*47–49*), *Trifolium repens* (*50*), *Medicago truncatula* (*51*) and potato (*52*).

The first account of the engineering of active and stable recombinant phytase from *Aspergillus niger* in tobacco seeds was reported by Pen *et al.* (*9*). The fungal phytase gene (*phy*A) was fused to a plant endoplasmic reticulum-targeting sequence and was placed under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter in a binary transformation vector. The gene was inserted into the tobacco genome by *Agrobacterium*-mediated transformation (*25*). In stably transformed plants, up to 1 % of soluble protein in seeds was active recombinant phytase. Subsequently, Ullah *et al.* (*26*) repeated the transformation of tobacco with *phyA* from *Aspergillus ficuum* and purified the recombinant protein for further biochemical characterization. Similarly, transgenic tobacco plants were generated that constitutively expressed β-propeller phytase from *Bacillus subtilis* (168 *phyA*) under the control of CaMV 35S promoter, and carrot extensin signal peptides were attached for root-specific secretion (*28*). Recombinant phytase accounted for 0.4 % of total soluble leaf protein. Recently, a heat-stable phytase gene from *Aspergillus fumigatus* (*fphyA*) has been introduced in tobacco by *Agrobacterium*-mediated transformation. Phytase gene expression was controlled by CaMV 35S promoter and secretion of recombinant phytase to extracellular fluid was established by use of signal sequence from tobacco calreticulin (*31*). In transformed plants, phytase accounted for 2.3 % of total soluble leaf protein.

The *phyA* gene was introduced in soybean using microprojectile bombardment of cell suspension culture (*10*). Cells were stably transformed with *phyA* construct containing a dually-enhanced CaMV 35S promoter fused to a signal peptide in order to secrete active phytase into the culture medium. Based on the estimated activity levels, up to 7 % of the secreted proteins were predicted to be phytase. Production of transgenic soybean expressing phytase has also been reported with linear vectorless construct without selectable markers constituting *phyA* gene and CaMV 35S promoter directly introduced through pollen tube pathway (*35*). In another study, *appA* gene from *E. coli* constructed with seed lectin promoter and a lectin signal sequence for specific expression of gene in vacuoles was transformed into soybean using *Agrobacterium*-mediated transformation (*36*). Subsequently, another *phy* gene from *Asperigillus ficuum* (*AfPhyA*) was transformed into soybean using *Agrobacterium*-mediated transformation. This *phy* gene was driven by the *Arabidopsis Pky*10 gene promoter and the carrot extension signal peptide was introduced for root-specific secretory expression of this gene (*37*). Recently, transgenic soybean plants have been generated using both *Agrobacterium*-mediated and pollen tube pathways with the *phyA* gene from *Aspergillus ficuum* (*38*).

The transgenic canola seeds overexpressing phytase gene (*phyA*) from *Aspergillus niger* were generated with the gene placed under the control of seed-specific cruciferin (*cru*A) promoter and fused to cruciferin signal peptide sequences for specific secretion into the seeds (*32*). These canola seeds expressed high phytase activity per mass of seed of 600 U/g. Later, transgenic canola expressing *A. niger* phytase gene (*phyA*) with codon modifications according to codon usage of *Brassica napus* was also developed resulting in a remarkable increase in phytase synthesis in seeds (*33*). For this purpose, synthetic *phy* gene was designed according to the codon usage of *B. napus* without altering the amino acid sequence of *A. niger phyA* gene. This codon modified *phyA* gene overexpressed in canola using a construct driven by CaMV 35S promoter, tobacco Ubi-U4 promoter for *GUS* expression,

tobacco PR-S signal peptide and KDEL sequence for retention of secreted proteins in the endoplasmic reticulum (ER). Phytase accumulation in transformed mature canola seed accounted for 2.6 % of the total soluble proteins.

The phytase gene (*phyA*) from *Aspergillus niger* has been applied to generate transgenic wheat plants using biolistic approach (*39*,*40*). This *phyA* gene was driven by maize *Ubi* promoter and fused to barley α-amylase signal peptide for apoplast localization (*39*). Similarly, a construct was generated for *phyA* gene driven by endosperm-specific 1DX5 promoter (wheat high molecular mass subunit gene, glutelin promoter *Glu-1D-1*) fused to barley α-amylase signal peptide for apoplast localization (*40*). Phytase gene (*phyA*) from *Aspergillus fumigatus* has been used to transform rice. The gene was placed under the control of glutelin promoter (*Gt1*) and barley β-glucanase signal peptide was introduced for secretion in the endosperm (*41*). Likewise, *phy* genes from bacteria *Selenomonas ruminantium* (*SrPf6*) and *E. coli* (*appA*) have been introduced in rice under the control of α-amylase gene (*aAmy8*) promoter fused to *aAmy8* signal peptide sequence for tissue-specific secretion (*42*). In a similar study, another *phy* gene from yeast (*Schwanniomyces occidentalis*) was introduced in rice (*4*). The codons of yeast phytase gene were modified according to their usage in rice plant without affecting the amino acid sequence and placed under the control of rice light-harvesting chlorophyll *a*/*b*-binding protein (cab) promoter together with a secretory signal sequence from rice chitinase-3 gene (*Cht3-SS*). This is the only report that describes the use of *phy* gene from yeast for development of transgenic plants. Furthermore, phytase gene (*phyI*) from *Aspergillus niger* was also employed for generating transgenic rice overexpressing phytase (*43*). The gene was driven by the maize ubiquitin (*Ubi*) promoter and tobacco pathogenesis related the protein signal sequence as well as the KDEL sequence for retention of secreted phytase into ER. This gene was transformed into a high-yielding rice cultivar *via Agrobacterium*-mediated transformation expressing phytase gene with an increase in phosphorus content in seeds.

Phytase gene (*phyA*) cloned from *Aspergillus ficuum* was expressed in leaves of transformed *Medicago sativa* (*44*). This gene was placed under the control of CaMV 35S promoter or *Arabidopsis thaliana* Rubisco small subunit (SSU) promoter with a signal peptide for apoplast localization. Phytase was produced in transgenic alfalfa at concentrations of over 1.5 % of the soluble protein. Similarly, *phyA* from *Aspergillus niger* was expressed in *Trifolium repens* (white clover), the gene was controlled by CaMV 35S promoter and a patatin signal peptide introduced for secretion of transgene products into intercellular space and rhizosphere (*50*). Furthermore, *phyA* from *Aspergillus niger* was expressed in *Medicago truncatula* transgenic cell suspension line (*51*). In another experiment, sweet potato sporamin promoter was used to control the expression of *appA gene* from *E. coli* and introduced in potato tubers, which encoded a bifunctional enzyme exhibiting both acid phosphatase and phytase activities (*52*).

A gene for phytase (*phyA*) from *Aspergillus niger* was stably expressed in maize seeds after transformation with the gene under the control of rice glutelin-1 seed-specific promoter, with murine immunoglobulin leader peptide sequence incorporated for better secretion (*47*). In a similar study, phytase gene from *Aspergillus niger* was also overexpressed in maize seeds when transformed with a construct constituting the maize embryo-specific globulin-1 promoter and synthetic barley α-amylase signal peptide gene for secretion of the expressed phytase into the intercellular space (*48*). Another *phyA* gene isolated from *Aspergillus niger* under the control of an endosperm-specific expression vector was also expressed in microprojectile-mediated transformed maize seeds (*49*). The details of the source of *phy* gene, promoters, signal sequences and vectors used in the development of transgenic crops overexpressing phytase enzymes are given in Table 1 (*4*,*6*,*10*,*25–29*,*31–33*,*35–50*,*52*,*53*).

Modifications of *phy* Gene for Expression in Plants

In most of the studies, *phy* gene from the microbial origin (bacterial/fungal/yeast) is used for transformation into the plants without any modifications. The expression of enzymes and proteins is often difficult outside the original host for the reason that codon usages vary significantly among different organisms (*54*). If more rare codons in a gene operate in the original host, it is less likely that the heterologous protein will be expressed in an alternate host at a high level (*55*). One of the strategies to improve the expression is to modify the codons of the gene according to the usage of host plant where the heterologous protein will be expressed. There are a few reports where the *phy* genes have been modified according to the codon usage in rice (*4*) and *Brassica napus* (*33*), without altering the amino acid sequence of native phytase gene. These studies revealed that phytase gene with preferred codons according to the target plant species leads to a remarkable increase in phytase yield as compared to the host plant. Consequently, codon modification can provide an alternative strategy to improve expression levels of non-plant-based phytases in various plant species.

Another strategy to increase the yield of the expressed protein is to direct the soluble proteins to a specific tissue (*56*). The fact that the functional phytase produced by fungi is a glycoprotein necessitated a strategy to direct the recombinant phytase to the plant ER for glycosylation. Almost all *phy* genes used for overexpression in plants were targeted to specific locations by attaching different signal sequences, such as rice chitinase-3 gene (*4*), tobacco PR-S (*10*,*25*,*35*,*43*,*45*), soybean VSPβ secretory signal sequences (*26*), cruciferin signal peptide (*32*), tobacco calreticulin signal peptide (*33*), barley β-glucanase signal peptide (*41*), barley α-amylase signal peptide (apoplast localizaton) (*6*,*39*,*48*), carrot extensin signal peptide (for root specific secretion) (*28*,*29*, *37*) and potato patatin signal peptide (*50*). The tetrapeptide KDEL is commonly found at the C-terminus of soluble proteins in the ER and it contributes to their localization and stable accumulation in plant ER (*57*).

Table 1. Expression of phytase gene in transgenic plants

Transgenic plant	Type of *phy* gene	Source of *phy* gene	Promoter	Signal sequence	Vector	Ref.
alfalfa	*phyA*	*A. ficuum*	CaMV 35S promoter or *Arabidopsis thaliana* Rubisco small subunit (SSU) promoter	signal peptide for apoplast localization	pBI binary vector	*44*
Arabidopsis	β-propeller phytase (*168phyA*)	*Bacillus subtilis*	CaMV 35S promoter	carrot extensin signal sequence for plasmid pCX-CAS and *Arabidopsis thaliana* extensin signal sequence (*AtExt3* gene) for plasmid pCX-A3S	pCX-CAS and pCX-A3S	*28*
	phyA	*A. niger*	*Pht1;2* phosphate transporter of *Arabidopsis* promoter	carrot extensin signal sequence (root-specific expression)	pPLEX502 *ex::phyA-1*	*46*
canola	*phyA*	*A. niger*	seed-specific cruciferin (*cru*A)	cruciferin signal sequences	pMOG425	*32*
	phyA	*A. niger*	tobacco ubiquitin-U4 (*Ubi*) promoter and CaMV 35S promoter	tobacco PR-S (pathogenesis-related proteins) signal peptide and ER retention signal KDEL for ER localization	pYP46	*33*
maize	*phyA*	*A. niger*	rice glutelin-1 seed-specific promoter	murine immunoglobulin leader peptide sequence	pTO126	*47*
	phy2A	*A. niger*	embryo specific globulin-1 (*Glb*) promoter	synthetic barley α-amylase signal peptide sequence	pSPHP3303	*48*
	phyA	*A. niger*	endosperm specific promoter	-	pCAMBIA1300	*49*
	appA	*E. coli*	-	-	-	*53*
potato	*appA*	*E. coli*	sweet potato sporamin promoter	α-factor signal peptide	pCAMBIA2301	*52*
rice	*phy*	*Schwanniomyces occidentalis*	cab promoter (P-cab)	signal sequence from rice chitinase-3 gene	pMOF	*4*
	phyA	*A. fumigatus*	embryo specific glutelin promoter (*Gt1*)	barley β-glucanase signal peptide	pGt1PF	*41*
	SrPf6	*Selenomonas ruminantium*	α-amylase gene (*αAmy8*) promoter	*αAmy8* signal peptide sequence	pPZP200	*42*
	appA	*E. coli*	α-amylase gene (*αAmy8*) promoter	*αAmy8* signal peptide sequence	pPZP200	*42*
	phyI	*A. niger*	maize *Ubi* promoter	tobacco PR-S signal sequence and KDEL sequence for ER localization	pYH582 (derived from pCAMBIA)	*43*
sesame	*phyA*	*A. niger*	CaMV 35S promoter	tobacco PR-S signal peptide	pMOG413	*45*
soybean	*phyA*	*A. niger*	CaMV 35S promoter	tobacco PR-S signal peptide	-	*10*
	phyA	*A. awamori*	CaMV 35S promoter	-	vectorless construct	*35*
	phyA	*E. coli*	soybean lectin promoter	lectin signal sequence	pZY101	*36*
	AfphyA	*A. ficuum*	*Arabidopsis Pyk10* gene	carrot extensin signal sequence	pYES2	*37*
	phyA	*A. ficuum*	CaMV 35S promoter	-	pBin438	*38*
tobacco	*phy*	*A. niger*	CaMV 35S promoter with double enhancer sequence	tobacco PR-S signal sequence	pMOG413	*25*
	phyA	*A. ficuum*	CaMV 35S promoter	soybean VSPβ secretory signal sequences	pTZ117	*26*
	β-propeller phytase (*168phyA*)	*B. subtilis*	CaMV 35S promoter	-	pCX-168phyA	*27*
	β-propeller phytase (*168phyA*)	*B. subtilis*	CaMV 35S promoter	carrot extensin signal sequence for plasmid pCX-CAS and *Arabidopsis thaliana* extensin signal sequence (*AtExt3* gene) for plasmid pCX-A3S	pCX-CAS and pCX-A3S	*28*
	phyA	*A. niger*	CaMV 35S promoter	carrot extensin signal sequence (*ex*)	pAER02	*29*
	fphyA	*A. fumigatus*	CaMV 35S promoter	tobacco calreliculin signal sequence	pBI121	*31*
Trifolium repens	*phyA*	*A. niger*	CaMV 35S promoter	patatin signal peptide	pCAMBIA 3301	*50*
wheat	*phy A*	*A. niger*	endosperm specific 1DX5 (promoter of the wheat high molecular mass glutenin gene *Glu-1D-1*)	barley α-amylase signal peptide for apoplastic localization	p1DX5SPPhyN	*6*
	phyA	*A. niger*	maize *Ubi*-1 promoter	barley α-amylase signal peptide for apoplastic localization	pUPhyN	*39*
	phyA	*A. fumigatus*	endosperm specific 1DX5 promoter	barley α-amylase signal peptide for apoplastic localization	p1DX5SPConPhyN	*40*

The KDEL sequence was introduced into the *phy* gene construct for its endoplasmic reticulum localization in transgenic canola and rice overexpressing phytase (*33*, *43*). It has been demonstrated that the addition of KDEL sequence with the *phyA* resulted in higher levels of phytase accumulation in these transgenic plants as compared to transgenic plants expressing *phyA* gene without KDEL sequences (*33*).

Glycosylation Pattern of *phy* Gene in Transgenic Plants

Glycosylation of phytase enzyme helps in folding of protein to generate active sites, and also aids in maintaining thermal stability and activity of the enzyme. The glycosylation has little effect on the substrate specificity of phytase but has a significant effect on the molecular mass, protein structure, thermal stability and biological synthesis. Most of the *phy* genes expressed in different transgenic crops exhibited lower mass as compared to the native phytase; this is attributed to the difference in the glycosylation pattern of this gene in plant and fungal systems. It has been reported that glycosylation is important for *Aspergillus* phytase activity, secretion and stability. The molecular mass of transformed tobacco phytase from leaves was found to be approx. 70 kDa as compared to *Aspergillus* enzyme of approx. 80 kDa. This difference in molecular mass of transgenic tobacco and donor of the gene *Aspergillus* was found to be due to the differences in glycosylation patterns (*25*). Phytase gene from *Aspergillus niger* expressed in tobacco was found to be 17 % less glycosylated as compared to the identical *phy* gene from fungi. Similarly, *Aspergillus fumigatus* phytase gene expressed in tobacco (*tfphyA*) was found to be 22 % less glycosylated than the native phytase gene (*31*). The yeast phytase expressed in rice, *i.e.* heterologous phytase, was 22 % less glycosylated than the original yeast phytase (*4*). The fungal phytase (*A. ficuum*) expressed in alfalfa was 16 % less glycosylated than its native form (*44*). In addition, similar results were obtained for the expression of fungal *phyA* gene in wheat, canola, rice and soybean, where recombinant phytase had low molecular mass as compared to the native phytase, revealing low glycosylation of plant-expressed phytase (*10*,*33*,*35*, *39*,*41*). Furthermore, *phy*2A gene of *Aspergillus niger* when expressed in maize showed a band of 60 kDa using Western blot analysis but the same gene when expressed in yeast exhibited a band of 75 kDa, clearly showing variations in enzyme glycosylation in maize and yeast (*48*).

It is well documented that *in planta* synthesized fungal phytase is glycosylated when targeted to the apoplast and ER but the pattern of glycosylation is less complex than in fungi (*6*). The importance of glycosylation in folding of phytase to generate the active site was studied using *A. ficuum* phytase gene expressed in *E. coli* and *Pichia pastoris.* The expressed fungal phytase in the bacteria was not only devoid of any activities, but it accumulated in the inclusion bodies (*58*). Despite the differences in glycosylation patterns, the plant expressed *phy* genes (tobacco, soybean, canola, wheat, alfalfa, rice and maize), conserved the catalytic properties of the native enzyme and was found to be functionally active.

Phytase Activity in Transgenic Plants

Transgenic tobacco plants overexpressing fungal *phyA* gene showed 2500 ng of phytase per mg of dry mass. Phytase was found to be biologically active and to accumulate in leaves up to 14.4 % of total soluble protein during plant maturation (*25*). Similar results were reported for transgenic tobacco overexpressing heat--stable phytase from *Aspergillus fumigates*, where phytase accumulation was 2.3 % of the total soluble protein in leaves (*31*). Transgenic wheat, rice, corn and soybean overexpressing fungal phytase showed phytase activity per mass of seed 1467, 16 500 and 125 U/kg and per mass of leaves 5000 U/kg (*4*,*32*,*35*,*39*,*42*,*58*). Phytase activity of leaves was found to be 389.3 nkat/g in transgenic alfalfa plants overexpressing fungal phytase (*44*). In transgenic canola seeds overexpressed phytase activity was as high as 15 600 U/kg and phytase accumulation was up to 2.6 % of the total soluble proteins (*33*). Similarly, transgenic rice overexpressing phytase gene (*phyI*) showed significantly higher (57 %) phosphorus content as compared to that in the untransformed wild type (*43*). Transgenic maize seeds expressed the fungal phytase *phyA2* gene in embryos without affecting the seed germination despite the phytase activity of virtually 2200 U/kg (*48*). The accumulation of the phytase in all the transgenic plants overexpressing fungal phytase was stable, and functionally active. Different transgenic plants (expressing bacterial, fungal and yeast phytase) with their respective phytase activities are presented in Table 2 (*4*,*6*, *25*,*26*,*28*,*29*,*31*,*33*,*35–37*,*39–42*,*45–50*,*52*,*53*).

Feed Trails for Transgenic Crops Overexpressing Phytase

The transgenic crops overexpressing phytase were used for feed trials for pigs and poultry. In a poultry feed trial, when phytase-containing alfalfa juice was added at the rate of 400 U/kg to the normal dietary supplements, the performance of chickens was at par with the group fed only with normal dietary supplements (*59*). The addition of phytase to the poultry and pig feed in the form of transgenic soybeans at 1200 U/kg and transgenic canola at 250, 500 and 2500 U/kg per day significantly improved the nutritional values and remarkably reduced phosphorus excretion (*34*,*60*,*61*). Germinated transgenic rice seeds overexpressing phytase from bacterial origin supplemented to poultry feed resulted in growth rates comparable to the growth rates of chickens fed with a standard meal supplemented with a commercial microbial phytase (*42*). When transgenic rice fresh leaves expressing yeast (*Schwanniomyces occidentalis*) phytase were ground and mixed with whole extract of seed-based feed for pigs, the release of orthophosphate increased significantly, due to phytase activity (*62*). For the efficacy of transgenic canola-derived phytase, the chicken trial results have shown that it is as effective as the commercial microbial phytase (*33*). Also, the chickens fed with phytase from transgenic canola performed at par to those fed with feed supplemented with inorganic phosphate. In another trial, differences were not observed in the utilization of P or Ca between pigs fed with 16 500 U/kg of either corn-based phytase (CBP),

Table 2. List of transgenic plants over-expressing phytase gene and their phytase activities

Transgenic crop expressing phytase gene	Phytase activity	Reference
Arabidopsis per mass of protein	431.49 mU/mg	*46*
canola per mass of seeds (2.6 % of the total soluble proteins)	15 600 U/kg	*33*
maize per mass of seeds	16500 FTU/kg	*53*
	2900 U/kg	*47*
	2200 U/kg	*48*
	20.67 U/kg	*49*
potato per mass of tubers	50 000 U/kg	*52*
rice		
per mass of fresh leaves (truncated gene)	10.6 U/g	*4*
per mass of fresh leaves (full-length gene)	5 U/g	*4*
per mass of seeds	72–9415 U/g	*41*
per dry mass	6000 (*SrPf6*) and 2500 U/kg (*appA*)	*42*
sesame per mass of protein	72 U/mg	*45*
soybean		
per mass of seeds	125 U/kg	*35*
n(P) per time per mass of protein	184 μmol/(min·mg)	*36*
per mass of protein	10.2 U/mg	*37*
tobacco		
mass of phytase per dry mass (1.7 % of total soluble protein in leaves)	2500 ng/mg	*25*
per mass of leaves	1494 nkat/g	*26*
per mass of protein	52.1 mU/mg	*28*
per mass of protein	3.8 nkat/mg	*29*
percentage of total leaf soluble protein (enzyme activity data not shown)	2.3 %	*31*
Trifolium repens per mass of protein	52 mU/mg	*50*
wheat per mass of seeds	1265 FTU/kg	*6*
	1467 FTU/kg	*39*
	3655 U/kg	*40*

i.e. transgenic corn expressing a phytase gene from *E. coli*, or Quantum® phytase derived from *E. coli* (*53*). These findings will perhaps extend the application of transgenic plants that produce heterologous phytases as an animal feed.

Conclusion and Future Prospects

Transgenic plants expressing microbial phytase are an innovative means of delivering phytases to non-ruminants to enhance the utilization of phytate phosphorus and mineral uptake. Hence, it could be concluded that molecular organic farming of stable hydrolytic enzymes like phytase is a practical proposition. It was found that the phytase produced by fungi, bacteria or yeast and the recombinant phytase produced in transgenic plants do not show much difference. Furthermore, the use of codon modifications adjusted to the host plant in seed- and endosperm-specific promoters and the addition of signal peptides for tissue-specific secretion would have an additional effect on the production and accumulation of phytases in plants. Therefore, production of stable phytase from microbial sources in crop plants could open a new venture for commercial purposes. There is a potential in the use of these transgenic seeds and fodder as additives for the improvement of digestibility of phytic acid in animal feeds, and the reduction of phosphate excretion from poultry, pigs and dairy cattle would substantially reduce phosphates in manure.

Only a limited number of phytase genes from bacteria and yeast have been used for developing transgenic crops. Further efforts for developing new technologies and identifying the most efficient phytases from various sources and their heterologous expression in appropriate host plants for improving animal feed must continue. Since the feed ingredients are often heated at 65–80 °C during the pelleting process, phytase stable at high temperature is desirable for animal feed applications. There is an urgent need to search for thermostable phytase from different microbial sources and efforts should be focused on employing phytase genes from such microorganisms for transformation of various crops. Another approach may be to limit the phytate content of seeds by developing low phytic acid (*lpa*) mutant plants through knocking out of genes involved in phytic acid biosynthesis. Thus, the purpose of investigations is to know if the low phytate trait results in undesirable side effects in crops such as yield reduction and loss of seed viability. Preferably both strategies, *i.e.* the use of *phy* gene for transformation of crop plants, and the production of *lpa* mutant plant seeds should go hand in hand either to improve phytate utilization by monogastric animals or to reduce their exposure to phytate.

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References

1. J.A. Maga, Phytate: Its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis, *J. Agric. Food Chem. 30* (1982) 1–9.
2. H. Brinch-Pedersen, L.D. Sørensen, P.B. Holm, Engineering crop plants: Getting a handle on phosphate, *Trends Plant Sci. 7* (2002) 118–125.
3. A. Vohra, T. Satyanarayana, Phytases: Microbial sources, production, purification, and potential biotechnological applications, *Crit. Rev. Biotechnol. 23* (2003) 29–60.
4. A. Hamada, K. Yamaguchi, N. Ohnishi, M. Harada, S. Nikumaru, H. Honda, High-level production of yeast (*Schwanniomyces occidentalis*) phytase in transgenic rice plants by a combination of signal sequence and codon modification of the phytase gene, *Plant Biotechnol. J. 3* (2005) 43–55.
5. M. Torre, A.R. Rodriguez, F. Saura-Calixto, Effects of dietary fiber and phytic acid on mineral availability, *Crit. Rev. Food Sci. Nutr. 30* (1991) 1–22.
6. H. Brinch-Pedersen, F. Hatzack, L.D. Sørensen, P.B. Holm, Concerted action of endogenous and heterologous phytase on phytic acid degradation in seed of transgenic wheat (*Triticum aestivum* L.), *Transgenic Res. 12* (2003) 649–659.
7. V. Raboy, Low phytic acid mutants and selection thereof. *US patent 5689504* (1997).
8. X.G. Lei, C.H. Stahl, Biotechnological development of effective phytases for mineral nutrition and environmental protection, *Appl. Microbiol. Biotechnol. 57* (2001) 474–481.
9. J. Pen, T.C. Venwoerd, P.A. van Paridon, R.F. Beudeker, P.J.M. van den Elzen, K. Geerse *et al.*, Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization, *Nat. Biotechnol. 11* (1993) 811–814.
10. J. Li, C.E. Hegeman, R.W. Hanlon, G.H. Lacy, D.M. Denbow, E.A. Grabau, Secretion of active recombinant phytase from soybean cell-suspension cultures, *Plant Physiol. 114* (1997) 1103–1111.
11. G.C. Whitelam, W. Cockburn, M.R.L. Owen, Antibody production in transgenic plants, *Biochem. Soc. Trans. 22* (1994) 940–944.
12. J.K.C. Ma, M.B. Hein, Immunotherapeutic potential of antibodies produced in plants, *Trends Biotechnol. 13* (1995) 522–527.
13. J. Kerovuo, M. Lauraeus, P. Nurminen, N. Kalkkinen, J. Apajalahti, Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis*, *Appl. Environ. Microbiol. 64* (1998) 2079–2085.
14. L.J. Yanke, H.D. Bae, L.B. Selinger, K.J. Cheng, Phytase activity of anaerobic ruminal bacteria, *Microbiology, 144* (1998) 1565–1573.
15. E. Rodriguez, Y. Han, X.G. Lei, Cloning, sequencing, and expression of an *Escherichia coli* acid phosphatase/phytase gene (*appA2*) isolated from pig colon, *Biochem. Biophys. Res. Commun. 257* (1999) 117–123.
16. A.H. Ullah, *Aspergillus ficuum* phytase: Partial primary structure, substrate selectivity, and kinetic characterization, *Prep. Biochem. 18* (1988) 459–471.
17. C.S. Piddington, C.S. Houston, M. Paloheimo, M. Cantrell, A. Miettinen-Oinonen, H. Nevalainen, J. Rambosek, The cloning and sequencing of the genes encoding phytase (*phy*) and pH 2.5 optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori*, *Gene, 133* (1993) 55–62.
18. W. van Hartingsveldt, C.M.J. van Zeijl, G.M. Harteveld, R.J. Gouka, M.E.G. Suykerbuyk, R.G.M. Luiten *et al.*, Cloning, characterization and overexpression of the phytase-encoding gene (*phyA*) of *Aspergillus niger*, *Gene*, *127* (1993) 87–94.
19. L. Pasamontes, M. Haiker, M. Henriquez-Huecas, D.B. Mitchell, A.P. van Loon, Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*, *Biochim. Biophys. Acta*, *1353* (1997) 217–223.
20. L. Pasamontes, M. Haiker, M. Wyss, M. Tessier, A.P. van Loon, Gene cloning, purification, and characterization of a heat-stable phytase from fungus *Aspergillus fumigatus*, *Appl. Environ. Microbiol. 63* (1997) 1696–1700.
21. R.M. Berka, M.W. Rey, K.M. Brown, T. Byun, A.V. Klotz, Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*, *Appl. Environ. Microbiol. 64* (1998) 4423–4427.
22. S.F. Lassen, J. Breinholt, P.R. Østergaard, R. Brugger, A. Bischoff, M. Wyss, C.C. Fuglsang, Expression, gene cloning, and characterization of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp., and *Trametes pubescens*, *Appl. Environ. Microbiol. 67* (2001) 4701–4707.
23. A.R. Wankhade, L.P.S. Rajput, K. Tantwai, A. Verma, Optimization of phytase production with submerged fermentation by *Aspergillus ficuum* using agri-waste materials, *JNKVV Res. J. 42* (2008) 124–127.
24. P. Gajbhiye, K. Tantwai, L.P.S. Rajput, Bioconversion of agri-waste material for fungal phytase production, *JNKVV. Res. J. 42* (2008) 226–228.
25. T.C. Verwoerd, P.A. van Paridon, A.J.J. van Ooyen, J.W.M. van Lent, A. Hoekema, J. Pen, Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves, *Plant Physiol. 109* (1995) 1199–1205.
26. A.H.J. Ullah, K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, S. Austin-Philips, Characterization of recombinant fungal phytase (*phyA*) expressed in tobacco leaves, *Biochem. Biophys. Res. Commun. 264* (1999) 201–206.
27. W. Yip, L. Wang, C. Cheng, W. Wu, S. Lung, B.L. Lim, The introduction of a phytase gene from *Bacillus subtilis* improved the growth performance of transgenic tobacco, *Biochem. Biophys. Res. Commun. 310* (2003) 1148–1154.
28. S.C. Lung, W.L. Chan, W. Yip, L. Wang, E.C. Yeung, B.L. Lim, Secretion of β-propeller phytase from tobacco and *Arabidopsis* roots enhances phosphorus utilization, *Plant Sci. 169* (2005) 341–349.
29. T.S. George, R.J. Simpson, P.A. Hadobas, A.E. Richardson, Expression of a fungal phytase gene in *Nicotiana tabacum* improves phosphorus nutrition of plants grown in amended soils, *Plant Biotechnol. J. 3* (2005) 129–140.
30. W.L. Chan, S.C. Lung, B.L. Lim, Properties of β-propeller phytase expressed in transgenic tobacco, *Protein Express. Purific. 46* (2006) 100–106.
31. Y. Wang, X. Gao, Q. Su, W. Wu, L. An, Expression of heat stable phytase from *Aspergillus fumigatus* in tobacco (*Nicotiana tabacum* L. cv. NC89), *Ind. J. Biochem. Biophys. 44* (2007) 26–30.
32. A.S. Ponstein, J.B. Bade, T.C. Verwoerd, L. Molendijk, J. Storms, R.F. Beudeker, Stable expression of phytase (*phyA*) in canola (*Brassica napus*) seeds: Towards a commercial product, *Mol. Breed. 10* (2002) 31–44.
33. R.H. Peng, Q.H. Yao, A.S. Xiong, Z.M. Cheng, Y. Li, Codon-modifications and an endoplasmic reticulum-targeting sequence additively enhance expression of an *Aspergillus* phytase gene in transgenic canola, *Plant Cell Repr. 25* (2006) 124–132.
34. D.M. Denbow, E.A. Graubau, G.H. Lacy, E.T. Kornegay, D.R. Russell, P.F. Umbeck, Soybeans transformed with a fungal phytase gene improve phosphorus availability for broilers, *Poultry Sci. 77* (1998) 878–881.
35. X.R. Gao, G.K. Wang, Q. Su, Y. Wang, L.J. An, Phytase expression in transgenic soybeans: Stable transformation with a vector-less construct, *Biotechnol. Lett. 29* (2007) 1781–1787.
36. K.D. Bilyeu, P. Zeng, P. Coello, Z.J. Zhang, H.B. Krishnan, A. Bailey *et al.*, Quantitative conversion of phytate to inorganic phosphorus in soybean seeds expressing a bacterial phytase, *Plant Physiol. 146* (2008) 468–477.

37. G. Li, S. Yang, M. Li, Y. Qiao, J. Wang, Functional analysis of an *Aspergillus ficuum* phytase gene in *Saccharomyces cerevisiae* and its root-specific, secretory expression in transgenic soybean plants, *Biotechnol. Lett. 31* (2009) 1297–1303.

38. S. Yang, G. Li, M. Li, J. Wang, Transgenic soybean with low phytate content constructed by *Agrobacterium* transformation and pollen-tube pathway, *Euphytica*, *177* (2011) 375–382.

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

40. H. Brinch-Pedersen, F. Hatzack, E. Stöger, E. Arcalis, K. Pontopidan, P.B. Holm, Heat-stable phytases in transgenic wheat (*Triticum aestivum* L.): Deposition pattern, thermostability, and phytate hydrolysis, *J. Agric. Food Chem. 54* (2006) 4624–4632.

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

42. C.Y. Hong, K.J. Cheng, T.H. Tseng, C.S. Wang, L.F. Liu, S.M. Yu, Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds, *Transgenic Res. 13* (2004) 29–39.

43. Q.Q. Liu, Q.F. Li, L.L. Jiang, D.J. Zhang, H.M. Wang, M.H. Gu, Q.H. Yao, Transgenic expression of the recombinant phytase in rice (*Oryza sativa*), *Rice Sci. 13* (2006) 79–84.

44. A.H.J. Ullah, K. Sethumadhavan, E.J. Mullaney, T. Ziegelhoffer, S. Austin-Phillips, Cloned and expressed fungal *phyA* gene in alfalfa produces a stable phytase, *Biochem. Biophys. Res. Commun. 290* (2002) 1343–1348.

45. U.H. Jin, J.A. Chun, J.W. Lee, Y.B. Yi, S.W. Lee, C.H. Chung, Expression and characterization of extracellular fungal phytase in transformed sesame hairy root cultures, *Protein Express. Purific. 37* (2004) 486–492.

46. S.R. Mudge, F.W. Smith, A.E. Richardson, Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables *Arabidopsis* to grow on phytate as a sole P source, *Plant Sci. 165* (2003) 871–878.

47. G. Drakakaki, S. Marcel, R.P. Glahn, E.K. Lund, S. Pariagh, R. Fischer *et al.*, 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* (2005) 869–880.

48. R. Chen, G. Xue, P. Chen, B. Yao, W. Yang, Q. Ma *et al.*, Transgenic maize plants expressing a fungal phytase gene, *Transgenic Res. 17* (2008) 633–643.

49. Y. Shen, H. Wang, G. Pan, Improving inorganic phosphorus content in maize seeds by introduction of phytase gene, *Biotechnology*, *7* (2008) 323–327.

50. S. Han, Y. Gu, K. Xiao, Improving organic phosphate utilization in transgenic white clover by overexpression of *Aspergillus niger phyA* gene, *Front. Agric. China, 1* (2007) 265–270.

51. A.S. Pires, M.G. Cabral, P. Fevereiro, E. Stoger, R. Abranches, High levels of stable phytase accumulate in the culture medium of transgenic *Medicago truncatula* cell suspension cultures, *Biotechnol. J. 3* (2008) 916–923.

52. Y.F. Hong, C.Y. Liu, K.J. Cheng, A.L. Hour, M.T. Chan, T.H. Tseng *et al.*, The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato, *Plant Mol. Biol. 67* (2008) 347–361.

53. E.K.D. Nyannor, P. Williams, M.R. Bedford, O. Adeola, Corn expressing an *Escherichia coli*-derived phytase gene: A proof-of-concept nutritional study in pigs, *J. Anim. Sci. 85* (2007) 1946–1952.

54. M. Gouy, C. Gautier, Codon usage in bacteria: Correlation with gene expressivity, *Nucleic Acids Res. 10* (1982) 7055–7074.

55. C. Gustafsson, S. Govindarajan, J. Minshull, Codon bias and heterologous protein expression, *Trends Biotechnol. 22* (2004) 346–353.

56. C.I. Wandelt, M.R. Khan. S. Craig, H.E. Schroeder, D. Spencer, T.J. Higgins, Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants, *Plant J. 2* (1992) 181–192.

57. J.L. Hadlington, J. Denecke, Sorting of soluble proteins in the secretary pathway of plants, *Curr. Opin. Plant Biol. 3* (2000) 461–468.

58. Y. Han, X.G. Lei, Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (*phy*A) in *Pichia pastoris*, *Arch. Biochem. Biophys. 364* (1999) 83–90.

59. R.G. Koegel, S. Austin-Phillips, M.E. Cook, R.J. Straub, Phytase from transgenic alfalfa for supplementation of poultry and swine rations, *U.S. Dairy Forage Res. Ctr. Res. Sum.* (1996) 43–45.

60. Z.B. Zhang, E.T. Kornegay, J.S. Radcliffe, D.M. Denbow, H.P. Veit, C.T. Larsen, Comparison of genetically engineered microbial and plant phytase for young broilers, *Poultry Sci. 79* (2000) 709–717.

61. Z.B. Zhang, E.T. Kornegay, J.S. Radcliffe, J.H. Wilson, H.P. Veit, Comparison of phytase from genetically engineered *Aspergillus* and canola in weaning pig diets, *J. Anim. Sci. 78* (2000) 2868–2878.

62. A. Hamada, K. Yamaguchi, M. Harada, K. Horiguchi, T. Takahashi, H. Honda, Recombinant, rice-produced yeast phytase shows the ability to hydrolyze phytate derived from seed-based feed, and extreme stability during ensilage treatment, *Biosci. Biotechnol. Biochem. 70* (2006) 1524–1527.