Annals of Plant Sciences
ISSN: 2287-688X
Review Article

Manipulating Microbial Phytases For Heterologous Expression In Crops For Sustainable Nutrition

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Received for publication: July 22, 2013; **Revised:** August 11, 2013; **Accepted**: September 10, 2013.

Abstract: Phytases are phosphohydrolases that facilitate the sequential removal of phosphate from phytate. Monogastric species such as swine, poultry and fish including humans need external phytase to digest phytate, the major form of phosphorus in plant-derived food products or feed. Eventually, dietary phytases are dispensed to liberate feed phytate-phosphorus to reduce the supplementation of inorganic phosphorus and to alleviate their phosphorus excretion. Phytases have emerged as one of the most efficient and productive feed additives. However, addition of commercially available phytase enzyme to the animal feed is expensive and laborious. To overcome these problems, development of genetically engineered plants for heterologous production of phytases improves the phosphorus and mineral bioavailability. Additionally, it reduces the phytic acid excretion and phosphate load on agricultural ecosystem and thereby alleviates eutrophication of aquatic environment. This review aims to summarize the recent information on plant-based phytase development for identifying an ideal phytase for transgenic plant production and its potential applications. It targets crucial and synergistic analyses on the global impression, innovative application and future prospects of phytases in promoting sustainable animal and human health.

Keywords: Monogastric species; Nutrition; Phosphorus; Phytase; Phytic acid; Transgenics

Introduction

Globally, cereal, legume and oil seeds are major dietary components for livestock and humans. Phytate (salts of phytic acid or PA) is a primary storage constituent of seeds that comprises of 80% of the total seed phosphorus. Monotonous consumption of cereal and legume seeds composed of high phytate chelates dietary minerals and vitamins causing micronutrient malnutrition in monogastric species including humans. Due to its high-affinity for cations, phytic acid forms insoluble phytate salts in solution (Crea *et al.,* 2008) thereby rendering the crucial dietary minerals such as iron, zinc, calcium and magnesium unavailable for absorption in the gut (Kumar *et al.,* 2010). Inefficient utilization of phytate by monogastric species (poultry, fish and swine) generates phosphorus management and environment impact issues in animal production. Phosphorus accessibility plays a key role in issue of soil prolificacy, crop production, animal health and nutrition as well as waste management and water quality. Phosphorus (P) is an essential non-renewable plant nutrient that may last for an estimated hundred years (Dawson *et al.,* 2011) and limits agricultural production on a global scale. For phosphorus to be usable to plants, it needs to be present as orthophosphate in soil solution. Orthophosphate accessibility is the prime determinant of enhanced plant productivity as P, exclusively in this form is absorbed by plants to cater their nutritional requirements. Nevertheless, limited orthophosphate availability for plant growth is prevalent in approximately 70% of cultivated soils. Poor availability of orthophosphate is significantly caused by high reactivity of orthophosphate with soil constituents and accelerated transformation of orthophosphate to organic forms (by soil bacteria) that remain unabsorbed by plants. Additionally, only up to 20% of fertilizer P is available for utilization by crops (Holford et al. 1997). Efficient consumption of fertilizer P is also required to avert the adverse environmental effects as a result of P pollution of the wider environment (Tunney *et al.,* 1997).

Reduction in phytic acid content can be addressed via low phytic acid mutants, silencing PA biosynthetic genes or over expressing phytases. The exudation of phosphatases or phytases that catalyze the hydrolysis of organic P is potentially an important way for plants to raise P availability (Fig. 1), especially as a large proportion of

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soil P (up to 80%) occurs in organic forms (Richardson *et al.,* 2004). For phosphorus bioavailability and dilution of eutrophication, microbe derived phytase is commonly added to the feed in areas with intense pig and poultry production (Brinch-Pedersen *et al.,* 2002). Eventually, phytases available commercially are expensive and intensive. Here we focus on the recent developments on classification and concept of ideal phytases and their heterologous expression in plants for cost-effective and sustainable animal and human nutrition.

Source, classification, molecular and biophysical characteristics of phytases

Phytase is a term used to describe the enzymes that hydrolyze phospho monoester bonds of PA (myoinositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate) in turn generating inorganic orthophosphates. Phytases are ubiquitously present in microorganisms, plants, and animals. Initially identified from rice bran (Suzuki *et al.,* 1907), phytases have been isolated from varied sources like bacteria, fungi and yeasts (Vats and benerjee 2004; Rao *et al.,* 2009; Yao *et al.,* 2012; Lei *et al.,* 2013). Depending on the source or expression host, phytases exhibit distinct biophysical and biochemical properties. Based on origin, there are microbial (bacterial, fungal and yeast), plant and gut associated microfloral phytases that may exist as intracellular (gram negative bacteria), extracellular (gram positive bacteria and fungi) and/or cell bound forms (Rao *et al.,* 2009). Phytic acid has six phosphate groups that may be released by phytases at differential rates and order. Phytases hydrolyze phosphates from phytic acid in a sequential manner, generating products that in turn become substrates for subsequent hydrolysis. Majority of the phytases are able to cleave five of the six phosphate groups from phytic acid (Konietzny and Greiner 2002). Depending on the site of initiation (or carbon in the myoinositol ring of phytate) of de-phosphorylation of the phytate molecule, the Enzyme Nomenclature Committee of the International Union of Biochemistry recognizes four phytases that may be classified as *3-phytases* (EC 3.1.3.8)*, 4-phytases* or *6-phytases* (EC 3.1.3.26)*, 5-phytases* (3.1.3.72). To date, most of the known phytases are 3-phytases that are majorly present in bacteria (*Klebsiella sp*. *Bacillus sp*. and *Methanobacterium thermophila*) and fungi (e.g., *Aspergillus niger (A. niger)*, *Neurospora crassa*, *Pseudomonas sp.*) that initiate de-phosphorylation at C1 and C3 (Sajidan *et al.,* 2004). The 4-phytases (or 6-phytases) have been extracted from grains and oil seeds of higher plants, in addition to bacteria like *Escherichia coli* (*E. coli)*, *Yersinia sp*., fungi (*A. niger*)and methylotrophic yeast (*Peniophora lycii*). However, phytases purified from lily pollen (Barrientos *et al.,* 1994) and *Selenomonas ruminantium* subsp. *Lactilytica* (Puhl *et al.,* 2008) has been assigned as 5-phytases. Most phytases exhibit broad substrate specificity with the ability to hydrolyze several phosphorylated components dissimilar in structure to PA like phenyl phosphate, glucose 6-phosphate, ADP and ATP. Nevertheless, few phytases from bacteria and fungi are recognized as highly specific for PA like *Bacillus sp*., *Aspergillus sp.*, *Escherichia coli* and *Selemonas ruminatium*.

Huge number of phytases has been identified with no structural similarities amongst all of them. Diverse catalytic machinery and requirements have been noted in phytases. Phytases have been categorised on the basis of their catalytic activity, position of the hydrolysis initiation site of phytate molecule and protein motifs. Phytases may be subdivided on the basis of differential catalytic machinery into four groups namely; histidine acid phosphate (HAP) (E.C.3.1.3.2), β-propeller phytases (BPP) (E.C.3.1.3.8), Purple acid phytases *(PAP)* (E.C.3.1.3.2) and Protein Tyrosine Phosphatase-like phytases (PTP) (Mullaney and Ullah 2003). The HAPs, PAPs and PTPs have acidic pH optima, whereas, the BPPs are characterized as alkaline phytases (Tye *et al.,* 2002). Many of the bacterial, fungal and plant phytases belong to HAPs (except lily pollen). The HAPs constitute a large group of acidic phytases, which share the catalytic mechanism as an N-terminal RHGXRXP motif and C terminal HD motif position together and form the active site (Lei *et al.,* 2007). This class of enzymes hydrolyzes PA in a two-step mechanism: nucleophilic assault on the phosphorus atom (Fig. 3A) by histidine in the active site ensued by hydrolysis of the resultant phospho-histidine intermediate (Vincent *et al.,* 1992). BPPs are alkaline phytases sharing 90-98% sequence identity and few characteristically conserved motifs (Fig. 3B) amongst themselves. However, they exhibited dissimilarity to other classes of phytases. The crystal structure of *Bacillus amyloliquefaciens*

displayed a six-bladed beta-propeller architecture with four- or five-stranded anti-parallel beta sheet and seven calcium-binding sites (Fig. 3 B). The Ca^{2+} (1), Ca^{2++}(2) and Ca^{2+} (3) are high affinity binding sites that confer thermo stability, whereas Ca^{2+} (4), Ca^{2+}(5) and Ca^{2+} (6) are crucial for catalytic function (Ha *et al.,* 2000; Fan *et al.,* 2013). Additionally, there are two phosphate binding sites; one is the "cleavage site" responsible for substrate hydrolysis and another represents the "affinity site" involved in enhanced substrate (with adjacent phosphate groups) binding affinity, thereby creating the seventh calcium binding site (Fig. 3B) in the presence of phosphate ions (Shin *et al.,* 2001). The PAPs are metallo hydrolases, which bind to two metal ions in the active centre one of the ions is usually Fe-III, while the second metal in plant PAPs can be Zn^{2+}, Mn^{2+} (Fig. 3C) that imparts specific colour to this enzyme (Vogel *et al.,* 2006). The three important fingerprint motifs that are specific to this class of phytases have been highlighted (Fig.3C). PAP gene was isolated from cotyledons of germinating soybean and PAP-like sequences have been revealed in several plants, mammals, fungi and bacteria. The PTPs or protein tyrosine phytases/cystiene phytases possess dual specificity with a conserved cysteine (C241) in PTP specific motif in their active site (Fig. 3D). This newly discovered class of phytase has been isolated from anaerobic ruminal bacteria that colonize their gut (Huang *et al.,* 2011). PTPs share very poor sequence homology with other microbial phytases with an active site comprising of a phosphate-binding loop (P loop) and substrate-binding pocket (WPD-loop) (Fig. 3D) (Puhl *et al.,* 2007). All characterized PTP-like phytases share an active site sequence motif (His-Cys-(X) 5-Arg: P loop), a two-step acid/base mechanism of de-phopshorylation and affinity towards phosphorylated tyrosine residues. The specific biological substrates and functions of bacterial PTP-like phytases have not been revealed. Interestingly, PTP-like phytases characterized from ruminal bacteria resemble in sequence and structural homology to the mammalian PTP-like Phosphoinositide/-inositol phosphatase PTEN (Puhl *et al.,* 2007; 2008). Recently, on the bases of fingerprint motifs, phytases are broadly classified into seven classes; HAPhys, PAPhys, BPPhys, BPRPhys, PTPhys, ALPhys and APPAs. According to this classification, these classes of phytases have specific or conserved motifs that are identical within each class. HAPs have 11 identical motifs, BPPhy has 13 motifs, PAPhy, PTPhy, BPRPhy and AppA (Fig. 3 E) classes have 4, 4, 4, 6 conserved motifs, respectively (Fan *et al.,* 2013). We have highlighted some of the importantly conserved or fingerprint motifs that are involved in phytase activity for five of the seven important classes as, crystal structures of the others were not available (Fig. 2). These motifs may provide insights on protein structure, function and evolution.

Ideal phytase for transgenic plant expression:

Phytase that possesses the ability to efficiently hydrolyze phytate-phosphorous at low pH (upper digestive tract of organism), has high thermostablity up to 65-80 °C for tolerating high feed pelleting temperatures, exhibits desirably high phytase activity and is cost effective may be termed as an "ideal phytase" for commercialized transgenic overproduction. The desired qualities of an ideal phytase are not found in any one single phytase variant so any one single phytase may not be ideal for all applications. A prerequisite is to identify a cluster of target-specific phytases that can complement the phytate hydrolysis requirements in the different species like swine, poultry, fish and humans or for different applications for instance soil P availability. Phytase with high activity at low pH is found in *Aspergillus niger* (2.2-5.5), whereas highly thermo stable phytases originate from *Bacillus amyloliquifaciens* (up to 80 °c) with enhanced stability during animal feed processing and storage. In addition, desirably high enzyme activity is exhibited by phytases from Citrobacter (3457 u/mg) and Yersinia intermedia (3960 u/mg). Therefore, different phytases may be employed for different targets. The search for ideal phytases may be achieved either by earmarking naturally existing new native phytases or by redesigning known phytases by manipulation of the desired qualities.

Earmarking innovative phytases

Prior to the use of recombinant DNA technology (RDT), initial studies for finding unique phytases concentrated on recognizing microbes possessing high phytase activity. Screening for high phytase activity was performed by using phytate as the only source of phosphorous for its hydrolysis. Identification of *Aspergillus niger* PhyA

(*AnPhyA*), HAP with a bimodal pH profile having optima at 2 and 5.5 was most notable (Irving and Cosgrove, 1972). With the incumbent RDT, the *AnPhyA* was cloned and over expressed for commercialization (Mullaney *et al.,* 1991) followed by several studies compiled by recent reviews (Konietzny and Greiner, 2002; Rao *et al.,* 2009; Yao *et al.,* 2012; Lei *et al.,* 2013; Fan *et al.,* 2013). A paradigm shift in phytase research has occurred with the advent of cutting edge technology for overproduction of target phytases in heterologous expression systems. The focus has shifted from finding novel phytases to application oriented studies on enzymatic characteristics of phytases that comprises efficiency of catalysis, pH profile, thermo stability and tolerance to proteolytic conditions. Eventually, the second generation phytases emerged for commercialization for example the *AppA* phytases (*AppA1* & *AppA2*) from *Escherichia coli* that are members of HAPs show high activity, tolerance to proteolysis and required pH profiles compatible to stomach conditions in several animals. Nevertheless, these *E. coli* phytases are less thermo stable than their fungal equivalents. Additionally, HAPs from *P. lycii* and *A. niger* (*AnPhyB*) have been commercialized. Among the four important groups of phytases, HAPs referred to as multiple inositol polyphosphate phosphatases are the only known commercialized ones. In the past decade, mining for new phytases for biotechnological applications have been in vogue. In depth information about novel phytases and their enzymatic properties have been reviewed (Rao *et al.,* 2009; Li *et al.,* 2010; Yao *et al.,* 2012). In line with this trend, attempts have been made to isolate phytases from extreme environments. For instance, phytases have been isolated from thermophilic fungi (Singh and Satyanarayana, 2011), microorganisms dwelling in glaciers (Huang *et al.,* 2009). Analyses of the fingerprint motifs and variation in phytase gene sequences available in public data banks have been performed by bioinformatics approach (Lim *et al.,* 2007; Fan *et al.,* 2013).

Feed for the monogastric animal industry essentially depends on plant products that have abundant phytate content. Commercial phytases have to be used for degrading phytate, which is cost-intensive. Therefore, an effective and simple solution is either to create transgenic plants with heterologous expression of phytases or to develop low phytate crops. Utilization of variants of phytases for specific function or application has been briefly discussed.

Livestock industry targeted:

Poultry diets specifically contain 2.5-4.0 gkg^{-1} phytate-P, amounting to one million tons of phytate-P consumption annually (Selle *et al.,* 2007). The site of phytate hydrolysis is predominantly in the fore-stomach (crop, proventriculus and gizzard) where the pH is acidic. However, some phytate hydrolysis may be indicated in the gastrointestinal tract. Studies on ileal and total tract assessments showed that degradation of phytate is incomplete in poultry, especially broiler chicks after phytase supplementation at standard inclusion rates (Leske and Coon 1999; Shirley and Edwards, 2003). Eventually, the form of residual phytate present in gut is important. Therefore, for poultry especially broiler chicks, HAP (*A. niger*) phytases with activity in broad pH spectrum, i.e, acidic (stomach), basic (ileum) would be beneficial (Riley *et al.,* 1984). In contrast to HAPs, the BPPs like *Bacillus sp.* displays basic pH profile, greater thermostability and higher activity for calcium-phytate complex. Due to this strict calcium substrate specificity, BPPs are suitable in diets for laying hens, which have high intake of dietary Ca^{2+} (Lei *et al.,* 2013). Since the feeds are often processed through a pelleting machine at 65 to 80°C, an ideal phytase for livestock must be able to withstand the high temperature and steam encountered during the pelleting process for instance the BPPs (*Bacillus* sp.), fungal or bacterial HAP phytases. Bacterial HAPs like *EcAppA* 1 and 2 phytases have desired pH profiles for stomach in young pigs (Jongbloed *et al.,* 1992), in addition to high activity and tolerance to proteolysis (Rodriguez *et al.,* 1999; Weaver *et al.,* 2009). In addition, for livestock industry, the phytase enzyme must able to tolerate long-term storage or transport at ambient temperature.

Aquaculture targeted:

Efficacy of microbial phytase for aquaculture is controlled directly or indirectly by several closely related factors. These may include fish species, dietary substrate levels, inclusion rate and source of phytase. For many fish species dosage of 250-2000 FTU/kg feed is generally advised to be advantageous (Cao *et al.,* 2007). Moreover, different phytase sources may cause diverse effects on growth parameters and nutrient deposition

(Liebert and Portz, 2005). The efficacy of phytase varies in agastric and gastric fish. The pH profile in digestive tract of agastric fish for instance carp is about 6.5-8.4 while gastric fish possess a much lower pH status (Ji, 1999). Phytases that works at neutral/alkaline pH (*Bacillus* sp.) are very suitable for aquaculture of agastric fish particularly for carp fish because their gut pH is alkaline (Zeng *et al.,* 2001). On the other hand, phytases like HAPs (bacterial and fungal) that have optimum activity in acidic or lower pH conditions are suitable for gastric fish like rainbow trout, catfish and other carnivorous fish is favourable for effective hydrolysis of phytate. HAPs may be suitable for agastric fish if an acidifier such as fumaric or citric acid is added in their diets for lowering the pH of the digestive tract and finally upregulating the activity of exogenous phytase (Baruah *et al.,* 2005; 2007). Moreover, phytases for aquaculture application require a low temperature optimum than for swine or poultry due to low body temperatures (16°-27°C) of organisms dwelling in aqueous conditions (Ramseyer *et al.,* 1999). Additionally, phytases utilized for aqua-feed should be heat-stable, resistant to aggressive feedmilling processes like extrusion followed by extended conditioning or drying and stable in treated feed during storage.

Human nutrition targeted:

Major concern over seed-derived dietary PA for humans has been in its contribution towards mineral diminution and deficiency. Developing and under developed populations worldwide, survive on whole grain and/or legume staple foods that comprise of high PA leading to micronutrient deficiencies. In India, the average PA intake ranges from 670 to 2500 mg (Schlemmer *et al.,* 2009). Ideal phytase for humans should be effective in releasing phytate-P in the digestive tract and be able to resist proteases (trypsin and pepsin) with low cost of production. In humans, as the stomach is the main functional site for supplemental phytases, those phytases (fungal and bacterial HAPs) that possess pH optima in the acidic range are desirable for improving nutrition like *A. niger* and *E. coli* phytases. AppA2 phytase from *E. coli* has eminent phytase activity and great proteolytic resistance (Weaver *et al.,* 2009), whereas *Bacillus subtilis* (BPPs with alkaline pH maxima) phytase is resistant to trypsin, pancreatin, and papain but highly susceptible to pepsin (Kerovou *et al.,* 2000).

Soil P availability targeted:

Poor availability of P in soils and subsequent P-deficiency are serious constraints to crop production throughout the globe. Most of the cultivated soils for agriculture possess 30-80% of organic P of which 60-80% exists in the form of phytate and is not directly available to plants (Schachtman *et al.,* 1998). Hence, improving phytate-P bioavailability is crucial for plant P nutrition and sustainable agriculture (Brinch-Pedersen *et al.,* 2002). Phytases with lower iso-electric point (PI) are desirable for better P assimilation from soil phytate. Phytase from *Peniophora lycii is* better for releasing soil P than its counterpart from *A. niger*, even though they share several characteristics (George *et al.,* 2007). For rhizhosphere expression, broad substrate specificity is more relevant as different kinds of ions are bound with phytate (Bohm *et al.,* 2010).

Plant nutrition:

Complete degradation of phytic acid in any system is not advisable. An ideal phytase should produce 3- or 4- myoinositol form of phytate as these intermediates play a crucial role in cell signalling and transport. Studying the significant roles of inositol tri- phosphates especially in signal transduction and regulation of cell function in plants and animals are an active area of research for comprehending the underlying mechanisms of signalling pathways (Vohra *et al.,* 2003; Rao *et al.,* 2009). Phytases with strict substrate specificities e.g. *Bacillus subtilis* would be ideal for transgenic production. Introduction of phytases with broad substrate specificity may cause disturbance in the metabolic pathways of plants leading to decreased yields (Rao *et al.,* 2009). Two strategies are suggested to improve phytase activity in plant system: (i) modifying the codon of the introduced phytase according to host system without altering the protein sequence. High phytase protein expression was achieved by this strategy in transgenic rice and canola (Hamada *et al.,* 2005; Peng *et al.,* 2006); (ii) second is by appending signal peptide sequence to the phytase gene for specific and localized expression. Several signal peptides have been used in studies for differential expression of phytases like in apoplast (Pederson *et al.,* 2002; Chen *et al.,* 2008) or roots (Lung *et al.,* 2005; Li *et al.,* 2009).

Protein remodelling for effective phytases

Nowadays, a huge demand exists for development of ideal phytases with the aid of protein engineering via directed evolution for targeted application. All features that enable a phytase to be "ideal phytase" are not present within a single phytase. Nevertheless, a consensus phytase could be designed based on the available sequences of phytases (Lehman *et al.,* 2000). Genetic engineering techniques such as site-directed mutagenesis could be employed for further ameliorating the properties via random or rational design. Mullaney *et al.,* (2012) achieved lower optimum temperature and enhanced heat tolerance through rational design based on charge interactions of polar and ionic residues in the active site (site-directed mutagenesis of two cysteine residues, which form a single disulfide bridge) in *A. niger* NRRL3135. These properties in phytases make them effective during feed pelleting process useful for live stock industry. In another study, the *AnPhyA* mutant protein (mutation E228K) shifted its pH profile from 5.5 to 3.5 making it compatible to stomach pH and leading to high phytase activity (266% compared to WT) in swines (Kim *et al.,* 2006). Based on sequence alignment and 3D structure, a hybrid phytase was synthesized from *A. niger* and *A. fumigatus,* which has all desirable qualities that includes high thermo tolerance (Bei *et al.,* 2009). Through multifactor rational design strategy, increased thermo stability of *E. coli* AppA (*EcAppA*) phytase was achieved thatcould tolerate high temperatures (upto 80ºC for 10 mins) with improved catalytic efficiency (Fei *et al.,* 2013). Interestingly, the double mutant (I142L/Y311K) showed maximal thermo stability, whereas the triple mutant (I142L/Y311K/Q260E) exhibited similar thermo stability as WT. Expression of *AnPhyA* and *BsPhyC* fused together in *Pichea* showed all desirable characteristics like broader pH profile (2-7) and higher thermostability up to 95ºC (Zou *et al.,* 2008). It is now realized that any single phytase may never be 'ideal' for transgenic plant production. For example *Yersinia* phytase activity is quite high but its pH is 4.5, which is not suitable to animal gastric pH. Similarly, *Bacillus* phytases are highly thermo tolerant and substrate specific but have relatively low activity. Choice of a system for phytase overproduction is thereby dependent on the target application**.**

In order to construct an "ideal phytase", redesigning of different phytases for specific requirements is a good strategy. Although, an ever increasing data for new and remodelled phytases exists but their commercial utilization is restricted. We need to explore for novel low temperature effective phytases that may be used for aquaculture. More research is needed in the area of "manipulation of protein-folding" processes so that the contribution of protein folding or/and high denaturation temperature towards thermo stable nature of proteins is unravelled. Therefore, for developing an agreeable chimeric "ideal phytase" endowed with thermostable nature that can tolerate high feed pelleting temperatures and performs effectively at low pH with high phytase activity is still far-fetched but yet not impossible.

Heterologous expression of phytases in crops and their impact on crop nutrition, soil phosphorus availability and animal health

Cereal and legume seeds constitute the primary staple food for livestock and humans. PA being highly negatively charged compound stored in large amounts in cereal and legume seeds is considered as the prime anti-nutritional factor for reducing the bioavailability of a range of essential micronutrients. PA forms complexes with cations rendering them unavailable for animal or human absorption. Scientists throughout the globe are attempting to reduce PA levels in grains and seeds of important edible crops. Three approaches exist to reduce PA that includes development of (i) low PA mutants through conventional breeding via induced mutations, (ii) transgenic plants expressing phytases in edible portions and (iii) transgenic plants by manipulating PA biosynthetic pathway. Amongst various strategies for alleviating phytate levels in crops, the heterologous expression of recombinant microbial phytase in the edible parts offers a great potential for improving phosphate and mineral bioavailability in food and feed. Transgenic plants can serve as bioreactors for the production of phytases (Table 1). Bio-farming of phytase is a cost-effective approach and its production in plants has tremendous potential in improving plant phosphorus acquisition and phyto-remediation. Microbial phytases are widely used over plant phytases for transgenic

expression due to their broader applications and the fact that most plant phytases have low efficacy and do not tolerate high temperatures. In this context, we have targeted two areas: firstly, improvement of monogastric animal nutrition and secondly, phosphorus availability to the plant. The widespread use of plant products as meal for livestock feed leads to serious environmental consequences. Introducing phytase genes into plants used as meal for livestock can improve the bioavailability of phosphates and proteins and reduce phytic acid excretion, providing a less expensive alternative to phytase supplementation. An informative outlay of transgenic plants expressing phytases developed so far is presented.

Medicago sativa:

Expression of phytase from *A. ficuum* in alfaalfa plant revealed that the recombinant phytase enzyme retained all characteristics of the benchmark enzyme, except for a slight variation in its pH optima (5.5 to 5) (Ullah *et al.,* 2002). Results demonstrated that molecular bio-farming could be a better option than commercially available phytases. In another study, Ma *et al.,* (2012) expressed native phytase (*MtPHY1*) and phosphatase (*MtPAP1*) genes from *Medicago truncatula* in *Medicago sativa* driven by two different promoters (CaMV35s-whole plant; MtPT1-root specific). Results revealed that transgenic expressing MtPT1:*MtPHY1* (promoter: gene construct) exhibited high levels of phytase expression and improved plant growth under natural soil conditions. Expression of phytase and acid phosphatase ameliorated P acquisition from natural agricultural soils. The study was performed on two different pH containing natural soils (pH: 5 and 6.7). Both the soils were maintained at P deficient conditions and as a result transgenic plants grown on both the soils showed improved phosphorus concentrations. Transgenic biomass was three-fold higher as compared to control plants (WT) when plants were grown in sand with phytate as a P source. While in natural soil without P supplementation transgenic lines showed two-fold increase in biomass than WT. Results suggest that these two transgenes have huge potential for enhancing plant P acquisition and biomass yield in P-deficient soils. However, some studies revealed that transgenic expression of phytase did not improve P acquisition in P deficient soils (George *et al.,* 2005; Wang *et al.,* 2009).

Brassica napus:

Modified *AnPhyA* with additional KDEL sequence improves the phytase expression levels (2.6% higher phytase activity) in host system (Peng *et al.,* 2006). The chicken feed trail also revealed that transgenic canola seeds were as effective as commercially available microbial phytase. Introduction of *AnPhyA* and *EcAppA* genes in *Brassica napus* significantly improved phosphorus uptake, plant biomass and seed yields when phytate was supplied as sole phosphorus (Wang *et al.,* 2013). Utilization of GM canola with phytase gene from *Aspergillus ficuum* in broiler chicks and weaning pigs showed that bone ash, P and Ca retention were comparable with that of feeds containing commercial phytase supplement. Results clearly indicate that transgenic expression of fungal and *E. coli* phytases improves P absorption in plants leading to increased plant production and P nutrition for monogastric animals and may prove as an alternative to commercial phytase.

Glycine max:

Introduction of *PhyA* gene using particle bombardment of cell suspension cultures resulted in high phytase activity in soybean i.e., approximately 920 pKa µg-1 in total soluble protein, which had similar physico-chemical properties like the native phytase. Moreover, their results revealed that ectopically expressed PhyA requires additional ability to sustain high temperatures (Li *et al.,* 1997). Native phytase expressed in soybean showed reduction of phytate (12.6-24.8%) levels in transgenic seed in comparison to WT seed and enhancement in phytase activity during seed development. In spite of high phytase activity the seeds germinated normally (Cheira *et al.,* 2004). In other study, transgenic soybean lines expressed *EcAppA* phytase in the cytoplasm of developing cotyledons exhibited little or no phytic acid in the seed. This kind of high-level phytase expression (1000u/g-1) may be ideal for in place of commercial phytase. Additionally, seed morphology and germination were unaffected (Bailey *et al.,* 2008). Transgenic soybean seeds expressing phytase gene from *Aspergillus awamori* developed by pollen-tube pathway transformation exhibited relatively higher thermo tolerance in comparison to WT seed (Gao *et al.,* 2007). Expression of *AtPAP1*

in soybean roots led to the substantial improvement of P use efficiency (Li *et al.,* 2009). Transgenic soybean lines showed increased plant dry weight when compared to WT in sand culture. Field experiments revealed enhanced pod and seed number per transgenic line in acidic soil. Another study found that over-expression of an Arabidopsis purple acid phosphatase gene or *APase* (*AtPAP15*) containing a carrot extracellular targeting peptide not only enhanced the secretion of *APase* from transgenic soybean plants, but also significantly improved intracellular *APase* activity in leaves, as well as P efficiency and yield on media of low organic P (Wang *et al.,* 2009). These strategies may lead to genetic improvement for phosphorus efficiency and yield improvement in soybean grown in low P-containing as well as acidic soils (Wang *et al.,* 2010). Genetically modified soybean that expressed phytase transgene *AnPhyA* was tested in broiler chicks in comparison with phytase-supplemented commercial feed (Denbow *et al.,* 1998) and performance revealed P retention and excretion. Results indicated that phytase from GM soybeans gave a similar positive effect as the one provided by commercial phytase supplement.

Nicotiana tabaccum:

Initial study of engineering *AnPhyA* in tobacco seed by Pen *et al.,* (1993) showed that phytase was expressed as 1% of the soluble protein in mature seeds. In vitro tests that simulated chicken crop and stomach conditions, released phosphate from feed by incorporating transgenic tobacco seeds. Supplementation of the broiler diets with recombinant seeds showed beneficial influence on P availability and improved growth rate in broiler chicks in comparison to diets comprising of commercially available phytase or inorganic phosphorus. On the same lines, Verwoerd et al. (1995) reported the extracellular constitutive expression of *AnPhyA*, using a signal sequence in tobacco transgenic plants. The specific phytase activity in extracellular fluid was reported to be 90-fold higher than in total leaf extract. High-levels of biologically active phytase in transgenic lines with stable phytase accumulation up to 14.4% of total soluble protein in maturing leaves (or 0.3% of dry weight) were observed. In addition, even in dried leaves, where most of the proteins were degraded, phytase was still present in high levels. Results clearly indicated that stable expression of phytase did not affect seed germination and transgenic seeds can be directly used as feed additive for livestock without processing (Verwoerd *et al.,* 1995). Another study showed that expression of *AnPhyA* in tobacco roots led to enhanced phytase activity in comparison to WT (George *et al.,* 2005). However, extracellular phytase activity in roots may not improve plant nutrition, as it requires phytate as P source. The results imply that recombinant plant exudates would use phytate successfully from phytate-rich soil. Expression of *Aspergillus ficuum* phytase in tobacco resulted in five-fold increase in phytase activity in comparison to WT. The recombinant purified enzyme retained all the characteristics of the native enzyme, except for a slight change in pH optima. Result proves that bio-farming of phytase could be a better alternative to the use of commercial phytase in a cost-effective manner (Ullah *et al.,* 1999). The introduction of *Bacillus subtilis* phytase (*BsPhyC*) led to change in the phenotype of transgenic tobacco lines like, small seed, increased flower number and enhanced levels of iP5, iP4 in transgenic seed. In P-deficient conditions, transgenic lines grew better with higher shoot and root dry mass than control (Yip *et al.,* 2003). Introduction of *A. fumigatus* phytase in tobacco exhibited high thermo stability and retained 28.7% of the initial activity even after incubation at 90°c for 15 minutes (Wang *et al.,* 2007). Findings from tobacco studies demonstrate that transgenic plants expressing phytase can mitigate seed phytic acid associated problems.

Solanum tuberosum:

First report of transgenic potato demonstrated stable phytase protein expression with retention of all characteristics of the benchmark enzyme, except size. Transformed potato leaves showed 24-fold enhancement in phytase activity in comparison to WT. Result denotes that recombinant potato expressing fungal phytase can replace the commercial enzyme and prove cost-effective (Ullah *et al.,* 2003). Another study on biochemical modification of root-soil interface showed the cell-specific expression of a synthetic gene encoding a secretory phytase driven by a trichoblast-specific promoter in root hair cells of potato plant for improved plant nutrition (Zimmermann *et al.,* 2003). Transgenic plants synthesized and secreted sufficient quantities of phytase to release phosphate

from phytate in media and accrued 40% additional P in leaves than WT plants. Trichoblast-targeted expression of desired enzymes holds promise for future applications in plant nutrition, phytoremediation and bio-farming.

Ipomoea batatas:

Phytase gene (*EcAppA*) expressed in sweet potato resulted in high levels of phytase expression (3.8 to 7.4% of total soluble proteins), which in turn switched on high expression levels of chlorophyll a and b, carotenoids, increased chloroplasts number and improved P utilization from phytate (Hong *et al.,* 2008). Subsequently, an increased number of tubers and higher yield was observed in comparison to WT plants. Recombinant phytase had broad pH range (from 4.5 to 1-6), but no effect on thermo-tolerance was observed. Animal feeding tests suggested that supplementation of feed with recombinant phytase from sweet potato was as effective as a commercially available microbial phytase in improving bio available phytate-P in weanling pigs. Moreover, sweet potato derived recombinant phytase may serve as alternative ideal feed additive for improving phytate-P digestibility in monogastric animals as well as improves tuber yield and plant growth by increasing P acquisition from organic fertilizers with a potential for phytoremediation.

Arabidopsis:

Fungal phytase genes (phyA) were expressed independently under the control of either the CaMV 35S promoter or the *A. thaliana* phosphate transporter promoter, with all constructs being modified for extracellular secretion by inclusion of an extracellular targeting sequence from the carrot extension (ex) gene (Richardson *et al.,* 2001; Mudge *et al.,* 2003; George *et al.,* 2005). Richardson *et al.,* (2001) showed that expression of *AnPhyA* in *Arabidopsis* roots enhanced growth and phosphorus nutrition of plants. Total enhancement of 20-fold in root phytase activity was noted in transgenic lines expressing *AnPhyA* gene with inclusion of signal peptide from carrot extensin gene and resulted in improved phosphorus nutrition. Data indicates that extracellular phytase activity of plant roots is a significant factor in the uptake of phosphorus from phytate. Ectopic expression of phytase gene from *Medicago truncatula* in *Arabidopsis* resulted in 4-fold increase in fresh and dry weights of the plant, and 5-fold increase in the total P content with no phenotypic change in comparison to WT (Xiao *et al.,* 2006). These studies suggest that phytase gene expression in transgenic plant roots can significantly improve P uptake from soil phytate sand enhances biomass in crops.

Oryza sativa:

In order to obtain transgenic rice plants with high phytase activity for animal nutrition, different yeast phytase genes constructs i.e., full length and truncated that included a secretary signal sequence from rice chitinase-3 were introduced in rice. Moreover, when transgenic rice plants were given ensilage treatment for 12 weeks, no decrease in heterologous phytase activity was reported. These results indicated that recombinant phytase could be used to deliver significant amounts of active and stable phytase (Hamada *et al.,* 2005; 2006). Expression of synthetic *AnPhyA* phytase gene in rice exhibited enhanced inorganic P content (57%) in comparison to WT plants (Liu *et al.,* 2006). Lucca *et al.,* (2001) showed highest *A. fumigatus* phytase activity (9415U/gm) in rice seeds i.e., 130-fold higher than WT. Heterologous phytase retained 90% of its activity after incubation at simulated stomach conditions. Transgenic plants were morphologically indistinguishable from control plants with no affects on plant growth and seed germination. Results demonstrate that phytase from *A. fumigatus* is one of the best alternative to the commercially available phytase enzyme. Recently, manipulating the PA biosynthetic pathway by using RNAi-mediated seed-specific silencing (using Oleosin 18 promoter) of IPK1 gene that catalyzes the last step of phytic acid biosynthesis was performed in rice. The oleosin 18 promoter driven silencing of IPK1 resulted in substantial reduction in seed phytate levels without hampering the development of transgenic rice plants and significant accumulation of iron in endosperm (Ali *et al.,* 2013). *E. coli* phytase gene introduced into rice showed results of safety and nutritional availability in experiments on rats (Cheng-Chih *et al.,* 2008). On the whole, these experiments demonstrate that regular phytase expression could be possible in transgenic plants without any special efforts replacing commercial phytases in future.

Triticum aestivum:

Constitutive expression of *AnPhyA* gene in wheat led to 4-fold higher phytase expression than control plants. To ensure secretion and glycosylation of the microbial phytase, an expression cassette comprising of α-amylase signal peptide sequence inserted between the promoter and phytase gene was designed. Findings indicate that functional phytase can be produced in substantial amount in wheat grains, which may be useful for efficient and improved phytate-phosphorus digestibility when wheat grains are used as feed for non-ruminants (Brinch-Pedersen *et al.,* 2000). HPLC analysis of inositol phosphate degradation in flour from wheat samples possessing endogenous (wheat) and exogenous fungal (*AnphyA*) phytases exhibited breakdown of PA in to intermediary forms (Brinch-Pedersen *et al.,* 2003). In another study, rationally designed *Aspergillus fumigatus* phytase was expressed in wheat seeds exhibited high thermo stability (89.3ºC) than native fungal phytase. This provides evidence for developing highly thermo tolerant phytases through rational engineering of the phytase sequences that shall be useful during animal feed processing. Genetically modified wheat lines that expressed fungal phytase transgene were tested in broiler chicks in comparison with phytase-supplemented commercial feed. On the basis of performance, P retention and excretion, the authors indicated that phytase from GM wheat showed similar effects as accrued due to use of commercial phytase supplement (Brinch-Pedersen *et al.,* 2006).

Zea mays:

In order to ameliorate PA levels for increased inorganic P, *Aspergillus niger* (*AnPhyA2*) phytase gene was successfully expressed in maize seeds with no effect on their germination percentage (Chen *et al.,* 2008). Phytase activity in recombinant maize seed rose up to approximately 50-fold higher as compared to controls seeds. The results clearly indicated that recombinant maize lines could be used to improve the phosphorus availability and alleviate the negative effect of phytate run-off on environment. Similarly, expression of *AnPhyA* directed by rice glutenin-1 promoter in maize endosperm, either alone or combination with iron binding protein ferritin exhibited reduction in endogenous PA by 95% and enhancement of P availability in transgenic maize seeds (Drakakaki *et al.,* 2005). Studies showed a significant increase in iron uptake levels in human intestine cells caco-2 cells. These results clearly show that decreased PA and enhanced ferritin levels lead to improved iron uptake. GM corn expressing the *Escherichia coli*-derived phytase gene when studied with broiler chicks showed that an increasing dietary level of transgenic maize concomitantly increased dry matter P, calcium (Ca) and nitrogen (N) retention (Nyannor and Adeola 2008). Results suggest that GM corn is as efficacious as the commercial microbial phytase in P- and Ca-deficient broiler diets and would thus minimize the need for supplemental dietary P (Nyannor and Adeola 2009). Additional studies using GM corn expressing *AnPhyA* as feed showed improved digestive tract physiology, elevated phytase activity, greater P retention, greater digestibility of energy and P as well as decreased PA content in growing pigs (Li *et al.,* 2013). We can infer from these findings that recombinant corn expressing fungal phytases if used as supplements may prove to be an effective replacement for commercial phytases in diets of animals or even humans for better nutrition and environment.

Solanum lycopersicon:

Fruit-targeted phytase expression in tomatoes using *BsphyC* gene resulted in 6-fold increase in phytase activity (1067 pmol/mg/min) as compared to WT (Reddy *et al.,* 2013). In addition, enhanced levels of lycopene and β-carotene were observed in transgenic tomato fruits with no effects on seed germination and transgenic plant performance. This strategy shall enable indirect degradation of dietary phytate for animals and specially humans.

Other crops with heterologous expression of microbial and plant phytases

Several other crops have been manipulated for heterologous phytase expression that has resulted in enhanced phytase activity and enabled them to survive in presence of organic phosphorus. For instance, plant phytase (*MtPHY*) and purple acid phosphatase (*MtPAP*) genes from *Medicago truncatula* were individually introduced in to *Trifolium repens*, which resulted in 3-fold enhancement of phytase activity in root apoplast in comparison to WT. Although both types of transgenic *Trifolium* plants showed improved P utilization from culture medium in comparison to controls, but

MtPHY transgenic plants showed better growth performance than *MtPAP* ones (Ma *et al.,* 2009). MtPHY transgenic in the presence of organic phosphorus exhibited increased shoot P and biomass, without any alteration in their phenotype. This is a clear demonstration of the advantage conferred on transgenic plants that express either native or heterologous phytase. The *MtPHY* transgene expressing transgenic can survive in existing organic phosphorus conditions, whereas WT plants could not survive (Ma *et al.,* 2009). Similar results were observed in case of alfa alfa transgenic expressing both the native *MtPHY* and *MtPAP* genes, independently (Ma *et al.,* 2012). In another study, Liu *et al.,* (2011) expressed fungal phytase in cotton root that enhanced the extracellular phytase activity by 3-fold during T2 and T3 generations in comparison to WT. P utilization from phytate was significantly improved due to extracellular secretion of recombinant high amounts of phytase in roots. Results indicate that transgenic plants expressing phytase in their roots can survive in the soil with low inorganic P or P content not accessible for direct absorption by plants.

In another study, six microbial phytase genes were expressed in microalgae *Chlamydomonas reinhardtii* by Yoon *et al.,* (2011) and they concluded that codon optimization and N-terminal signal peptide plays a crucial role in phytase expression in host system. They aimed to deliver the phytase orally to the animals, which may decrease the P faecal excretion as well as phosphorus pollution in a significant manner. They conducted the experiment of chick-feed trail by supplying commercial *E. coli* phytase, *Chamydomonas* sp. WT and transgenic and the results showed 25.4%, 25.5% and 43% reduction in the overall phytate excretion (41% increased iP content). The results clearly demonstrated that recombinant microbial phytase produced in microalgae may serve as a better alternative to the commercial feed additives for monogastric animals. The recombinant fungal phytase expressed in sesame hairy root on purification and characterization showed enzyme properties similar to native phytase, which can be used as an alternative to the commercial phytase (Jin *et al.,* 2004).

Global phytase impression, current challenges and future prospects

Present research in phytase and its application is directed towards alleviation of its drastic environmental impact due to manure phosphorus excretion by monogastric animals i.e., livestock. Ecological studies on open extensive level and restrained experiments on animals have shown favourable outcomes. Worldwide, phytase supplementation in animal diets have considerably reduced total P loads and associated water quality problems (Sharpley *et al.,* 2009; Patoine *et al.,* 2012). Few aspects exist that shall lead to extensive use of transgenic plants expressing phytases as a tool for resolving future environmental issues. Firstly, the P load on environment in regions with intensive animal agriculture is so high that enormous amount of commercial phytase application would be required, which is cost-intensive (approximately $350 million per annum). Secondly, there is a limitation of the usual sources of feed-phosphorus supplements, meat and bone meals as well as inorganic phosphorus due to growing demands throughout the Southeast Asia especially India and China. In addition, the present rate of use of inorganic phosphate for feed application shall hasten the rate of predicted depletion of P, a non-renewable resource, in the future. Finally, industrial applications of various WT plants (like biofuels) or transgenic plants expressing phytases shall render huge amounts of phytate-rich biomass to be used as future feed resource. Globally 65% of the elemental phosphorus marketed as fertilizers is accounted from worldwide yield of plant phytate that tantamount to an estimated 51 million metric tons per annum. Thus, an accelerated demand for transgenic plants expressing phytases in the near future shall exert a wholesome socio-economic impact rather than for just improving animal nutrition.

Innovative roles and applications of phytases:

Amongst the well-known roles of phytases is release of P from phytate and in turn enhancing the bio available levels of calcium, iron, zinc and improving protein digestibility. Recently, the role of phytase has been studied with respect to biochemical, molecular and metabolic affects in vivo. For instance on decreased expression of sodium-dependent glucose transporter 1 in pig small

intestines due to PA was regained by dietary phytase (Woyengo *et al.,* 2011). Bacterial or *E. coli* phytases have been utilized to release P from PA that binds to the uranium forming a uranium-phosphate precipitate on the bacterial cells that can be harvested to recover the uranium. Phytase supplementations ameliorate endogenous impairment of carbohydrase activity, liver insulin-receptor responsiveness and digestive competence (Liu *et al.,* 2008; Jozefiak *et al.,* 2010). Phytases play crucial role in food processing (Meyer 2010), biofuel or alcohol production (Fujita *et al.,* 2001; Hubenova and Mitov 2010), improvised bread making (Haros *et al.,* 2001), production of dephytinized soy-milk (Ushasree *et al.,* 2012) and in paper and pulp industries as strong additives (Nampoothiri *et al.,* 2004).

Inclusion of phytases in human diet is an important application as PA chelates most of the essential micronutrients leading to micronutrient malnutrition or hidden hunger lingering in one third of the globe. Efficiency of phytase or phytase producing bacteria has been demonstrated in human efficacy or interventional trials (Troesch *et al.,* 2011), de-phytinization of infant cereals (Hurell 2004) and improvement in iron bioavailability in bread (Porres *et al.,* 2001). Somehow, inadequate consumer awareness of recombinant phytase and paucity of promptly available native phytases has restrained liberal implementation of phytase in human nutrition. Moreover, the controversy over direct intake of phytase or consuming low-phytate crops as well as potential health risk of losing the antioxidant functions of phytate and other intermediary forms of inositol phosphates in diet and gut (Kumar *et al.,* 2010) is another drawback. Introduction of phytase in diets for human health and medicine poses a new arena for research. Phytase supplementation in diets of broilers (Karimi *et al.,* 2011) and pigs that are best models for clinical trials that can be extended to humans have shown enhancement in bone development (Pagano *et al.,* 2007). A potential role of zinc and/or phytase supplementation increased the degree and span of botulinum toxin effect as therapy for cosmetic facial rhytids, benign blepharospasm and hemifacial spasm in humans (Koshy *et al.,* 2012).

"Generation-next" phytases

The first generation of fungal phytases and second generation of bacterial phytases (*AnPhyA, EcAppA1* and *EcAppA2)* have proved beneficial for their nutritional and environmental issues and compatibility with different species. However, any individual phytase may not perform at its maximum efficiency in all species or animal types. Poultry and aquaculture industry demands a heat stable phytase as *Bacillus sp.*, but it has lower activity than *AnPhyA* fungal phytase. For expression in plant roots, phytase with a lower pI value and broad substrate-specificity is optimal. On the other hand, for expression in green tissues of plants, phytase with strict substrate-specificity is required. Similarly, due to difference in body temperatures of terrestrial and aquatic animals, the phytase applied would differ in property. This is due to the fact that each phytase has unique properties i.e., temperature maxima, pH, thermo stability, metal requirement that may not be compatible with different dietary needs and stomach conditions of all species. Therefore, any one phytase may not possess all the desired characteristics to fulfil the tag of an "ideal phytase". Need of the hour is to develop custom-made polymorphic or chimeric phytases with desired features for commercial use either directly or by expressing in crops. Additionally, presently available or new phytases should be tried for co-adjuvancy with other feed enzymes to effectuate the aspired global responses.

Conclusions

Modern genetic engineering and molecular biology may be utilized for the development of foods and feeds with enhanced iron, phosphorus and zinc content with improved bioavailability of the minerals and proteins. The prime anti-nutrient that is the major hurdle is the phytic acid content in food grains and seeds that may be ameliorated via use of phytase for improved mineral absorption from food based diets. Phytases have been proved to play a crucial role in nutrition for monogastric animals including humans. In addition, to maintain sustainability of agriculture, it is imperative to reduce the dependency of crops on inorganic phosphorus fertilizers. One of the approaches is to improve the ability of crop plants to acquire P from organic reserves in present abundantly in soil. Expressing microbial phytases such as fungal and bacterial in crop plants hold significant potential in achieving

this goal. Transgenic plants with low phytic acid or expressing recombinant phytase could be a novel approach for lowering the rate of malnutrition with concomitant reduction in phosphorus content in animal waste. Moreover, direct manipulation of rhizosphere biochemistry through genetic engineering of microbial phytases in roots of transgenic plants had negligible impact on the microbial community structure in comparison to WT (*George et al., 2009*). The utilization of phytases in enhancing mineral bioavailability in diets of simple stomached animals (that includes humans) and in improving the uptake of inorganic P from acidic soils and soil fertilization for enhanced productivity renders these enzymes indispensable for nutritional health, sustained agriculture and environmental issues. Transgenic plants expressing adequate phytase shall provide higher levels of orthophosphates leading to enhanced bioavailability of minerals and aid in replacing the use of exorbitant commercial microbial phytases for supplementation of feed and food, thereby reducing the downstream processing and formulation costs involved in its production. Alternatively, transgenic plants could be used as bioreactors to enhance the phosphorus bioavailability for monogastric animals, eventually diluting eutrophication. Incidentally, intensive research is needed to study the mechanism of accumulation of phytic acid during seed development and efficient execution of this approach at the community level (Singh and Satyanarayana 2011). Globally scientists working on different aspects of phytases should coordinate for the biotechnological development of an ideal phytase for improving animal nutrition, human health, and environmental protection.

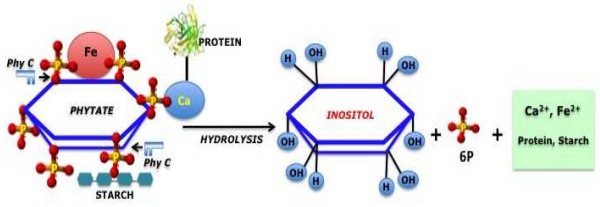

Figure.1: Structure of phytic acid showing its affinity towards diverse classes of molecules. Phytase (*PhyC*) hydrolyzes phytate into inositol phosphates (P) and releases cations or micronutrients, proteins and starch rendering them bioavailable for absorption in gut of animals and humans.

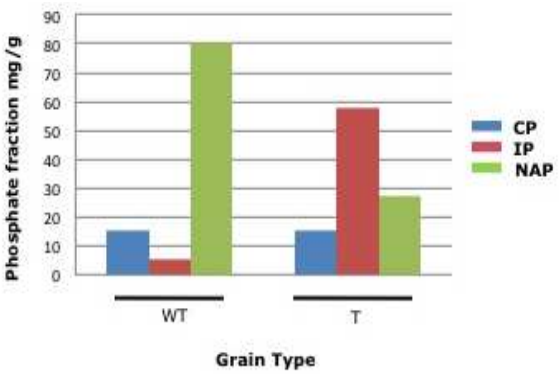

Figure.2: Comparative analysis of the seed phosphate levels in wild type (WT) and low phytate or phytase expressing transgenic (T) lines. The bioavailable P content (CP+ IP) in low phytate or transgenic (T) grains is higher than in WT grains. [CP: Cellular Phosphorus; IP: inorganic Phosphorus and NAP: Non Available Phosphorus].

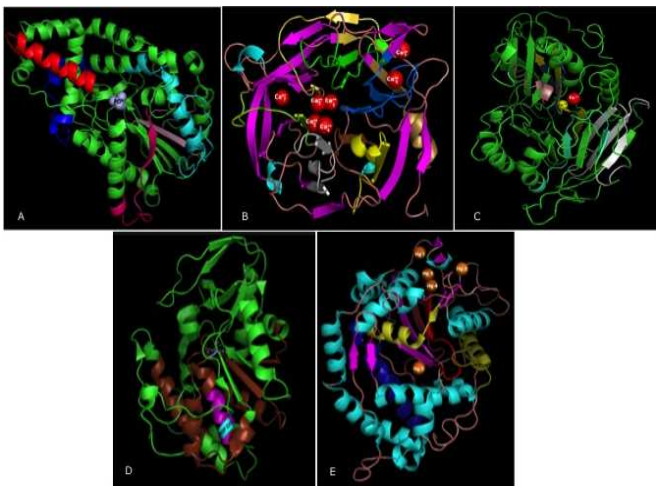

Figure.3: Three-dimensional protein structures of important classes of phytases revealing their importantly conserved motifs and residues (shown in different colors) essential for phytase activity using the PYMOL software (http://www.pymol.org/):
(**A**) HAP from *A. fumigatus* (Swiss PDB Sequence ID: *1QWO*): (i)*IPEGCELEHVHILSRHGVRYP* (66-86), Red; (ii)*SPFCDLFTQEEWHSYEYYQDLQWYYCYGP GNPLMAAQGVGYVNELLSRLT* (277-326), Cyan; (iii) (*VRASSQQRVRKSAQWFLKGFF* (107-127), Hot pink; (iv) *PGMNLTAMDVSHMMDMCPYET* (246-266), Blue; (v) *YVHSWIVPFAARMYIEKMSC* (395-414), Light blue;
(**B**) PAP from *Phaseolus vulgaris* (Swiss PDB Sequence ID: *1KBP_D*): (i) *YKSGIIHHCRVDGLEYGTKYYYKC*, violet; (ii) *RWDYWGRFMERVTAYQPWMWNEGNHEIEQ*, Light purple; (iii) *WLIVGWHAP WYNSNKAHYMEGECMRVAMEKWFYKYKIDIVFTGHVHAYER*, Purple blue; (iv) *CWDRQPDYSAFREASFGHGILQVKNKWHRNDDGKH,* Grey; (**C**) PTP from *Selenomonas ruminantium* (Swiss PDB sequence ID: *1U25*): (i) P-loop (251-258), Purple; (ii) WPD loop, wheat; (iii) *KNGMHYVRIPATDHKWPSYQMIDDFVNF* (211-238), Orange, Phytate binding loop; (iv) (MPKHAWLHFHCQAGQGRTTTFMIMYDIMK (242-270), Chocolate, Phosphorus binding loop; (**D**) BPP from *Bacillus*

sp. **(**DS11) (Swiss PDB sequence ID: *2POD*): (i) *GDDADDPAIWVHPTDPEKSLIIGTDKK* (51-77), Green, includes Ca^{2+} binding sites); (ii) *VYDLDGKQVQYLPVGRMNNVD* (82-102), Yellow orange; (iii) *IPTDMNEPYGMCLY* (151- 164), Limon; (iv)*SQIEGCVVDEETGQLYIGEEDVG* (208-230), Light grey; (v) *LVADVEGLTIY* (255-265), Brown; (vi) *NGQGYLIVSSQGNNSYAVYRR* (269-289), Yellow; (vii) *IDGVSETDGIEVTNVPLGEHFPHGLFVVQDG* (307-337), Marine; (viii) *QNFKYVDWRDIAKAF* (348-362), Sand; (**E**) AppA (Subclass of HAPs) from *E. coli* (Swiss PDB sequence ID: *1DKN*): (i) *QPMDQVAWGKITSEQQWSQLLSLHNAQYDL MNKMPYIAQH* (210-250), Density; (ii) *MQQVTPRKWPKWPVPYGWLTPRG* (40-52), Deep Olive; (iii) *QCDNIPPGGKLVFERWQ* (325-329), Ruby; (iv) *IPEGCELEHVHILSRHGVRYP* (10-20), Red; (v) *KWTFLVGHDTNIAYIRTMLGFKWQL* (300-310), TV yellow.

Table.1: List of various microbial phytases used for transgenic expression in crops showing maximum phytase expression in optimum assay conditions.

Source	Expression Vector	Promoter	Plant system	Signal Peptide	pH; Temp.	Specific Activity	Reference
A. ficuum	pPC-KSA	Arabidopsis Pky10	Soybean root	Carrot extensin	____	10.2 U mg^{-1}	Li et al., 2009
A. niger	pMOG413	CaMV35s P	Tobacco leaf	TobaccoPR-s	2 & 5.5; 58 ºC	144 µg mg^{-1}	Verwoerd *et al.,* 1995
A. ficuum	pTZ117	CaMV 35S P	Tobacco leaf	Soybean VSPb	2 & 4; 58ºC	203 nKAT mg^{-1}	Ullah *et al.,*1999
A.niger	pUBARN & pUPhyN	Maize ubiquitin-1	Wheat grain	α-amylase	____	1471 FTU kg^{-1}	Brinch-Pedersen et al.,2000
A.ficuum	pTZ117	CaMV 35S P	Alfalfa leaf	Soybean VSPb	5; 58 ºC	3761 nKAT/mg	Ullah et al.,2002)
A. niger	pPLEX502	AtPt	Arabidopsis root	Carrot extension	____	____	Mudge *et al.,* 2003
A. niger	pMOG413	CaMV 35Ps	Sesamame root	Tobacco PR-s	4/5; 50-60 °C	0.51 U ml^{-1}	Jin et al.,2004
Glycine max	pPHY35P	Dual ECaMV 35s P	Soyabean	Soybean patatin	5; 63 °C	920 pKat mg^{-1}	Cheira *et al.,* 2004
A. niger	pLPL	Rice glutelin-1	Maize endosperm	Murine Ig	5	200–3000 FTU kg^{-1}	Drakakaki 2005
M.trunculata	pCAMBIA3301	Root specificMtPT1	Arabidopsis root	Native	____	22-36 fold> WT	Xiao *et al.*,2005
S. cerevisiae	pOriF, pModF, pOriT	Rice chlorophyll *a*/*b*binding(cab)	Rice leaf	Rice chitinase-3	5.5; 70 °C	10.6 U g	Hamada *et al.,*2005
A. niger	pAER02	CaMV35s	Tobacco	Carrot extensin	____	3.7 fold > WT	George *et al.,* 2005
A. niger	pYU159	Maize ubiquitin	Rice seed	Tobacco *PRs*	____	57% >WT	Liu et al.,2006
Aspergillus	pYP46	D35s omega	Canola seed	Tobacco PR-S	____	41 FTU g^{-1}	Peng *et al.,* 2006
A.fumigatus	p1DX5SPConPhyN	Wheat HMW 1DX5 GS	Wheat endosperm	Barley α-amylase	87 °C	4777 FTU kg^{-1}	Brinch-Pedersen et al 2006
A.awamori	pBI121-phy	35s promoter	Soybean	____	5.5; 37 °C	150 U mg^{-1}	Gao *et al.,* 2007
A. niger	pSPHP3303T-Phy	Maize globulin-1 P	Maize embryo	Barley aamylase	5.5; 37 °C	2,200 U kg^{-1}	Chen *et al.,* 2008
E. coli	pZY101	Soybean lectin	Soybean seed	S.P removed	____	53µmol/min/mg	Bilyue *et al.,* 2008
E. coli AppA	pCAMBIA2301	Sporamin promoter	Potato tubers	SPO signal peptide	2.5-5.5, 37 °C	36,000–58,000 U kg^{-1}	Hong et al., 2008
A.thaliana	pBa002a	AtPAP15 promoter	Tobacco and *Arabidopsis*	____	4.5, 23° / 37°C	10 U mg^{-1}	Kuang *et al.,* 2009
Peniophora lycii & *A.niger*	pPLEX502	CaMV 35S &AtPt)	Tobacco	Carrot extensin	____	____	George et al., 2009
Arabidopsis	pTF101.1	CaMV35S, MtPT1	Soybean	Carrot extensin	____	____	Wang etal 2009
M.truncualata	____	Root-specific (pyk10)	Clover (*Trifolium repens* L.)	____	____	3 fold > WT	Ma *et al.,* 2009

A.ficuum	pC-KSA2300	CaMV 35S P	Cotton root	Carrot extensin	____	3.2-fold >WT	Liu *et al.,* 2011
A.ficuum	pTZ117	CaMV35s promoter	Potato leaves	Soybean VSPb	5, 58 °C	3000 nkat mg^{-1}	Ullah *et al.,* 2003
A.ficuum	pBI121	CaMV35s	Tobacco	____	2 & 5.5, 50 °C	109 U mg^{-1}	Wang *et al.,* 2005
A. niger	pM0G413	CaMV35s promoter	Tobacco seed	____	____	15 FTU g^{-1}	Pen *et al.,*1993
A. niger	pPhy35s	CaMV35s, Soybean seed specific	Soybean seed	Patatin peptide	2; 55 °C	920 pKat μg^{-1}	Li *et al.,*1997
E.coli AppA	pATPA	____	*Chlamydomonas*	____	5; 60 °C	10 U/g	Yoon *et al.,* 2011
A.niger	pBI binary vector	Arabidopsis rubisco small sub-unit	Tobacco and Alfalfa leaf	Apoplast targeted	2.5; 55 °C	1-2% of TP	Koegel *et al.,* 1996
M.trucualata	____	CaMV35s, MtPT1	*Arabidopsis* root	____	____	16.2 fold > WT	Xiao *et al.,* 2006
B.subtilus	pCAMBIA1300	CaMV35s	Tobacco	____	____	____	Yip *et al.,* 2003
B.subtilus	pMDC100	E8 promoter	Tomato	Native signal peptide	5-8	6 fold >WT	Unpublished
A.niger & *E.coli*	pBI121	CaMV35s promoter	*Brassica napus*	Carrot extensin	2-8	1605 U/kg^{-1}	Wang *et al.,* 2013

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Source of support: Nil
Conflict of interest: None Declared