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Engineering crop plants: getting a handle on phosphate

Henrik Brinch-Pedersen, Lisbeth Dahl Sørensen and Preben Bach Holm

In plant seeds, most of the phosphate is in the form of phytic acid. Phytic acid is largely indigestible by monogastric animals and is the single most important factor hindering the uptake of a range of minerals. Engineering crop plants to produce a heterologous phytase improves phosphate bioavailability and reduces phytic acid excretion. This reduces the phosphate load on agricultural ecosystems and thereby alleviates eutrophication of the aquatic environment. Improved phosphate availability also reduces the need to add inorganic phosphate, a non-renewable resource. Iron and zinc uptake might be improved, which is significant for human nutrition in developing countries.

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Phosphate is an essential macronutrient for all living organisms. In natural ecosystems, phosphorus is returned to the soil and converted to inorganic phosphate (P_i) via biological and chemical processes, after which it is available for a new cycle of plant growth. In agricultural ecosystems, two additional factors are introduced: a tapping of the phosphate cycle by the harvest of plant materials for animal and human consumption, and the reintroduction of phosphate in the form of manure or industrially produced fertilizers (Fig. 1).

Because of the importance of P_i as a macronutrient for plant growth and livestock production, and the ability of phosphate compounds to bind important minerals, there is increasing interest in understanding the factors that underlie phosphate uptake and bioavailability in plants used for animal feed and human consumption. Moreover, there is a strong demand for optimization of feeding regimes because of the effect of excess P_i on the aquatic environment (eutrophication). A central component in these contexts is the enzyme phytase, which can release bioavailable phosphate from one of the most important compounds in the phosphate cycle, phytic acid [phytate, or *myo*-inositol-(1,2,3,4,5,6)-hexa*kis*phosphate] (Fig. 2).

Phytic acid is largely indigestible by monogastric animals because they have no or limited phytase activity in their digestive tract. Moreover, it is considered to be an important antinutritional factor, preventing the uptake of a range of important minerals. Phytic acid and phytases are

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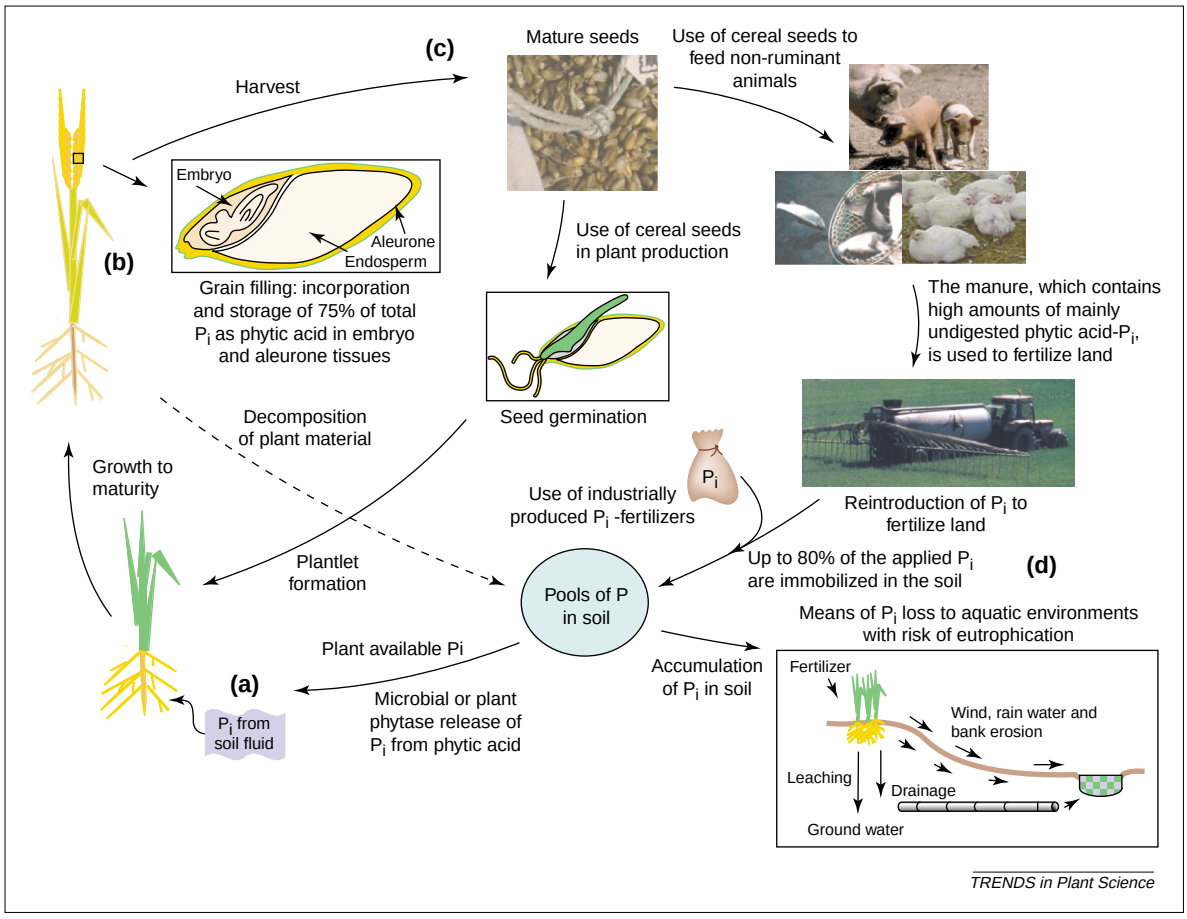

Fig. 1. Phytic acid phosphate cycles in natural and agricultural ecosystems. (a) Inorganic phosphate (P_i) is absorbed by plant roots from the soil fluid and translocated by the xylem and the phloem to all parts of the plant. Only a limited amount of the soil P_i is available to the plant because the mobility of P_i is low and it has high affinity for organic and inorganic compounds, and for soil particles. (b) Phytic acid is the major phosphate storage compound in seeds. In natural ecosystems, seeds germinate or decompose like vegetative parts of the plant do, returning P_i back to the soil by chemical, microbial and plant enzymatic actions on organic phosphorus compounds. (c) In agricultural systems, seeds are used either for plant production or as feed for livestock production. In particular, feeding of non-ruminant animals causes the excretion of large amounts of undigested phytic acid because the digestive system of these animals lacks phytases. (d) Up to 80% of phosphorus supplied via fertilizers becomes fixed in the soil. Consequently, to ensure crop productivity, a surplus of fertilizer P_i is often applied. Many years of intense applications with industrially produced P_i and manure phosphorus has resulted in strong phosphorus accumulation in the soil. Increased soil phosphorus content increases the P_i loss to the aquatic environment.

also of major economic importance. The yearly global production of phytic acid is estimated to be >51 million metric tons, almost 65% of the elemental phosphorus sold worldwide for use in mineral fertilizers [1], and the production of phytase as an additive to animal feed is rapidly expanding. Here, we discuss the roles of phytase and phytic acid in the phosphate cycle, review available knowledge on phytases, and focus on the potential of plant biotechnology for improving phosphate and mineral bioavailability in feed and food, and for reducing the environmental phosphate load.

Phytic acid cycle

Deposition and biosynthesis

Phytic acid is the primary phosphate storage compound in seeds, and typically ~70% of the phosphate reserves is sequestered in this compound [2]. In small-grained cereals, ~90% of the seed phytic acid is in the aleurone, and the remaining 10% in the scutellum; by contrast, in maize, 90% is found in scutellum and 10% in aleurone. In dicots, phytic acid is deposited in the endosperm and cotyledons [3]. Almost all phytic acid is present as phytin, a mixed salt (usually with K^+, Ca^{2+}, Mg^{2+} or Zn^{2+}) that is deposited as globoid crystals in single-membrane vesicles together with protein [2]. Phytic acid deposition is restricted to cells that remain alive through the quiescent phase of seed development, but it is also found in vegetative tissues and in pollen [4].

Phytic acid is synthesized from *myo*-inositol via a series of phosphorylation steps (Box 1, Fig. 2). There is only limited information available about the intracellular location of the intermediates in phytic acid biosynthesis. In developing castor bean endosperm, phytin particles could be detected in single-membrane vesicles in the cytosol or associated with the endoplasmic reticulum (ER) and protein storage vesicles. It was hypothesized that phytic acid biosynthesis takes place in the cytoplasm or in the ER, after which the phytin particles are transported in vesicles to the protein bodies [5]. In the slime mould

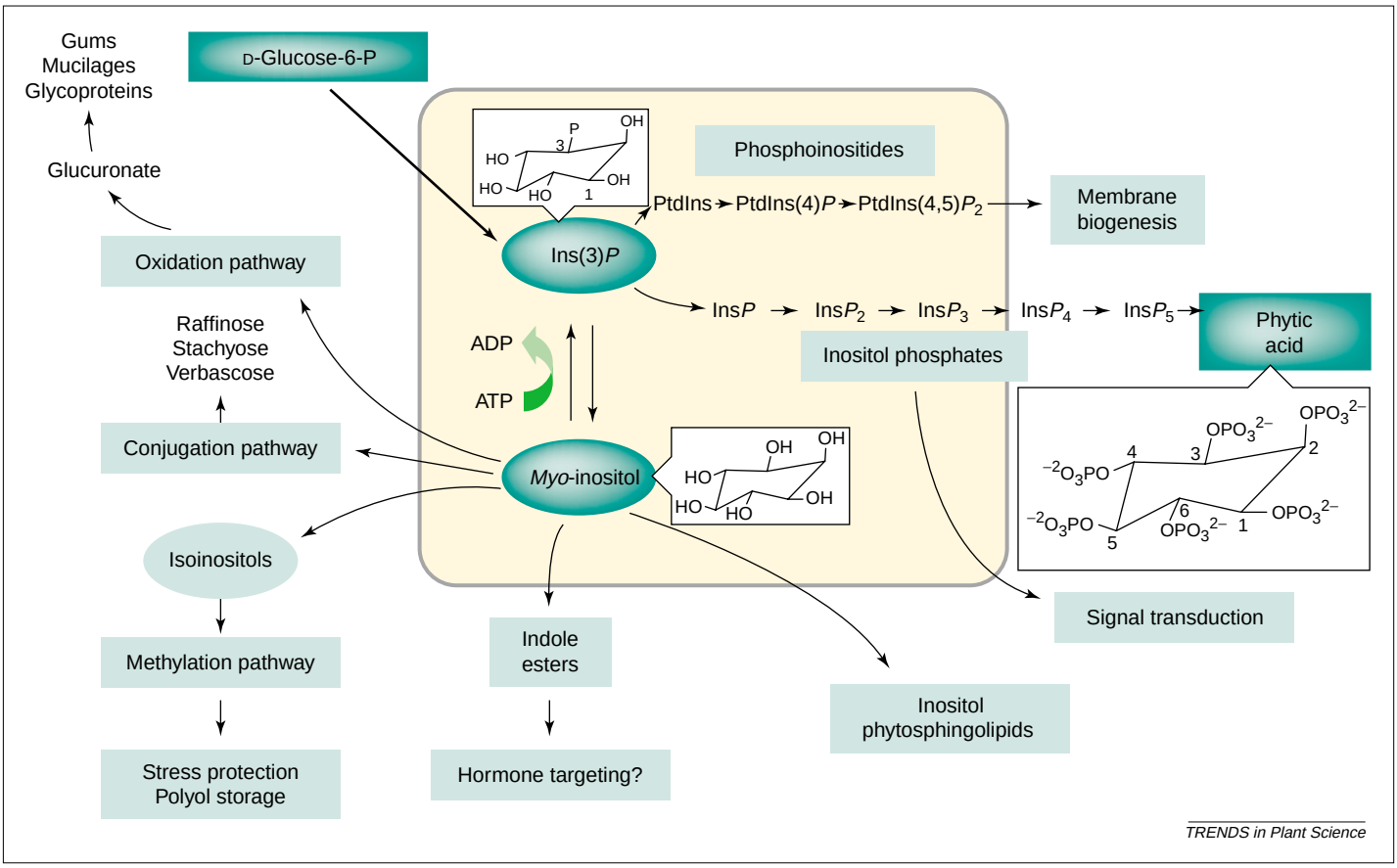

Fig. 2. Apart from being phosphorylated to phytic acid, *myo*-inositol plays a central role in several metabolic processes as well as signal transduction in the plant cell. Figure modified from Ref. [53]. Abbreviations: D-glucose-6-P, D-glucose-6-phosphate; Ins, inositol; PtdIns, phosphatidylinositol.

Dictyostelium, analyses of lysates indicated a cytosolic route for the biosynthesis of phytic acid [6]. Other studies have shown that the stepwise phosphorylation of the secondary messenger *myo*-inositol-1,4,5-trisphosphate to phytic acid can occur in the nucleus [7].

Degradation

Phytic acid is degraded during seed germination by a specific group of enzymes – the phytases (Box 2). In plants, the characterization of phytases at the molecular level is in its infancy and only a few plant phytases have been purified and extensively characterized (Table 1). Most plant phytases belong to either an acidic group with a pH optimum around pH 5.5 or to an alkaline group with a pH optimum around pH 8.0. Acidic phytases have a broad affinity for various phosphorylated substrates. Wheat (*Triticum aestivum*) bran phytase and barley (*Hordeum vulgare*) seedling phytase catalyse the hydrolysis of a large range of *myo*-inositol-phosphate isomers from phytic acid to *myo*-inositol monophosphate [8–10]. Mung bean (*Vigna radiata*) phytase exhibits the enzymatic properties of the acidic group in spite of having a pH optimum of 7.5. Unlike the acidic phytases, alkaline phytases identified from pollen of *Typha latifolia* and *Lilium longiflorum* show high specificity for phytic acid and cannot degrade *myo*-inositol phosphates containing three or fewer phosphate groups [11,12].

Several cereal phytases have been purified and characterized (Table 1). Except for the wheat bran F2 phytase, they all belong to the acidic group. Phytase from wheat, spelt (*Triticum spelta*), barley, rye (*Secale cereale*) and oat (*Avena sativa*) are all monomeric enzymes, whereas maize (*Zea mays*) seedling and root phytases consist of two subunits, each with a molecular mass of 38 kDa. One phytase-encoding cDNA (*phy S11*) and two genomic clones (*PHYT1* and *PHYT2*) have been reported for maize [13,14]. *PHYT1* and *phy S11* show 99.2% identity at the nucleotide level and probably originate from the same gene. Recently, a third phytase gene (*GmPhy*) has been isolated from soybean (*Glycine max*) [15]. It shows no homology to maize or microbial phytases (Box 2).

Dry seeds have a phytase potential that is realized at the onset of germination. Phytase potential is widely different between species; for example, rye, triticale, wheat, barley, oat, maize, rapeseed meal and soybean meal have activities of 5130 U kg^{-1}, 1688 U kg^{-1}, 1193 U kg^{-1}, 582 U kg^{-1}, 41 U kg^{-1}, 15 U kg^{-1}, 16 U kg^{-1} and 40 U kg^{-1}, respectively [1 U is the amount of enzyme that liberates 1 µmol orthophosphate (min^{-1}) from phytin] [16]. Phytase activity increases greatly during germination. The initial activity during the first hours of germination is assumed to be due to the activation of preformed enzyme. The presence of

Box 1. Phytic acid biosynthesis

Phytic acid is generated by a stepwise phosphorylation of *myo*-inositol. The sole *de novo* route to *myo*-inositol is the conversion of D-glucose-6-phosphate to D-*myo*-inositol-3-phosphate by D-*myo*-inositol-3-phosphate synthase (MIPS) (EC 5.5.1.4). Conversion of D-*myo*-inositol-3-phosphate to free *myo*-inositol is catalysed by *myo*-inositol monophosphatase (EC 3.1.3.25) [a]. As well as being phosphorylated to phytic acid, the *myo*-inositol moiety is the precursor for many other compounds (see Fig. 2 in main text).

There seems to be a close relationship between the biosynthesis of phytic acid and the formation of D-*myo*-inositol-3-phosphate by MIPS. Northern blot analysis and *in situ* hybridization of a rice cDNA (*RINO1*) with high homology to the MIPS of yeast and other plants showed that high levels of transcripts accumulated in embryos but were undetectable in shoots, roots and flowers. Strong signals were detected in the scutellum and aleurone at day four, increased until seven days after anthesis, and gradually decreased thereafter. Phytin-containing globoids appeared four days after anthesis in the scutellum and aleurone, coinciding with the presence of *RINO1* transcripts [b]. Also, in soybean, transcripts encoding MIPS (*Gm*MIPS1) were mainly located in the developing seeds and only early during seed development [c].

The temporal and spatial patterns of accumulation of *RINO1* transcripts and globoid formation in rice, and expression data for *Gm*MIPS1 suggest that the conversion of D-glucose-6-phosphate to D-*myo*-inositol-3-phosphate constrains phytic acid biosynthesis. However, both soybean and rice phytic acid levels have been reported to increase steadily until late in seed maturation, suggesting that phytic acid biosynthesis in rice and soybean is not exclusively regulated via RINO1 and *Gm*MIPS1 [d,e]. Multiple MIPS genes probably supplement each other to influence the formation of D-*myo*-inositol-3-phosphate. In *Arabidopsis*, two individual genes encoding MIPS have been identified [f,g]. Four differently expressed forms of MIPS have been suggested in soybean [c]. Previous studies in soybean showed that a 33 kDa polypeptide with MIPS activity was active during the globular stage of embryogenesis, in mature roots and in thylakoid membranes of fractionated leaf protoplasts. A second 56 kDa MIPS was active in green cotyledons and in young roots [h]. In maize, seven MIPS-homologous sequences have been identified. A loss-of-function mutation in the low phytic acid 1 locus (*lpa-1*) that causes a 50–95% reduction of phytin in seeds has been mapped to the position of one particular MIPS gene on chromosome 1S [i].

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long-lived mRNA synthesized during grain filling cannot be ruled out but phytase transcripts could not be detected in dry maize seeds. Phytase mRNA accumulates during the first day of germination, reaches a maximum after two days and decreases in young seedlings [13]. Similarly, the concentration of immunodetectable phytase increases during germination, indicating *de novo* synthesis of the enzyme [17]. In barley, one phytase (P2) appears to be present at the onset of germination, whereas the other (P1) is activated or synthesized during germination, resulting in a 35-fold increase in phytase activity during the first four days of germination [8]. Like barley, pea has one form of phytase in ungerminated seeds and two additional forms in the germinating seed [18].

The subcellular location of seed phytase is not known. However, in lily pollen, cytochemical studies indicated that the pH 8 phytase is an integral part of the membrane of the phytic acid storage body, whereas the pH 5 phytase is more loosely attached [19]. Interestingly, the C-terminal region of the maize Phy S11 sequence is hydrophobic and might constitute a membrane anchor [13].

Transfer to soil

In natural ecosystems, phytin is transferred to soil via vegetative plant tissues and seeds. Many microorganisms secrete phytases that allegedly mobilize phosphate from organic phosphate compounds. The question of whether plant roots secrete phytase has attracted much attention. It has been shown by cytochemical techniques that phytases in maize roots grown under phosphate-sufficient conditions are confined to the root endodermis [20]. Studies of root cross-sections of 7-day-old maize seedlings localized the phytase and its transcript to the rhizodermis, endodermis and pericycle [14]. The subcellular location of the maize root phytase is unknown but, because the two isolated maize phytase genes lack a signal sequence, the enzyme is probably not secreted. In other plants, the root phosphate starvation response comprises increased activity of phosphatases, including phytases, in intracellular

Box 2. Phytase: enzymatic characters and sources

Phytase [*myo*-inositol-(1,2,3,4,5,6)-hexa*kis*phosphate phosphohydrolase] is a phosphatase that catalyses the stepwise hydrolysis of phytic acid to orthophosphate, a series of lower phosphate esters of *myo*-inositol and eventually *myo*-inositol. Phytases can be roughly categorized as 3-phytases (EC 3.1.3.8) or 6-phytases (EC 3.1.3.26). The 6-phytases initially hydrolyse the phosphate ester bond at the L-6 (D-4) position of phytic acid, whereas the initial activity of the 3-phytases is directed towards position D-3. In general, plant acid phytases are considered to be 6-phytases, whereas phytases from microorganisms are often referred to as 3-phytases.

However, this classification might be too rigorous because there are several exceptions; for example, the *E. coli* P2 phytase, is a 6-phytase [a] and the alkaline phytase from lily pollen initially hydrolyses phytic acid at position D- or L-5 [b]. The site of initial hydrolysis of phytic acid is an important determinant for the sequence of further hydrolysis. Subsequent attacks on the penta*kis*phosphate are nonrandom, occurring adjacent to the free hydroxyl group. As a consequence, wheat phytase, which is a 6-phytase, generates D- or L-*myo*-inositol-(1,2,3,5,6)-penta*kis*phosphate and D- or L-*myo*-inositol-(1,2,3,6)-tetra*kis*phosphate plus D- or L-*myo*-inositol-(1,2,5,6)-tetra*kis*phosphate as the first and the second intermediates after hydrolysis of phytic acid [c]. *Aspergillus* phytase, a 3-phytase, preferentially hydrolyses phytic acid at position D-3, generating D-*myo*-inositol-(1,2,4,5,6)-penta*kis*-phosphate and subsequently D-*myo*-inositol-(1,2,5,6)-tetra*kis*phosphate as the major tetra*kis*phosphate [d,e].

Most known phytases are histidine phosphatases, with a phosphorylated histidine as an intermediate in their phosphoryl-transfer reaction [f]. The active site (RHGxRxP, where x represents any amino acid) is highly conserved and is believed to be the phosphate receptor region. After X-ray analysis of crystallized phytase from *Aspergillus niger* var. *ficum*, a model for substrate binding and attack was proposed to involve the formation of hydrogen bonds to the 3-phosphate group of phytic acid and attack by His59 [g]. Soybean phytase is a purple acid phosphatase, as are the phytases of *Arabidopsis* and wheat, which are currently under investigation (K.S. Johansen *et al.*, unpublished) [h].

Phytase activity has been reported from a large variety of bacteria, fungi and yeasts [i]. Of the 29 species listed, 21 produced extracellular phytase. The filamentous fungus *Aspergillus niger* is the most efficient producer of active phytase. In spite of limited heat stability [j], all commercial available phytases have until recently been based on *A. niger* phytase. The heat stability of phytases is an essential parameter for animal feed production because the enzyme should be able to withstand the temperatures of 60–90°C that are reached during the feed-pelleting procedure. Plant phytases have a temperature optimum of ~50°C (see Table 1 in main text) and are rapidly denatured and inactivated at temperatures >60°C. Several reports have described microbial phytases with improved thermostability and catalytic properties [k,l]. Another fruitful strategy has been the design of a completely novel phytase (Bio-Feed®, Novozymes, Bagsværd, Denmark) based on sequence information from other phytases with good heat stability [m].

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[21,22] and extracellular [23–25] fractions. Extracellular phytases appear to be root associated or to be released to the rhizosphere. In barley, the root-associated phytase activity has been reported to be about ten times higher than the root-released phytase activity [23].

The phytase response has been reported to differ between plant species and genotypes of the same species [21,23,24]. The activity of phytases constitutes only a small proportion of the total phosphatase response and is considered to be inadequate for ensuring sufficient phosphate acquisition [22,25,26]. Application of a fungal phytase to sterile cultures of *Trifolium subterraneum* seedlings enabled the plants to use phytic acid as the only phosphate source and the plants grew as well as P_i-supplied plants [26]. An improved ability to grow on phytic acid was also observed for sterile cultures of wheat inoculated with a phytase-secreting soil bacterium [22]. *Arabidopsis* lines engineered to secrete an *Aspergillus niger* phytase had a 20-fold increase in root phytase activity and grew better than the wild type on medium with phytic acid as the only phosphate source [27].

Table 1. Characteristics of plant phytases

Plant	Plant tissue	Molecular weight (kDa)	pH optimum	Temperature optimum (°C)	Refs
Barley (*Hordeum vulgare*)	Seedlings	67 (P1) 67 (P2)	5.0 (P1) 6.0 (P2)	45 (P1) 55 (P2)	[8]
Oat (*Avena sativa*)	Seedlings	67	5.0	38	[54]
Maize (*Zea mays*)	Seedlings	Two 38 kDa subunits	4.8	55	[17]
	Roots	Two 38 kDa subunits	5.0	40	[20]
Rye (*Secale cereale*)	Grains	67	6.0	45	[55]
Spelt (*Triticum spelta*)	Grains	68	6.0	45	[56]
Wheat (*Triticum aestivum*)	Bran	48 (F1) 48 (F2)	5.6 (F1) 7.2 (F2)	55	[10]
Mung bean (*Phaseolus aureus*)	Cotyledons	160	7.5	57	[57]
Soybean (*Glycine max*)	Cotyledons	60 70–72	4.5–4.8 4.5–5.0	55 58	[58] [15]
Tomato (*Lycopersicon esculentum*)	Roots	Two 82 kDa subunits	4.3	45	[59]
Lily (*Lilium longiflorum*)	Pollen	88	8.0	55	[60]

Abbreviations: F1, fraction 1; F2, fraction 2; P1, phytase 1; P2, phytase 2.

Strategies for solving the phytic acid problem in agricultural ecosystems

Ruminant animals can use phytin because of microbial phytase activities in the rumen, whereas non-ruminants have little or no phytase activity in their digestive tract. Phytase activity has been reported in the intestinal mucosae of humans, chickens and calves but appears only to be of significance in rats [28]. To compensate for the phytase deficiency, bioavailable phosphate in the form of P_i or fishmeal is added to the feed. Consequently, non-ruminants excrete large amounts of phosphorus, primarily in the form of phytic acid, which is subsequently transferred to the agricultural soils. Although organic and inorganic phosphate are tightly bound to soil particles, soils can become saturated and phosphate can be transferred to the aquatic environment via drainage, surface runoff and wind erosion (Fig. 1).

At least five different strategies have been devised to improve the phosphate bioavailability in animal feed and to reduce the environmental load. (1) Many pig farmers incubate the feed in water (steeping), whereby the endogenous phytase potential is activated. (2) Mutation breeding for impaired phytic acid biosynthesis has proved to be useful in maize, barley and rice [29]. (3) Addition of phytase to feed significantly enhances the release of phosphate from phytin. The commercial potential of microbial phytases has stimulated a large body of research and development activities to identify microbial phytases with high thermostability and better catalytic properties (Box 2). (4) Recently, pigs have been engineered to produce heterologous phytase in their salivary glands [30]. (5) Plants can be transformed for increased phytase production in the seeds. We consider that the transgenic approach will, in the long run, prove to be the most versatile and cost-effective.

Transforming plants for improved phosphate bioavailability

An expression strategy was implemented in tobacco [31] that has subsequently been shown to function in soybean [32], oilseed rape [33,34], rice [35] and wheat [36]. In the initial study [31], tobacco was transformed with constructs comprising the *phyA* gene from *A. niger* controlled by the 35S promoter. To ensure secretion of the heterologous phytase, a signal sequence from the tobacco gene encoding PR-S was inserted at the 5′ end of the *phyA* gene. Phytase constituted ~1% of soluble proteins in the tobacco seeds. The molecular mass was 67 kDa, compared with 80 kDa for the native enzyme, a difference that could be attributed to differences in glycosylation. The tobacco seeds could be stored for at least a year without loss of phytase activity. Seeds were tested under simulated digestive conditions and shown to release P_i from standard feed in a more efficient way than when adding equivalent amounts of phytase. Feeding trials with broiler chickens showed that the addition of milled transgenic seeds to a basal diet resulted in higher growth rates than the addition of non-transgenic seeds. Diets supplemented with transgenic seeds were comparable to those with P_i addition [31].

Detailed molecular and biochemical characterizations of the expression of the *phyA* gene are available for tobacco [31,37,38], soybean [39] and wheat [36]. In all cases, design for secretion via the default secretory pathway resulted in a heterologous phytase with a molecular mass comparable to that reported for tobacco. Localization studies in tobacco confirmed that the phytase had been secreted to the apoplast [38]. Apparently, however, the full glycosylation potential is not realized for phytase produced *in planta*. Later studies involving the isolation and purification of the heterologous phytase from tobacco leaves showed it to be catalytically indistinguishable from endogenous *A. niger* phytase, to possess the same temperature optimum but to have a shift in pH optimum from 5 to 4 [37].

It is apparent that glycosylation is essential for ensuring the function of the *A. niger* phytase. When the *phyA* gene was expressed in *E. coli*, most of the heterologous phytase was in the form of inactive aggregates but the residual activity displayed the same kinetic properties as the *A. niger* enzyme [40]. In *Pichia pastoris*, the *A. niger* phytase had the same characteristics as that produced in *Aspergillus*, although the molecular weight was slightly increased. Deglycosylation of the enzyme resulted in a reduction of thermostability by 34%, whereas prevention of glycosylation resulted in a reduction of both intracellular and extracellular phytase activities. Glycosylation of the *A. niger* phytase is thought primarily to affect thermostability [41].

In wheat, omission of the barley α-amylase signal sequence gave rise to a phytase with a molecular weight of 50 kDa, indicating absence of glycosylation [36]. The phytase activity was only slightly above background, whereas lines expressing a secreted phytase showed up to four times more phytase activity than wild-type wheat.

Detailed feeding studies have been performed with transgenic soybean and canola (*Brassica napus*) producing the *A. niger* phytase. Addition of phytase via transgenic soybeans at 1200 U kg^{-1} in the diet caused a 50% reduction in phosphorus excretion from broilers when compared with a diet supplemented with an intermediate level (0.16%) of dietary non-phytic acid phosphorus [32]. The canola feeding trials were performed using the cultivar Phytaseed® (BASF, Ludwigshafen, Germany) and compared with the effect of adding a commercial version of the same enzyme produced by microbial fermentation. Three levels of each of the two sources of phytase [250 U $(kg\ diet)^{-1}$, 500 U $(kg\ diet)^{-1}$ and 2500 U $(kg\ diet)^{-1}$] were added to a corn–soybean meal basal diet and fed to broilers [33] and piglets [34]. At the end of the trial, several of the experimental animals were randomly selected for gross necropsy and histological evaluation of liver, kidney and bone tissues. In broilers as well as piglets, there were no adverse affects observed for either source of phytase. There were similar increases in body-weight gain, feed intake, the gain:feed ratio, apparent retention of dry matter, P and Ca, and toe (broilers) and rib (pig) characteristics. For all parameters, there were significant increases compared with broilers and pigs fed on a conventional low phosphate diet, and a major decrease in phosphate excretion.

Implications for human nutrition

It is estimated that >2 billion people, primarily women and children in developing countries, suffer from iron deficiency. A similar number of people might lack zinc. These deficiencies are mainly because of a low content of the two minerals in commonly consumed foods, a lack of vegetables and meat in the diet, and inhibitors of iron and zinc uptake in the staple diet [42]. Phytic acid is considered to be the most important antinutritional factor for the availability of minerals such as zinc and calcium, and possibly also iron [43]. Phytic acid readily forms complexes with Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} (in decreasing order of stability) [44]. The antinutritional properties of phytic acid have been supported by several feeding experiments. Dietary supplementation with microbial phytase improves Zn use in rats [45] and broiler chickens [46]. In pigs, phytase supplementation increases the apparent absorption of Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} [47].

Several initiatives have been launched to alleviate iron and zinc deficiencies, involving screening for cultivars with a higher iron and zinc content or mutation breeding for low phytin levels [48]. Small-scale feeding experiments using tortillas prepared from low-phytin corn enabled better iron absorption in humans [49]. Genetic modifications of food staples for expression of phytase could facilitate iron and zinc absorption. However, the heat stability of the phytases will have to be improved to realize their full potential, particularly in crops for which prolonged cooking is required before consumption. By contrast, bread-making, in which phytase is active during the fermentation, might allow a realization of the phytase potential [50].

The practical implications of the lack of phytase thermostability have been illustrated by engineering rice for endosperm-specific expression of the *Aspergillus fumigatus* phytase, which is known to be able to refold into an active configuration after thermal denaturation [51]. Unfortunately, when the transgenic rice grains were cooked for 20 min, only 8% of the phytase activity was retained after cooling. By comparison, the fungal enzyme boiled with rice flour for 20 min retained 59% of the activity. This implies that, somehow, the *in planta* production or the cellular environment of the rice endosperm interferes with the capacity of the *A. fumigatus* enzyme to refold into an active form when the temperature is reduced [51].

Perspectives

Increased knowledge of phytic acid biosynthesis, deposition and degradation, combined with more information about phytase heat stability and catalytic properties, should provide plant biotechnologists with new options for improving phosphate and mineral uptake and bioavailability. This might benefit human and animal nutrition, and the environment. Cloning the genes involved in phytic acid biosynthesis should enable specific antisense approaches to reducing phytic acid content. The introduction of genes encoding specific *myo*-inositol- or phytic acid-degrading enzymes might, in combination with targeting to the compartment of synthesis and deposition for these compounds, also be used to reduce the phytic acid content. Engineering plants to secrete phytases from their roots might also be an important strategy for mobilizing phosphate reserves in the soil.

Although phytic acid is generally considered to reduce phosphate and mineral bioavailability, there is now evidence to suggest a beneficial role for phytic acid. A few studies have indicated that it is a potential antioxidant and that it has anti-neoplastic activity in the large intestine [52]. The potential benefits of phytic acids must be carefully evaluated. In this context, the generation of a range of mutagenized or transgenic materials and the testing of these materials in feeding trials or cell culture experiments might prove to be essential.

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