

## GENETIC IMPROVEMENT OF CEREALS WITH LOW PHYTIC ACID CONTENT

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### Summary

During their development, seeds accumulate phosphorus and other nutrient supplies necessary to subsequently support germination and early seedling growth. Normally about 75% of seed total phosphorus is found as a single compound called phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate) that serves as the seed's storage form of phosphorus. However, phytic acid can contribute to nutritional and environmental problems when seeds are used in feeds in foods. Phytic acid binds tightly to mineral cations like iron, zinc and calcium. Humans and non-ruminant animals, such as poultry and swine, excrete nearly all the phytic acid they consume. This process can contribute to mineral deficiency, particularly iron deficiency, in human populations that rely on grains and legumes as staple foods. In the context of livestock production, this phenomenon means that non-ruminant animals excrete a major fraction of the seed phosphorus they consume. This is an inefficient use of seed phosphorus stores, and the resulting animal waste phosphorus can contribute to water pollution. As one approach to these issues, low phytic acid mutations in major grain and legume crops have been isolated, and are being used to breed new “Low Phytate” versions of these crops. These new Low Phytate types are very useful in studying the potential benefits, in terms of mineral nutritional health, of reducing phytic acid in human diets. It is possible that by simply substituting Low Phytate versions of crops for normal versions in foods, iron, zinc and calcium nutritional status may be significantly improved in humans. Also, when compared with traditional crops, a much larger fraction of total phosphorus in seed produced by Low Phytate crops is “available phosphorus” for non-ruminants, such as poultry and swine. These animals therefore obtain a much larger fraction of the phosphorus they need from the grain, and excrete a correspondingly smaller amount of waste P. This can represent a significant improvement in the efficiency of phosphorus

management in animal production. The goal of continuing genetics and breeding research is to develop “optimized” Low Phytate crops that are both highly productive and more nutritious.

## 1. Introduction

During their growth and development, seeds accumulate basic carbon, nitrogen, phosphorus (P) and mineral nutrient stores. During germination, these basic stores are utilized to support early seedling growth. In most cases “building block” nutrients like sucrose or amino acids accumulate in the developing seed and are then synthesized into more complex storage forms like starch or storage proteins. In the case P, phosphoric acid ( $H_3PO_4$ ; also referred to as “phosphate”, or “inorganic P”) is taken up by the plant, translocated to the developing seed and there synthesized into a storage form of P called phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate or Ins  $P_6$ , Figure 1A). In mature seeds of most cultivated species phytic acid P represents about 75% of seed total P (for an example see the bars labelled “W.T.” or “wild-type” in Figure 2). The remaining 25% of seed total P represents all other P-containing compounds that together comprise “cellular P” like DNA, RNA, starch, and lipids, and inorganic P (Figure 2). Inorganic P typically represents from 2% to 7% of seed total P.

Phytic acid synthesis is also an important part of seed storage processes for minerals like magnesium, potassium, iron and zinc. Phytic acid is a “polyanion” at cellular pH, and can have from six to eight negative charges per molecule. This polyanion can bind tightly to positively charged mineral cations like  $K^+$ ,  $Mg^{2+}$ , or  $Zn^{2+}$ . In the mature cereal grain crop seed, phytic acid is primarily found as a “mixed salt” of K and Mg, but these “phytate” salts may also contain other mineral cations like Zn, or Fe. The mineral cation content of phytates may represent a major fraction of the mineral content of seeds.

Phosphorus is an essential nutrient required by all life. Just as growing seedlings and plants require a certain level of P for vigorous and healthy growth, so do human beings, and all animals, bacteria, fungi, and algae. P is an important part both of our bones and our DNA. In most cases, more P means more growth. This is why P is a basic component of fertilizers, but is also one of the most important reasons why increased human population growth, and the increased agricultural and industrial growth that accompanies it, leads to water pollution. Human and animal wastes, fertilizer runoffs, and industrial waste all contribute P to surface waters like rivers, lakes, and estuaries. The elevated levels of P lead to increased growth of algae and other microorganisms. The metabolic activity of these microorganisms can then deplete the water of oxygen, ultimately leading to the killing off of fish and other life, in a process called “eutrophication”.

The fact that seeds synthesize most of their P into phytic acid ultimately contributes to eutrophication. The amount of P synthesized by seeds into phytic acid represents a sum equivalent to about ½ of all P applied as fertilizer worldwide, but human beings and other non-ruminant animals (poultry, swine and fish), do not efficiently digest and utilize phytic acid. Instead, phytic acid P consumed by non-ruminants is excreted. This waste P can contribute significantly to water pollution. This is also a highly inefficient use of P in terms of animal nutrition.

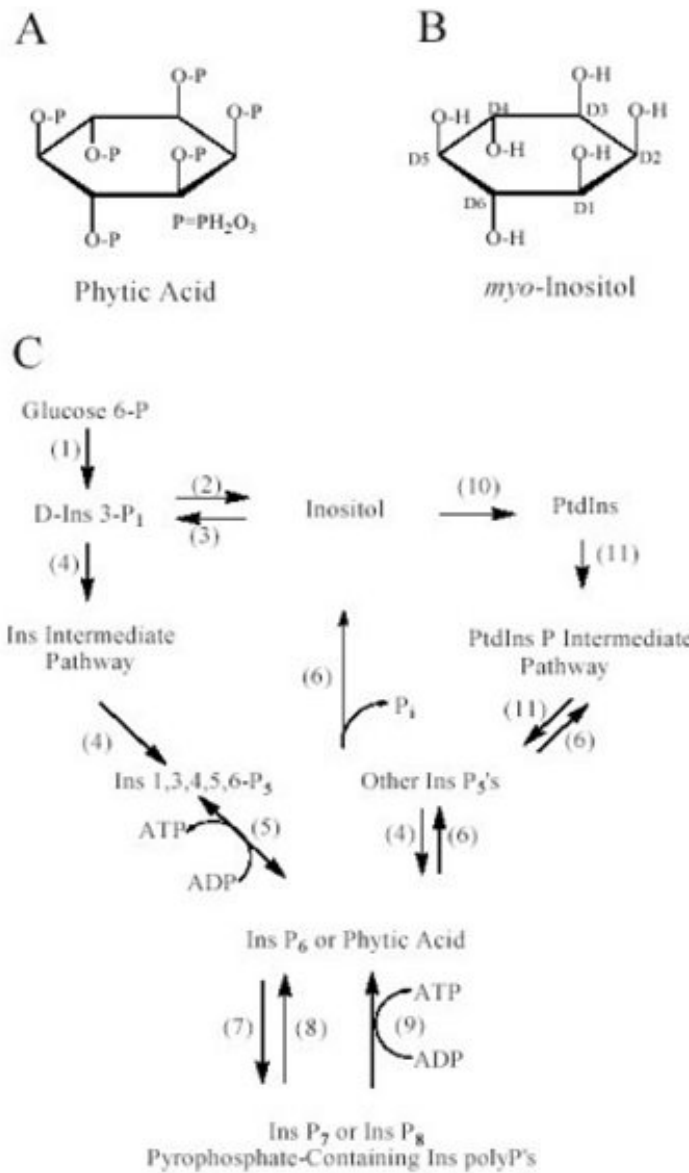


Figure 1. A summary of biochemical pathways involving phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakisphosphate or Ins P<sub>6</sub>) in the eukaryotic cell. (A) Structure of phytic acid. (B) Structure of *myo*-inositol. The numbering of the carbon atoms in the ring is given following the D-convention. (C) Biochemical pathways. Numbers at arrows indicate the following enzymatic activities: (1), D-*myo*-inositol 3-P<sub>1</sub> synthase (MIPS); (2), D-Ins 3-P<sub>1</sub> phosphatase, or Ins monophosphatase; (3), D-Ins 3-kinase or Ins kinase; (4), Ins P or polyP kinases; (5), Ins 1,3,4,5,6 P<sub>5</sub> 2-kinase or phytic acid-ADP phosphotransferase; (6), phytases and phosphatases; (7), Ins P<sub>6</sub> or pyrophosphate-forming kinases; (8) pyrophosphate-specific phosphatases; (9), pyrophosphate-containing Ins PolyP –ADP phosphotransferases; (10), phosphatidylinositol (PtdIns) synthase; (11), PtdIns and PtdIns P kinases followed by PtdIns P-specific phospholipase C.

If grain and legume crops are used in poultry, swine or fish feeds, their phytic acid content contributes little or nothing to the animal's nutritional requirement for P. Often the 25% of a normal seed's P that is “non-phytic acid P”, or “available P” in terms of animal nutrition, does not meet the nutritional requirements of livestock. To meet this requirement, feeds can be supplemented with a form of inorganic P. However, this practice does nothing to reduce the amount of phytic acid P ending up in waste. A more recent practice is to supplement feeds with an industrially-produced enzyme called “phytase”. When poultry, swine or fish consume feeds supplemented with phytase, it breaks down the phytic acid, thus using more of the P in seeds to supply the animal's nutritional requirement, and reducing the amount of phytic acid P in animal waste. The issue of P and eutrophication has become so important in the developed world that new regulations and standards are being put in place to require better management of P in agricultural production.

In historical terms, the greatest interest in seed phytic acid has been in its impact on human nutrition and health. While the question of seed phytic acid P and P management in animal agriculture is relatively straightforward, the question of dietary phytic acid in human nutrition and health is much more complex. Dietary phytic acid may have both positive or negative effects on human health, and whether the primary concern is over a positive or negative role depends on the specifics of population, diet and methods of food preparation. At first, this interest concerned the possible “anti-nutrient” effects of seed-derived phytic acid. Dietary phytic acid forms salts with minerals it encounters in the digestive tract, both seed-derived minerals consumed in the meal, or in cases like zinc, from “endogenous stores” already in the individual consuming the meal. The P and mineral content of these salts may pass through the digestive tract and be excreted. This phenomenon can contribute to mineral depletion and deficiency, depending on the population, diet, and method of food preparation. For example, in those populations that rely on certain whole-grain cereals and legumes as staple foods, children and child-bearing women may be at risk for iron and zinc deficiency, due in part to chronic consumption of phytic acid (see *Iron Nutrition in Man: Global Perspectives on Iron Deficiency and Malnutrition* and *Global Importance of Zinc Deficiency in Humans: its Relation to Malnutrition and Strategies for its Prevention*). Again, depending on population and diet, in some cases dietary phytic acid may negatively impact calcium nutrition, and the nutrition of other mineral elements.

More recently, there has been growing interest in the possible positive roles for dietary phytic acid as an anti-oxidant and as an anti-cancer agent. Phytic acid is a particularly effective chelator of iron. Three of phytic acid's phosphate esters, the ones found at positions “D1”, “D2”, and “D3” on the inositol ring that forms phytic acid's backbone (Figure 1B), can bind to iron in such a way as to completely neutralize its charge. Each iron ion so bound cannot participate in the “Fenton reactions” that lead to free radical formation and oxidation. Phytic acid may also impact cell growth, and thus cancer growth, in other ways by binding to additional minerals as well. For those populations where the incidence of colon cancer or degenerative effects of oxidation in aging are of greater concern than general mineral nutritional health, dietary phytic acid may be beneficial to health. However, for those populations where mineral nutritional health represents a more pressing need than the incidence of cancer, perhaps dietary phytic

acid may have a negative impact on health. Clearly, the question of dietary phytic acid in human health and nutrition must be evaluated on a case-by-case basis.

In the last decade plant genetics and breeding researchers have developed new types of maize, barley, and rice that produce seed containing normal amounts of total P, but greatly reduced amounts of phytic acid P (Figure 2). This began with the isolation of low phytic acid (or *lpa*) mutants of maize, and was followed by the isolation of similar mutants of barley, and rice. These mutants are the result of recessive alleles of single genes encoding information important to phytic acid synthesis. In some cases, these alleles can be used to breed new hybrids or cultivars that have yields approaching that observed in contemporary, high-yielding types, at least when grown under production conditions ranging from nominal to optimal. This first generation of “low phytate” cereals were developed using classical genetics; the isolation of mutants followed by standard plant breeding methods. No molecular genetics or transgenics were used. Using these techniques the first maize “Low Phytate” maize hybrids were developed in the late 1990s. Whether or not these are of any value in the real world of agricultural production or human nutrition remains to be determined. Continuing genetics research is developing more advanced molecular approaches to producing the “low phytate” trait. These approaches may prove necessary to produce high-yielding “low phytate” cultivars and hybrids. Use of these latter types will depend on the outcome of the current worldwide debate on “Genetically modified organisms”.

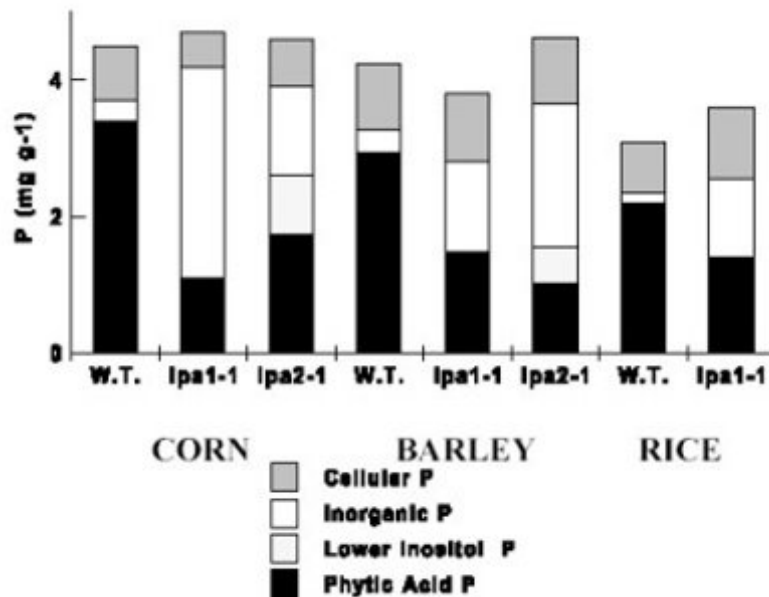


Figure 2. Seed phosphorus fractions in wild-type (non-mutant) and *low phytic acid 1* and *low phytic acid 2* lines of maize and barley, and wild-type and *low phytic acid 1* lines of rice. All fractions are expressed as their phosphorus (P, atomic weight 32) contents. W.T.=wild-type; lpa1-1=*low phytic acid 1-1*; lpa2-1=*low phytic acid 2-1*. “Cellular P” represents all seed P-containing compounds other than inorganic P, phytic acid P and “lower inositol P”. The “lower inositol Ps” primarily consists of Ins tri-, tetra- and pentakisphosphates.

A great deal remains to be studied concerning these new “low phytate” types of maize and other crops. Little is known about any potential problems concerning disease susceptibility, pest or stress tolerance, or storage and handling problems. The current status of low phytic acid genetics, and breeding of “low phytate” crops, will be reviewed here. Agronomic and nutritional studies using the first generations of these crops will also be reviewed. The potential value of these plant genetic resources both in studies of seed phytic acid's role in animal and human nutrition, and as a tool in addressing problems associated with seed phytic acid, will be discussed.

## 2. Phytic Acid Genetics

### 2.1. Background

Up until the 1990s, there were no reports of single-gene mutants that had large and specific effects on seed phytic acid content. Thus, there was little or no “Mendelian” genetics of seed phytic acid. The molecular genetics relevant to this field was also in its infancy. One early objective was to attempt to isolate mutations that greatly reduce seed phytic acid content, but that are non-lethal and have little or no other effects, such as on seed total P or seed function. These then could be used in basic studies of phytic acid physiology, metabolism and molecular genetics, to study phytic acid's role in nutrition, and to breed first-generation “low phytic acid” crops.

Predicting the physiological or metabolic outcome of a heritable change or “lesion” in a given gene or biochemical pathway can help in designing methods for screening for such a mutation. What effect would a block in the synthesis of inorganic P into phytic acid during seed development have on other metabolic pathways important in this tissue, such as on protein, oil or starch synthesis? A number of lines of evidence had indicated that phytic acid metabolism is important to the regulation of inorganic P concentration, or “P homeostasis”, during seed development. This homeostasis may be important to the normal functioning of the developing seed's major metabolic pathways, such as protein, oil or starch synthesis, and might be important for other processes like RNA export from the nucleus. Would a “low phytate” mutant also be “low starch”, or have germination problems, or be sensitive to cold stress?

Phytic acid also represents a major store of *myo*-inositol (also referred to as “Ins”; Figure 1B) in the mature seed. Since mutants were sought whose seed had normal levels of total P but greatly reduced levels of phytic acid P, such mutations would probably be in Ins or Ins P metabolism. A brief review of the metabolic pathways producing both Ins and phytic acid would therefore help to predict the potential phenotypes of “low phytate” mutants, but can also help identify genes that can be used as targets in a molecular genetics or biotechnological approach to developing “low phytate” crops. All pathways to phytic acid begin with the synthesis of Ins, phytic acid's backbone, and ultimately proceed via its phosphorylation to yield the hexaphosphate ester of Ins. The sole synthetic source of Ins is via a simple two-step pathway: 1) the synthesis of D-*myo*-inositol 3-monophosphate (here we will use the “D”-numbering convention for the carbons of Ins, so this compound would be Ins 3-P<sub>1</sub>) from glucose 6-P catalyzed by the enzyme D-*myo*-inositol 3-P<sub>1</sub> synthase (MIPS; Figure 1C, Step 1); 2) the breakdown of Ins monophosphate to Ins catalyzed by a specific monophosphatase (Figure 1C, Step 2).

If seed-specific MIPS activity is required for phytic acid synthesis, one question is whether the product of MIPS is used directly for further phosphorylation to phytic acid, or whether the intermediate steps of dephosphorylation to yield Ins, followed by re-phosphorylation catalyzed by a *myo*-inositol kinase (Figure 1C, Step 3), is required. The product of *myo*-inositol kinase is the same as MIPS; D-*myo*-inositol 3-P<sub>1</sub>.

The pathway to phytic acid may then proceed via step-wise phosphorylation of soluble Ins 3-P<sub>1</sub> through a series of soluble Ins phosphate intermediates to phytic acid, catalyzed by two or more specific Ins P kinases (Figure 1C, Steps 4 and 5). Phytic acid synthesis may also proceed in part or in whole via an alternative pathway that uses Ins phosphate intermediates produced from phosphatidylinositol phosphates (Ptd Ins phosphates; Figure 1C, Steps 10 and 11). This alternative pathway begins with Ins and the first step is the synthesis of phosphatidylinositol catalyzed by a phosphatidylinositol synthase (Figure 1C, Step 10). The relative contribution of either of these two alternative routes to more highly phosphorylated inositols in the developing seed, via “free” Ins phosphates or via Ptd Ins P intermediates, has not been unequivocally determined at present.

Under the current view, phytic acid is seen not simply as a P-storage product or end product for Ins phosphorylation, but as a pool for both P and Ins phosphates. Ins phosphate metabolism is possibly of importance to many cellular processes, such as signal transduction, RNA export, DNA repair, and ATP regeneration. Ins phosphates more highly phosphorylated than phytic acid, such as Ins P<sub>7</sub> and Ins P<sub>8</sub>, have been documented to occur widely in eukaryotic cells (Figure 1C, Steps 7, 8, and 9). These compounds contain pyrophosphate moieties, and may be involved in ATP regeneration. For students of plant and seed biology, this is reminiscent of the decades-old hypothesis that phytic acid itself may serve as a P-donor in ATP regeneration. It now appears that this early conjecture may have been closer to the truth than originally believed. A classic question in seed biology is how ATP regeneration is accomplished in seeds immediately following imbibition, prior to establishment of membrane integrity: perhaps pyrophosphate-containing Ins polyphosphates do in fact serve in this role. It is possible that Ins polyphosphate-pyrophosphate metabolism actually plays a key role in phytic acid synthesis and breakdown. However, there has to date been very little progress in the study of these pyrophosphate-containing Ins compounds in plant systems.

Genomic and/or cDNA sequences encoding Ins 3-P<sub>1</sub> synthases and monophosphatases have been isolated from a number of plant species. However, to date little is known about genes encoding an Ins kinase, or genes encoding plant Ins polyphosphate kinases specifically involved in phytic acid synthesis. The genetic resources and methods developed in studies of Ins P<sub>6</sub> in non-plant systems will probably lead to breakthroughs in the isolation of plant genes encoding these latter functions. At this point in the near future the genetics of both Ins and Ins P<sub>6</sub> will begin to reach maturity. A mature understanding of phytic acid metabolism and biology will then follow.

At present, however, a great deal remains to be accomplished concerning the phytic acid pathways in seeds. There is still really very little that is definitely known concerning these pathways. In addition to the questions concerning the central biosynthetic

pathway discussed above, other aspects of phytic acid metabolism in seeds, such as transport, localization and compartmentalization functions, and pathway regulation, remain obscure at the molecular genetic level. As the molecular genetics in this field advances, more tools will become available that are useful in engineering the “low phytate” trait using molecular genetics approaches.

Since Ins is an important component of numerous pathways in plant tissues, a mutation that completely blocks its synthesis, throughout the plant and seed, would probably be lethal as a homozygote. However, a mutation in a MIPS gene whose expression is seed-specific and serves mostly to supply Ins for phytic acid synthesis may not be lethal. A mutation that reduces MIPS activity but does not abolish it may also not be lethal and may reduce phytic acid accumulation. Perhaps a mutation early in the pathway to phytic acid, such as a MIPS null, may have a more deleterious effect on the plant and seed than a mutation in the latter Ins P or Ptd Ins P pathways. Since phytic acid metabolism may be central in both P and Ins/Ins P pathways, at the onset of this work it was a very real possibility that no “low phytate” mutant could be found that produced physiologically or phenotypically normal seeds, with normal storage of other nutrients like starch and protein, and normal germination and early seedling growth. It turned out that mutations in both Ins and Ins P pathways can have small to large effects on both plant and seed function, and that specific low phytic acid mutations or alleles that have little or no effect on other aspects of plant or seed function can be found.

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### **Biographical Sketch**

**Victor Raboy** received his Bachelor of Science Degree (1978) in Plant and Soil Science, from the University of Massachusetts, Amherst. He then received a Masters Degree (1980) and a PhD (1984) while studying in the Department of Horticulture, at the University of Illinois. At Illinois, he first worked on “slow release” formulations of herbicides for use in horticultural crops (for his Masters Degree), and then studied the physiology and genetics of seed phytic acid in the soybean and its wild-relatives (for his PhD). He was then a Post Doctoral Research Associate (1984 to 1987) in the Laboratory of Genetics, University of Wisconsin, where he studied the genetics of maize transposable elements. Since 1987, Dr. Raboy has been a Research Geneticist for the USDA's Agricultural Research Service, first in Bozeman Montana, and since 1996, as part of the National Small Grains Germplasm Research Facility, Aberdeen Idaho. His main research area continues to be the genetics of seed phosphorus and phytic acid. His laboratory's main contribution has been the isolation, study and use of cereal and legume “low phytic acid” mutants. This work led to the first generation of “low phytate” crops. These are currently the subject of agronomic study, and are being employed in studies of both animal and human nutrition.