Mutations in genes controlling the biosynthesis and accumulation of inositol phosphates in seeds

Søren K. Rasmussen1, Christina Rønn Ingardsen and Anna Maria Torp

Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark

Abstract

Most of the phosphorus in the resting seed is stored inside protein storage vacuoles as PA (phytic acid; InsP6). The biosynthesis and accumulation of PA can be detected beginning from a few days after anthesis and seem to continue during seed development until maturation. The first step in PA biosynthesis is the formation of Ins3P by conversion of glucose 6-phosphate. This is then followed by a sequential and ordered phosphorylation of the remaining five positions of the inositol ring by a number of kinases, resulting in PA. Identification of low-PA mutants in cereals, legumes and Arabidopsis is instrumental for resolving the biosynthetic pathway and identification of genes controlling the accumulation of PA. Mutations in seven genes involved in the metabolism of PA have been identified and characterized among five plant species using induced mutagenesis and insertion elements. Understanding the biosynthetic pathway and genes controlling the accumulation of PA in plant seeds and how PA may balance the free phosphate is of importance for molecular breeding of crop plants, particularly cereals and legumes.

Key words: inositol phosphate, inositol phosphate kinase, molecular breeding, phytic acid, seed.

Introduction

PA (phytic acid; InsP6) is the primary storage compound of phosphorus in seeds (Figure 1). Typically, a cereal seed contains 1.5% PA. PA poses a number of challenges in husbandry of fodder and for staples in human nutrition [1]. PA is stored in globoïds as mixed salts (phytate) with minerals such as potassium, magnesium, calcium and iron, in decreasing concentration order [2]. Analysis of isolated phytate globoïds from rice bran shows that, although iron co-purifies with PA, zinc does so to a much lesser extent [2]. Recently, it was found that, whereas iron is mainly associated with PA, zinc appears to be mainly bound to peptides [3]. PA reduces the bioavailability of iron and thus contributes to the ‘hidden hunger’, while people still obtain sufficient energy from the starchy cereal grains. In the case of foodstuffs, the main issue is phosphate management in animal production systems. PA cannot be dephosphorylated in the digestive system of monogastric animals, and excreted phytate might contribute to environmental phosphate pollution with eutrophication of aquatic ecosystems as a result. Identification of low-PA (lpa) mutants impaired in PA biosynthesis or transport is one way to solve some of the problems outlined above. Such mutants have been identified in barley, rice, maize, wheat and soya bean [4-9]. In the present paper, we review the pathway of PA biosynthesis in plants, including the mutations which have contributed significantly to our understanding of this pathway.

Pathway overview

PA can be synthesized by a lipid-dependent as well as a lipid-independent pathway. As the lipid-independent pathway is predominant in seeds of cereals and legumes, this pathway will be reviewed in the present paper. The first step in the pathway is the formation of Ins3P (Figure 2), followed by a stepwise and ordered phosphorylation of the remaining carbons, with position 2 as the last.

MIPS (myo-inositol-3-phosphate synthase; EC 5.5.1.4)

The first committed step of PA biosynthesis involves the conversion of glucose 6-phosphate into Ins3P catalysed by MIPS. The reaction is the only de novo source of the inositol ring in all organisms [10]. The number of MIPS genes seems to differ in plant genomes: barley has one MIPS gene and rice has two, whereas several are found in Arabidopsis, maize and soya bean [11-15]. In both monocotyledons and dicotyledons, MIPS genes are temporally and spatially expressed [11,14]. One of two MIPS genes in rice and one of four in soya bean respectively were highly expressed in the developing seeds [11,13].

Lpa mutants caused by down-regulation [antisense or RNAi (RNA interference)] or mutations in the MIPS gene are described in soya bean, Arabidopsis, potato and rice (Table 1). Although wild-type PA levels are not essential [4,12], abolition of MIPS gene expression by knockout is lethal [16]. Mutations in the MIPS gene(s) often give lower yield and reduction in seed viability, altered morphology or
Figure 1 | Structure of PA
PA (InoP₆) in boat formation showing the five equatorial and the single axial group (2-position). The inositol ring is shown in the α-conformation, and carbon atoms have been numbered according to the IUPAC-IUB recommendation.

Figure 2 | The lipid-independent pathway of PA in higher plants
Enzymes catalysing important steps in this pathway (1–8) are illustrated. See the text for details.

increased susceptibility to pathogens [17–19]. One strategy to avoid these effects is to specifically down-regulate seed-specific MIPS expression. An lpa transgenic rice line was successfully generated using a seed-specific promoter and antisense against the rice seed-specific MIPS gene RINO1. This resulted in a seed PA reduction of 68%, whereas no pleiotropic effects on seed germination, yield or plant growth were observed [20]. Interestingly, whereas mutant atips2 plants showed higher susceptibility towards pathogens, this was not the case with plants having a mutation in the seed-expressed atips1 gene [18].

ITPK (inositol tris/tetrakisphosphate kinase; EC 2.7.1.159)
The ITPKs belong to a large family of ATP-grasp fold proteins. The ITPK enzymes are also called ITP5/6K (inositol-1,3,4-trisphosphate 5/6-kinase) after the first molecular description of enzymes of this class in plants. In the present paper, we use the term ITPK to distinguish the enzymes of this family from the multikinase family. ITPKs have been isolated from several plants. Four different ITPKs are found in both Arabidopsis [21–23] and soya bean [24]. One of the four soya bean ITPKs, GmITPK3, showed higher expression in early stages in seed development. Studies in rice revealed six different ITPKs. Two of these, OsITP5/6K-4 and OsITP5/6K-6, showed seed-specific expression [13]. In addition, a barley ITPK, HvIPK, as well as a rice ITPK, OsIPK, has been cloned [25]. So far, only one gene, ZmIPK, has been reported from maize. This gene showed embryospecific expression. Mu (Mutator) transposon insertion mutants as well as EMS (ethyl methanesulfonate)-induced mutations in this gene gave a reduction of 30–50% in seed PA content [6,26]. This suggests that other parts of the interwoven inositol phosphate pathways are also involved in the formation of seed PA. ITPKs are divided into three phylogenetic subgroups [13,24,25]. The three seed-specific monocotyledon ITPKs, ZmIPK, OsITP5/6K-4 and OsITP5/6K-6, group together, whereas the dicotyledon seed-specific GmITPK3 from soya bean is found in another subgroup, indicating that the subgrouping cannot be used as a guide to tissue specificity of gene expression. The observed grouping is not believed to reflect differences between monocotyledons and dicotyledons as both monocotyledon and dicotyledon ITPKs are found in all three phylogenetic subgroups.

The substrate specificity of many ITPKs has been evaluated, on both plant extract and heterologously expressed enzymes. Getting a clear picture of the biologically relevant substrate(s) used by ITPKs in the formation of seed PA is severely hampered by the fact that these enzymes show differences in tissue and temporal expression and that enzymes extracted from plants might be a mixture of several enzymes. Furthermore, different subsets of substrates are tested, and enantiomers often cannot be discriminated. Finally, at least some ITPKs show phosphatase activity towards several inositol phosphates [25]. Generally, however, the ITPKs phosphorylate InsP₃s [Ins(1,3,4)P₃, Ins(3,5,6)P₃, and/or Ins(3,4,6)P₃] and InsP₄s [Ins(3,4,5,6)P₄, Ins(1,3,4,5)P₄, and/or Ins(1,2,5,6)P₄] [21,24–26]. HvIPK interconverted the two substrates Ins(1,3,4,5)P₄ and Ins(1,3,4,6)P₄ by isomerase activity, and, when Ins(1,3,4)P₃ is used as substrate, this and other enzymes often produce a mixture of Ins(1,3,4,5)P₄.
and Ins(1,3,4,6)P₄. This isomerase activity is not seen in AtITPK4, belonging to the third subgroup of ITPKs [22]. Analysis of heterologously expressed HvIPK showed that this enzyme is not restricted to phosphorylation of higher inositol phosphates. On the basis of these in vitro studies, HvIPK is so far the only example of a kinase able to phosphorylate all steps from InsP to PA [25].

### IPK1 (inositol-pentakisphosphate 2-kinase; EC 2.7.1.158)

The last step in the synthesis of PA (InsP₄) is the conversion of Ins(1,3,4,5,6)P₅ into InsP₄ (Figure 2) performed by IPK1. Two nearly identical paralogues, ZmIPK1A and ZmIPK1B, were identified in maize. ZmIPK1B was expressed in roots, whereas ZmIPK1A was expressed in a range of tissue including immature ears, seeds at 12 days after pollination, middle-stage endosperm and maturing embryos [27]. A single IPK1 gene was found in rice (OsIPK1) showing the highest expression in the aleurone layer 10 days after anthesis [13]. At least two IPK1 genes are expressed in Arabidopsis [28,29]. One of these, AtIPK1, is expressed in developing flower buds, siliques, leaves and cauline leaves [29]. A loss-of-function mutant due to a T-DNA (transferred DNA) insertion in the AtIPK1 gene (Table 1) resulted in a reduction in the seed phytate level of 83% [28].

### MRP (multidrug-resistance-associated protein) ABC (ATP-binding cassette) transporter

A maize lpa mutant (lpa1-1) was shown to be defective in an MRP ABC transporter [30]. Since then, other lpa mutants defective in MRP genes have been described in maize [31], rice [32] and soya bean [33,34], confirming involvement of this gene and its product in the accumulation of PA in plants. These MRP mutants are characterized by strong reductions in PA content, matched by a similar increase in Pi. In addition, such mutants generally show accumulation of Ins, whereas inositol phosphate intermediates are undetectable [30,32,34]. Mutations affecting MRP can be lethal in both rice and maize [31,32]. At present, it is not completely clear how MRP regulates accumulation of PA in seeds, but two hypotheses have been proposed. MRP could affect transport and compartmentalization of PA. In this case, a mutation in the gene is speculated to result in accumulation of PA in the cytosol and a subsequent reduction in PA biosynthesis through a negative-feedback mechanism. Alternatively, MRP

---

#### Table 1 |克隆lpa突变体和转基因线

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Plant species</th>
<th>Name of mutated gene/allele</th>
<th>Origin of mutation</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>Arabidopsis</td>
<td>vtc4-2, vtc4-4</td>
<td>T-DNA insertion</td>
<td>[39]</td>
</tr>
<tr>
<td>IPK1</td>
<td>Arabidopsis</td>
<td>atipk1-1</td>
<td>T-DNA insertion</td>
<td>[28]</td>
</tr>
<tr>
<td>ITPK</td>
<td>Maize</td>
<td>lpa2-1, lpa2-2</td>
<td>EMS Mu insertion</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zmtpk-mum1-2, -3, -4</td>
<td>Mu insertion</td>
<td></td>
</tr>
<tr>
<td>MIK</td>
<td>Maize</td>
<td>lpa3-1, -2, -3</td>
<td>Mu insertion</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>lpa N15-186</td>
<td>Sodium azide+MNU</td>
<td>[36]</td>
</tr>
<tr>
<td>MIPS</td>
<td>Arabidopsis</td>
<td>atips1</td>
<td>T-DNA insertion</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>Potato</td>
<td>Anti-IPS</td>
<td></td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>G-107</td>
<td>Antisense</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Soya bean</td>
<td>∆GmMIPS</td>
<td>RNAi</td>
<td>[15,16,19]</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>Gm-lpa-T1W-1</td>
<td>γ-Rays</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>GM m1 1-PS-1A</td>
<td>MNU</td>
<td></td>
</tr>
<tr>
<td>MRP</td>
<td>Maize</td>
<td>lpa1-1</td>
<td>EMS</td>
<td>[30,31]</td>
</tr>
<tr>
<td></td>
<td>Maize</td>
<td>lpa1-mum1-2, -3</td>
<td>Mu insertion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maize</td>
<td>lpa1-241</td>
<td>Gene silencing</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>XS-lpa2-1, XS-lpa2-2</td>
<td>γ-Rays+sodium azide</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>Rice</td>
<td>OsMRP5</td>
<td>T-DNA insertion</td>
<td>[30,33,34]</td>
</tr>
<tr>
<td></td>
<td>Soya bean</td>
<td>GM event</td>
<td>Gene silencing</td>
<td>[30,33,34]</td>
</tr>
<tr>
<td></td>
<td>Soya bean</td>
<td>lpa1-a</td>
<td>EMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Soya bean</td>
<td>lpa2-a lpa2-b</td>
<td>EMS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ZPGK</td>
<td>lpa1-1</td>
<td>γ-Rays</td>
<td>[41,42]</td>
</tr>
<tr>
<td></td>
<td>ZPGK</td>
<td>lpa1-2</td>
<td>γ-Rays</td>
<td></td>
</tr>
</tbody>
</table>

*Name of mutant line.*
mutations may somehow reduce the supply of phosphate used by inositol phosphate kinases to synthesize inositol phosphates [30].

**IMP (inositol-phosphate phosphatase/inositol monophosphatase; EC 3.1.3.25)**

As described above, phosphorylation of InsP is one route to PA in the lipid-independent pathway. However, in plants, a second route seems to exist where InsP is first dephosphorylated by IMP to produce Ins which is subsequently phosphorylated to produce InsP by the enzyme MIK (myo-inositol kinase/inositol 3-kinase). Reduction in PA content of up to 75% in mutants defective in MIK [35,36] (see below) has provided direct evidence for the importance of this route in plants. Apparently, these two enzymes maintain exchange between InsP and Ins supplies. This may be an important control point as Ins is the precursor for a number of compounds other than PA.

IMP genes are classified into IMP genes similar to the mammalian IMP genes and IMPL (IMP-like) genes, which are more similar to prokaryotic IMP genes [13,37–39]. The number of IMP and IMPL genes identified in plant genomes at present includes one IMP gene in barley [37], one IMP and one IMPL gene in rice [13], one IMP and two IMPL genes in Arabidopsis [39] and three IMP genes in tomato [40]. Experimental evidence from Arabidopsis [39] shows that plant IMP enzymes may have several functions in plants. Thus L-Gal1P, D-Ins3P and D-Ins1P were shown to be good substrates for recombinant IMP protein, and two T-DNA insertion mutants for the VTC4 gene were shown to accumulate less Ins and ascorbate than wild-type. This indicates that IMP proteins could be involved in both de novo synthesis and recycling of Ins, as well as in ascorbate synthesis in plants. Evidence on the involvement of the IMPL genes in biosynthesis of PA in plants is relatively scarce; however, Arabidopsis IMPL2 may have a dual function with similar substrate specificities as IMP, whereas recombinant IMPL1 enzyme may be D-Ins1P-specific [39].

**MIK (EC 2.7.1.64)**

To date, one MIK gene has been identified in each of the genomes of maize and rice. Mutations in both of these genes result in non-lethal mutants with a significant reduction in PA content, accompanied by increased contents of P3 and InsP [35,36]. Studies with a recombinant maize MIK enzyme showed that, when supplied with InsP as substrate, the enzyme produced a mixture of InsPs, with the exception of Ins2P. However, MIK showed no kinase activity on any of the InsP or InsP2 substrates tested [35].

A double mutant for ITPK (ZmITP) and MIK in maize showed a higher reduction in PA content than either of the two single mutants; however, the reduction was smaller than the sum of the two single mutants. This indicates that ITPK participates in both of the Ins3P and Ins phosphorylation routes in plants and that both routes play a role in PA biosynthesis [35].

**2PGK (2-phosphoglycerate kinase)**

Studies in rice have shown that mutated forms of a 2PGK-related gene give rise to an lpa phenotype with approx. 40% reduction of PA in the seeds [41,42]. The seeds accumulated InsP, whereas no inositol polyphosphate intermediates were observed. The 2PGK from bacteria catalyses the ATP-dependent phosphorylation of 2-phosphoglycerate to form 2,3-BPG (2,3-bisphosphoglycerate). 2,3-BPG is a strong inhibitor of inositol polyphosphate 5-phosphatases, and removing this inhibition might lead to immediate breakdown of the inositol polyphosphate intermediates. However, a regulatory domain is not conserved and there is a structural similarity of both the substrates 2-phosphoglycerate and Ins3P and of the products 2,3-BPG and Ins(3,4)P2. This suggests that the gene encodes a kinase that functions in the early phosphorylation steps of inositol (Figure 2).

**Remobilization of stored PA**

Seed phytases have been purified and characterized from a number of plant species, showing a wide range of activities among plant species (for review see [1]). At germination, phytase carries out sequential and ordered dephosphorylation of PA (Figure 2) to provide phosphorus for the emerging plant [2]. Information on phytase expression throughout the plant life cycle is limited, and mutants with altered phytase activity remain to be identified. Seeds also contain phosphatase activity, which, by definition, does not accept PA as substrate, but they can unspecifically hydrolyse the lower forms of inositol phosphates.

**Other functions of inositol phosphates**

Ins is a precursor not only for the formation of PA (InsP3), but also for a large range of other compounds (Figure 2). Free Ins is required for example in the formation of the raffinose series of oligosaccharides [43], in the formation of the methyl inositols, ononitol and pinitol [44], and for the generation of cell wall polysaccharides via the Ins oxidation pathway (for a review, see [45]). Mutants that affect the amount of Ins in plants might give phenotypes due to imbalances in these pathways rather than a direct effect of a lowered level of PA.

The lipid-independent pathway functions in close interaction with the lipid-dependent pathway, where Ins is transformed into PtdIns, a lipid that has Ins as its headgroup. This pathway is involved in the regulation of several cell processes such as function of guard cells and tip growth of pollen tubes and root hairs, response to gravity stimuli and different types of abiotic stresses (reviewed in [46]). PtdIns also plays a role in membrane trafficking [47]. Thus changing the balance in the pools between the different components in the PA pathway would most certainly have an influence on these functions as well. Furthermore, in yeast and mammals, inositol polyphosphates play a role in the regulation of gene
expression and inositol pyrophosphates ($\text{InsP}_3$ and $\text{InsP}_6$) are involved in signalling, but whether these compounds play a similar role in plants is still unknown (for reviews, see [48,49]). $\text{InsP}_3$ are also involved in signalling [50,51], pathogen defence [18] and auxin-mediated gene expression [52].

Conclusions
Induced mutations have successfully been pursued in many crop plants aiming at reducing the PA concentration in the mature seed of cereals and legumes (Table 1). This has led to mutant lines with PA content ranging from 80% down to 10% of the parent line. In some cases, these mutants have been advanced to the stage where they have been registered as cultivars or germplasm in the U.S.A. [53,54]. In many of these mutants, characterization and identification of the underlying genes resulting in the lpa phenotype has contributed significantly to our understanding of the PA biosynthetic pathway in plants, even if there are still gaps in our knowledge. It is still an open question which enzymes carry out the phosphorylation of $\text{InsP}_2$ (and $\text{InsP}_3$) substrates. A single ITPK enzyme from barley has been shown to be able to carry out all steps from $\text{InsP}_2$ to PA in vitro [25]; however, it is very likely that other as yet unidentified enzymes contribute to the phosphorylation of $\text{InsP}_3$ substrates. In conclusion, with the help of mutations, our understanding of the biosynthesis and deposition of PA in plants has over the last 10 years created new resources for molecular plant breeding and our basic understanding of functions in which PA takes part.

Funding
Research on PA in our laboratory was supported by The Danish Council for Strategic Research: Improved Quality and Disease Resistance in Cereals (iKORN) 2008–2013 and the European Union Framework Programme 6 ERA-PG (European Research Area Network in Plant Genomics): Exploring Genomics-Assisted Analysis and Exploitation of Barley Diversity (EXBARDIV) 2007–2010, as well as the Danish Directorate for Food, Fisheries and Agri Business.

References


33 Gillman, J.D., Pantalone, V.R. and Bilyeu, K. (2009) The low phytic acid genotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome 2, 179–190


Received 27 December 2009
doi:10.1042/BST0380689