Chapter 4

Genetics of Inositol Polyphosphates

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1. INTRODUCTION

myo-Inositol (Ins, Figure 1 top center) is the cyclic alcohol that, in terms of the distribution of hydroxyl groups around the 6-carbon ring, retains the basic stereochemistry of glucose, the "D-gluco" configuration (Loewus and Murthy, 2000). The Ins ring is basically one synthetic step away from glucose, in that glucose 6-P is converted to Ins (3) P_1 in a reaction catalyzed by one enzyme (see below). Therefore it would seem to make biological common sense that this compound would itself in turn represent a major metabolite, the parent of all other cyclic alcohols and of many other compounds, and that its use would be important to a number of biological processes and functions. While this is true for most organisms, plant cells in particular do a lot with Ins (Loewus and Murthy, 2000; Figure 1). This review will focus on Ins and one branch of derivatives of Ins, the soluble Ins phosphates and their related lipid phosphatidylinositol (PtdIns) phosphates. The canonical pathway for transient production of Ins phosphates and PtdIns phosphates, central as second messengers in cellular signaling, proceeds via the production of PtdIns(4,5)P_2 and two of its derivatives, Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3 (Berridge and Irvine, 1989). In particular, this review will focus on the genetics of what is now known as an important additional pool in cellular Ins/PtdIns phosphate metabolism, Ins-1,2,3,4,5,6-P_6 (Ins P_6, Figure 1 bottom right; Sasakawa *et al.*, 1995), the hexamonoester of Ins. Ins P_6 is the most abundant Ins phosphate in nature.

In the fields of agriculture and nutrition Ins P_6 is commonly referred to as "phytic acid" (Raboy, 2001). This compound was first identified as a major P-containing component of seeds, and thus called phytic acid (Raboy, 1997), but subsequently its occurrence was found to be widespread in eukaryotes, and its metabolism to have many functions (Shears, 2001, 2004). It represents a major

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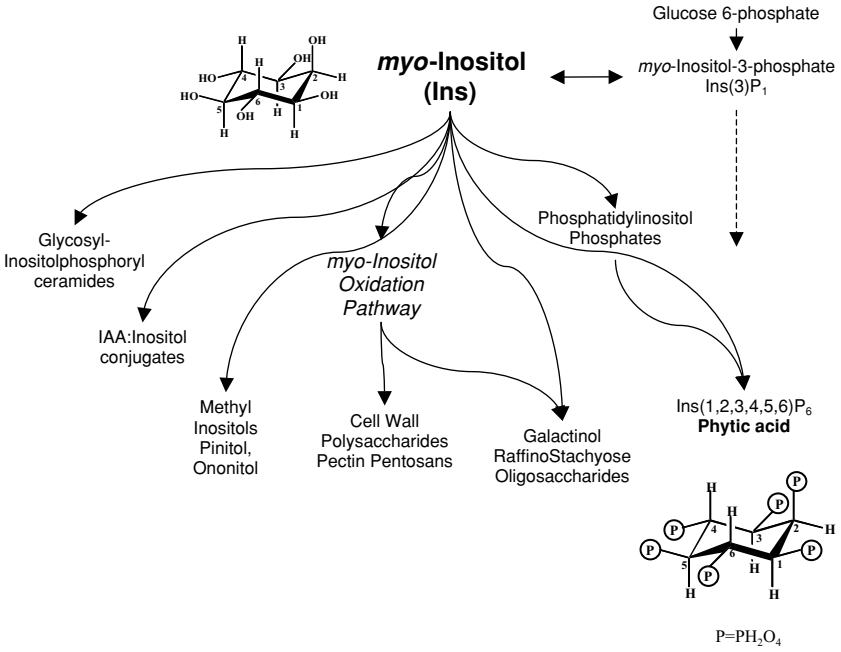

Figure 1. Pathways that utilize *myo*-inositol (Ins). The six carbons of the Ins ring are numbered according to the "D-numbering convention."

metabolic pool in the Ins phosphate/PtdIns phosphate pathways, of importance to development, cellular structure, membrane cycling, signaling, DNA repair, and RNA export (Hanakahi *et al.*, 2000, 2002; Odom *et al.*, 2000; Saiardi *et al.*, 2000a, 2002; York *et al.*, 1999). Ins P_6 might function itself as an effecter molecule, a second messenger, in signal transduction. Studies in plant systems (Lemtiri-Chlieh *et al.*, 2000, 2003) have shown that Ins P_6 might function as a signal regulating K^+-inward rectifying conductance in guard cells, and in mobilizing calcium flux in guard cells. These processes are critical to the function of guard cells in the regulation of transpiration and the maintenance of turgor in plant tissues. Of particular relevance to this review is that Ins P_6 is the most abundant phosphate in seeds, representing from 65% to 80% of seed total P (Raboy, 1997). To put this simple fact in perspective, consider the following: most of the phosphate taken up by crop plants is ultimately translocated to seeds during their development; most of phosphate translocated to seeds is ultimately synthesized into Ins P_6. As a result, the amount of Ins P_6 that accumulates in seeds represents a sum equivalent to more than 50% of all fertilizer phosphate applied annually worldwide (Lott *et al.*, 2000). Thus Ins P_6 represents a bottle-neck in the flux of P through the world-wide agricultural ecosystem.

Applied interest in seed phytic acid concerns its roles, both negative and positive, in human and animal nutrition, and health (Raboy, 2001). The fact that most seed P is found as phytic acid P can have a negative impact on P utilization by livestock. Excretion of dietary phytic acid can contribute to mineral deficiencies in humans. On the other hand, dietary phytic acid might play a positive role as an anti-oxidant and anti-cancer agent. This review will not directly address in detail questions relating to dietary phytic acid, other than to point out that studying and possibly dealing with these issues represents the applied rational for understanding the genetics and biology of seed Ins P_6 metabolism, and for the isolation of the so-called *low phytic acid* (*lpa*) mutations in a number of major seed crop species. The second rational for isolating *lpa* mutations is to advance basic knowledge concerning Ins P_6 metabolism. Initially *lpa* mutations were thought of as falling into two classes, reflecting the fact that the structural metabolic pathways to Ins P_6 can be thought of as consisting of two parts; the first part being the synthesis and supply of Ins substrate, the second being the conversion of Ins to Ins P_6. Thus plant *lpa* mutations were initially hypothesized to be mutations that perturb Ins supply, or those that impact the conversion of Ins to Ins P_6 (Raboy *et al.*, 2000).

At the beginning of the 1990s there was essentially no Mendelian genetics of the Ins phosphate pathways (Raboy and Gerbasi, 1996). There were few if any reports at that time demonstrating that a particular mutant phenotype was due to altered Ins phosphate pathway metabolism, or that altered levels of Ins phosphates were due to a particular mutation. The molecular genetics of these pathways was still only partially developed. Sequences encoding Ins phosphate kinases and phosphatases were just beginning to be identified. Furthermore, at least one important component of the Ins phosphate pathways, the pyrophosphate containing Ins phosphates, had not yet been discovered (Europe-Finner, 1989; Safrany *et al.*, 1999). One decade later a great deal of progress has been made in the biochemistry and molecular genetics of these pathways. There have also been numerous studies describing mutants and mutations in most components of these pathways, such as the plant *lpa* mutations mentioned above and others in many organisms ranging from *Dictyostelium* to the yeast *Saccharomyces*, *Drosophila*, and mammals. Studies that include in-part classical genetics of simply inherited mutant phenotypes continue to contribute to our understanding of these pathways, often yielding unexpected insights. With the acquisition of the complete genome of a number of species, along with large amounts of genomic sequence data for a number of additional species, and large amounts of information concerning expressed sequences, genomics has now also contributed greatly to this field. A selection of recent genetics and genomics studies that provide new insights into the field of Ins and Ins phosphate biology will be reviewed here.

2. THEMES

This review will use in sequence Ins and the conversion of Ins to $InsP_6$ as focal points for a discussion of questions into the metabolism of Ins phosphates, their biological function, and its evolution. Throughout this discussion there will be a few recurrent themes. First, rapidly advancing genomics has shown that with a few important and informative exceptions, most eukaryotes inherit a similar, ancestrally related set of sequences, most of which are expressed, encoding a relatively similar set of enzymes involved in Ins and Ins phosphate metabolism. This raises the question; is the biology of these pathways across evolutionarily divergent microbes, plants, and mammals, largely similar, or greatly different? Is there a conserved similarity, a conserved set of multiple functions, overlooked in some important ways?

An increasing number of studies indicate that, across these divergent organisms, Ins phosphates, and metabolites involved in their lipid precursor metabolism can serve in signal transduction and the regulation of development, according to the well-known signal-transduction paradigm (Berridge and Irvine, 1989; Irvine and Schell, 2001; Shears, 2004). But they can also serve in other functions that don not perfectly fit the signal-transduction paradigm. Thus Ins phosphates or related lipids also have functions in basal cellular metabolism and housekeeping. A given compound can be an "essential ubiquitous and abundant intermediate," in the same cells, tissues, or organs that it also serves via transient production in second messenger metabolism (Loewen *et al.*, 2004). Recent developments will be discussed that reveal the linkage of cellular Ins, Ins lipid and phosphate metabolism, demonstrating that they together represent a coordinated component of basic cellular nutrition and structure. Does the ability of cells to sense changes in internal concentrations of phosphate, phosphatidic acid, or its breakdown product glycerophosphoinositol, even if those changes are an outcome of external conditions, represent "signal transduction," or simply an example of the molecular mechanisms for feed-back regulation and maintenance of cellular nutritional status? In other words, phosphate, Ins phosphate, Ins lipids and their metabolites probably *first* are important in the own right, and *second*, sometimes important as second messengers.

A third theme concerns the biological significance, or occasional lack thereof, of observed differences in Ins phosphates or their lipid intermediates, or of observed differences in the copy number of genes encoding enzymes in these pathways. These differences might exist between cells within a species, or across species, families, or kingdoms. There may be differences in the apparent presence or absence of a given protein or differences in gene expression. Does this always serve some selective advantage in a Darwinian sense? Does it always have some important biological purpose? Some of these differences, however real, might be neutral in terms of fitness of an organism. Perhaps there is a propensity for researchers to "over interpret" the differences in genes, gene products, and metabolites that they observe. However, in some

cases differences between divergent species are highly informative. One aspect of this third theme is the observation that when researchers probe the Ins/PtdIns phosphate pathways via analysis of mutations that block specific components, they often find that alternative metabolic routes still allow the cell to produce important metabolites (Acharya *et al.*, 1998). The Ins/PtdIns pathways sometimes appear to perform a metabolic balancing act.

3. *MYO*-INOSITOL

A simple, two-step biosynthetic pathway to Ins has been established for all organisms. The sole synthetic source of the Ins ring is via the conversion of glucose 6-P to D-Ins(3)P_1 (Figure 2 top), catalyzed by the enzyme D-*myo*-inositol(3)P_1 synthase ("MIPS," EC 5.5.1.4). This enzyme and its activity's product were originally referred to using the "L" numbering convention, thus as an L-Ins(1)P_1 synthase (for a review, see Loewus and Murthy, 2000). However, it has become the accepted convention to refer to Ins phosphates using the "D"-numbering convention, where D-1 and D-3 refer to the same positions on the Ins ring as L-3 and L-1, respectively. The D-numbering convention will be used uniformly here. The ubiquity and importance of Ins has resulted in studies of MIPS from a large number of prokaryotes and eukaryotes (Majumder *et al.*, 2003). In addition to cytosolic forms, chloroplastic isoforms of MIPS have also been reported (Lackey *et al.*, 2003; reviewed in Majumder *et al.*, 2003). The second step in the pathway to Ins is to hydrolysis of Ins(3)P_1 to Ins and P_i (inorganic P), catalyzed by Ins monophosphatase (IMP, EC 3.1.3.25). A summary of the variety of pathways in plant cells that utilize Ins is illustrated in Figure 1.

For the purposes of this review, it is informative to compare the history of our understanding of the regulation of Ins supply in the budding yeast (*Saccharomyces cerevisiae*), versus higher plants. In yeast studies a major focus of research has addressed the regulation of Ins supply as central in the regulation of general phospholipid metabolism (Carman and Henry, 1989). The yeast genome contains one gene, *INO1*, encoding MIPS (Donahue and Henry, 1981). *INO1* as well as a large fraction of all yeast genes involved in phospholipid synthesis, are regulated at the transcriptional level via a feedback-loop system sensitive to cellular Ins and to a lesser extant choline levels (Loewen *et al.*, 2004). The machinery of this regulatory system consists of the *cis*-acting Upstream Activating Sequence (UAS^{INO}) found in the 5′-untranslated sequence of *INO1* and lipid pathway genes, and three transcription factors that interact with UAS^{INO} (Greenberg and Lopes, 1996). *INO1* and the lipid pathway genes are activated via binding to UAS^{INO} of two transcription activators, Ino2p and Ino4p. These in turn are repressed via binding by the transcription factor Opi1p, encoded by the *Overproduction of Inositol1* (*OPI1*) gene. How does this system sense Ins levels? Opi1p is itself repressed via binding by phosphatidic acid, and localization in the ER. At low cellular Ins levels, phosphatidic acid binds to Opi1p, inactivating it in

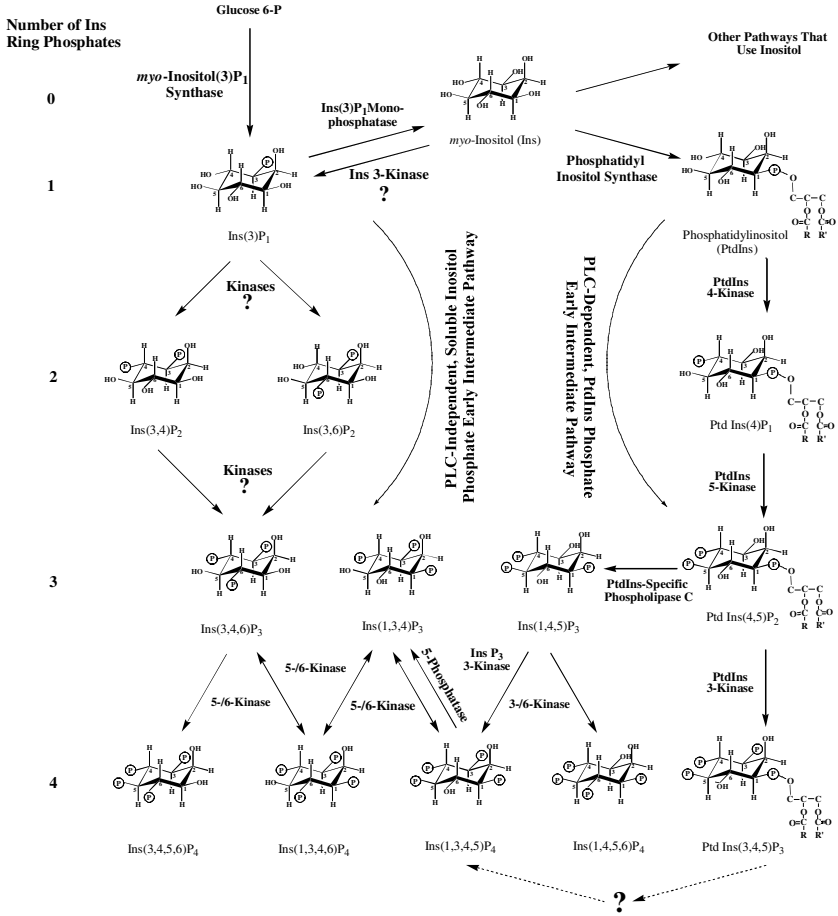

Figure 2. Biosynthetic pathways from glucose 6-P to *myo*-inositol tetra*kis*phosphates. The six carbons of the Ins ring are numbered according to the "D-numbering convention." The pathways to the center-left proceed entirely via soluble *myo*-inositol (Ins) phosphates. Pathways to the center-right proceed via phosphatidylinositol (PtdIns) phosphates. Questionmarks indicate synthetic steps that have not been confirmed in chemical, molecular or genetic analyzes. In addition, the hydrolysis of PtdIns(3,4,5)P_3 to yield Ins(1,3,4,5)P_4 has not been observed in any experiments, and is entirely speculative. P = PH_2O_4.

a localization in the ER, thus in turn allowing the activation of *Ino1* transcription via Ino2p and Ino4p, the translation of MIPS and production of Ins. Ins production in the presence of PtdIns synthase activity (EC 2.7.8.11), in turn consumes phosphatidic acid. Consumption of phosphatidic acid releases Opi1P which is transported to the nucleus, where it represses Ino2p and Ino4p, repressing Ins synthesis. This story is relevant to how compounds involved in signaling pathways, and Ins phosphates in particular, are viewed. Phosphatidic acid "appears

to be both an essential and ubiquitous metabolic intermediate and a signaling lipid" (Loewen *et al.*, 2004). Perhaps it will become apparent that, in terms of Ins phosphates, the eukaryotic cell has not evolved separate systems for basal, housekeeping metabolism and transient signaling metabolism, but rather manages to use components of one system for both.

The feedback-loop system links cellular Ins, phosphatidic acid, and lipid levels, but is it the only system in place in yeast that regulates *INO1* expression and cellular Ins levels? In addition to transcriptional regulation via binding of the Ino2p/Inso4p/Opi1p complex, *INO1* is also transcriptionally regulated via the "chromatin-remodeling" complexes *SNF2*, and *INO80*. Shen *et al.* (2003) and Steger *et al.* (2003) together demonstrated that in yeast, induction of genes in response to phosphate starvation, such as *PHO5*, requires the same chromatin-remodeling complexes as does *INO1*. *PHO5* represents a gene encoding one of the structural components of the *PHO* system, in this case a secreted phosphatase, and the regulation of its expression is particularly relevant to this discussion. The *SNF/INO80* remodeling in response to phosphate starvation requires the synthesis of Ins P_4 and Ins P_5 produced by the activity of an Ins(1,4,5)P_3 3-/6-kinase, alternatively called Ins phosphate multikinase (IPMK), in yeast encoded by the *Arg*82/*IPK2* gene (Shears, 2004; York *et al.*, 1999). This observation was confirmed and extended by El Alami *et al.* (2003), who showed that *PHO* gene repression by phosphate (i.e., repression of gene expression when cells are grown in "high" nutrient phosphate) requires both the Ins phosphate kinase catalytic activity of *Arg82*, a kinase that ultimately produces an Ins P_5, and Ins P_6 kinase activity encoded by the *KCS1* gene, that converts Ins P_6 to pyrophosphate-containing Ins P_7. These Ins phosphate kinases and the gene(s) encoding them will be discussed in more detail below. The relevant observation here is that Ins, phosphate, and Ins phosphate/PtdIns phosphate pathways clearly are interlinked in yeast. However, the authors of these studies uniformly interpret roles and results in terms of signal transduction, even when it appears clear that the sensing mechanisms have to do with cellular nutrition and metabolism.

The compartmentalization/transport mechanism for reversible inactivation of the transcription factors Ino2p and Ino4p has a parallel in the regulation *PHO*5, in this case via compartmentalization of the transcription factor Pho4p in the cytoplasm. Studies of *PHO5* contribute to a developing understanding that in yeast, it is not just Ins and global lipid metabolism that are intimately linked, but there is coordination of phosphate and lipid metabolism via the common component Ins. The *PHO5* gene is regulated in part by the transcription factors encoded by the *PHO2* and *PHO4* genes, Pho2p and Pho4p, respectively. Pho4p is maintained in a phoshorylated state when yeast is grown under high nutrient P, and is localized in the cytoplasm. When yeast is grown under low nutrient P, Pho4p is not phosphorylated and is translocated to the nucleus, where it binds cooperatively with the second transcription factor Pho2p, and thus activates transcription of *PHO5* (O'Neill *et al.*, 1996).

The linkage in coordination of gene expression and metabolism of the phosphate, Ins phosphate, and PtdIns Phosphate pathways in yeast, in response to either phosphate or Ins nutritional status change, is supported and extended by two additional recent studies of the metabolite glycerophosphoinositol and the *Glycerophosphoinositol transporter1* (*GTI1*) gene (Almaguer *et al.*, 2003, 2004). Glycerophosphoinositol is a breakdown product of PtdIns, and is excreted from the yeast cell. When yeast is grown either under P or Ins limitation, *GTI1* is expressed, and glycerophosphoinositol is transported into the cell. This can provide yeast cells with sufficient Ins or P. The transcription factors Pho2p and Pho4p are required for the induction of *GTI1* in response to P starvation. In addition to Pho2p and Pho4p, the transcription factors Ino2p and Ino4p are required for transcription induction in response to Ins starvation.

Thus in studies in yeast, initial interest in the regulation of Ins synthesis concerned its role in phospholipid metabolism, but recent studies have demonstrated clear links between phosphate and lipid metabolism via the common component Ins, and with a role for Ins phosphates. How does this compare with studies in plant systems? While Ins is clearly important in plants as a head-group for the Ins lipids, in plant systems the regulation of expression of MIPS-encoding genes has not been viewed as central to the regulation of phospholipid metabolism. Instead, interest in the regulation of Ins production has ranged from roles of Ins in pathways unique to the plant cell, such as in cell wall polysaccharide synthesis, raffinose/stachyose synthesis, and IAA conjugation (reviewed in Loewus and Murthy, 2000), to roles in hormone and stress response (Ishitani *et al.*, 1996; Smart and Fleming, 1993), and also to the localization of Ins P_6 synthesis and accumulation (Yoshida *et al.*, 1999). Thus, the coordinated expression of the pathway from glucose 6-P, through Ins, to its cyclitol derivatives D-ononitol and D-pinitol, involving MIPS, IMP, and also methylases and epimerases, is central to how the ice plant (*Mesembryanthemum crystallinum*) achieves salt/drought/cold tolerance (Ishitani *et al.*, 1996). The ice plant is used as a model system for studies of stress tolerance, but this role of Ins metabolism is presumed to be widespread in plant stress response. One component of the signal transduction pathway involved in plant stress response, plant guard cell response, and plant development, is the plant hormone abscisic acid, or ABA. Ins synthesis via rapid MIPS induction in response to ABA has been observed in studies of *Spirodela polyrrhiza* (Smart and Fleming, 1993).

Recently the *PINO1* gene of the salt-tolerant wild rice *Porteresia coarctata* (Roxb.) was found to encode a salt-tolerant MIPS enzyme, as compared with the MIPS encoded by the salt-sensitive cultivated rice (*Oryza sativa* L.) (Majee *et al.*, 2004). Comparison of MIPS sequences in these species revealed distinct differences in a 37 amino acid stretch, which upon deletion from the salt-tolerant MIPS rendered it salt-sensitive. Introgression of the *PINO1* gene via transformation rendered tobacco salt-tolerant. The genome of cultivated rice contains one gene encoding MIPS, termed *RINO1*. In addition to constitutive and

induced expression that must be essential for the variety of cellular, tissue, and organismal functions discussed above, *RINO1* expression is also induced at the site of $InsP_6$ accumulation in the developing seed (Yoshida *et al.*, 1999). Thus localized *de novo* synthesis of Ins contributes substrate, at least in part, for Ins P_6 accumulation in developing seeds. This was confirmed by the isolation of a mutation, "LR33," in a soybean MIPS gene that confers both a "low phytic acid" and "low raffinose/stachyose" seed phenotype (Hitz *et al.*, 2002). The soybean genome contains at least four MIPS-encoding sequences, one of which appears to have seed-specific expression (Hegeman et al., 2001). One interesting and at present unexplained observation concerning the LR33 mutation is that both germination rate and field emergence in a temperate environment like Iowa USA is greatly reduced when the seed itself was produced in a sub-tropical versus temperate environment (Meis *et al.*, 2003). Thus, subsequent field emergence of non-mutant control lines was 77% for seed produced in temperate environments and 83% for seed produced in sub-tropical environments, whereas field emergence was 63% for LR33 seed produced in temperate environments and only 8% for LR33 seed produced in sub-tropical environments. A similar effect of seed production environment, sub-tropical versus temperate, on subsequent germination rates was also observed. These studies reveal that seed-specific Ins production is critical to both germination and subsequent seedling emergence, but the exact effect of the block in Ins production on these processes is not yet known.

The maize genome contains up to seven sequences with homology to the canonical MIPS (Larson and Raboy, 1999). One of these, the maize chromosome 1S-MIPS, is linked to the maize *low phytic acid* 1 (*lpa*1) locus, also found on chromosome 1S (Raboy *et al.*, 2000). Homozygosity for recessive alleles of the maize *lpa*1 locus result in reductions in seed Ins P_6 ranging from 50% to >90% (Raboy *et al.*, 2001), but have little or no effect on seed total P, nor result in the accumulation of Ins phosphates with five or fewer phosphate esters, compounds that might be precursors to Ins P_6. Instead, the reductions in seed Ins P_6 are accompanied by increases in inorganic P, so that the sum of Ins P_6 and inorganic P in normal and *lpa*1 seed is similar. In contrast, maize *low phytic acid* 2 (*lpa*2) mutations cause reductions in seed Ins P_6 accompanied by increases in both seed inorganic P and in Ins phosphates with five or fewer esters, such as specific Ins *tris*-, tetra*kis* and penta*kis*phosphates (Raboy *et al.*, 2000). Based on these biochemical phenotypes, it was hypothesized that the maize *lpa*1 locus plays some role in substrate Ins supply in the developing seed, whereas maize *lpa*2 plays some role in the conversion of Ins to Ins P_6. This hypothesis has been confirmed by subsequent studies. In maize *lpa*1-1 seed (seed homozygous for the first recessive allele of the *lpa*1 locus), MIPS expression is reduced in proportion to the reduction in seed Ins P_6 (Shukla *et al.*, 2004). However, no change in the maize chromosome 1S MIPS-encoding sequence has been found. Thus maize *lpa*1 is a gene that is closely linked to the 1S MIPS and that impacts 1S MIPS expression. It might be a regulatory locus

that plays some role in the expression of many genes in the Ins and Ins phosphate pathways, or a sequence that specifically regulates MIPS expression. Maize *lpa*2 and the enzyme it encodes, an Ins(1,3,4)P_3 5-/6-kinase (Shi *et al.*, 2003), will be discussed in more detail below. Of relevance here is that maize *lpa*2 mutations represent blocks in the conversion of Ins to Ins P_6. As a result *lpa*2 mutant seed have elevated levels of free Ins, indicating that Ins P_6 synthesis in seed does represent a sink for seed Ins.

In terms of copy number of MIPS-encoding sequences, the barley genome is similar to rice, but differs from maize and soybean. The barley genome contains only one sequence with homology to the canonical MIPS gene, which maps to barley chromosome 4H (Larson and Raboy, 1999). Four barley *lpa* mutations have been studied and genetically mapped in some detail to date (Dorsch *et al.*, 2003; Larson *et al.*, 1998; Ockenden *et al.*, 2004): barley *lpa*1-1 whose expression has been shown to be aleurone-specific and which maps to barley chromosome 2H; barley *lpa*2-1 which maps to barley chromosome 7H and which is phenotypically similar to maize *lpa*2-1, in that reductions in seed Ins P_6 are accompanied by increases in Ins tetra*kis*- and penta*kis*phosphate; barley *lpa*3-1 which maps to barley chromosome 1H; and barley M955 (a gene symbol has not been assigned to this mutation yet), which also maps to chromosome 1H and which may be allelic to barley *lpa*3-1. Barley M955 is of particular interest since seeds homozygous for M955 have Ins P_6 levels reduced by $>$90%, as compared with non-mutant seeds, but retain near-wild type viability.

These chromosomal mapping results clearly demonstrate that none of these barley *lpa* mutations are MIPS mutations. Barley *lpa*1-1 seed have Ins levels similar to non-mutant seed, whereas in barley *lpa*2-1, *lpa*3-1, and M955 seed, Ins levels are twice that observed in non-mutant seed (Karner *et al.*, 2004). This confirms the results with maize *lpa*2 mutations (Shi *et al.*, 2003) that indicate that seed Ins P_6 synthesis does represent a significant sink for seed Ins.

In the barley *lpa* mutants *lpa*2-1 and M955, the elevation in seed Ins is accompanied by elevations in galactinol, raffinose and to a lesser extent sucrose (Karner *et al.*, 2004). This result is relevant to understanding the regulation of the synthesis of raffinose family oligosaccharides in seeds. This class of compounds is important in its own right in seed biology, protecting seed cellular structure during desiccation and serving as carbon reserves during germination. It is also important to the quality of seeds for their major end-use in foods. The synthesis of raffinosaccharides was thought to be regulated via the activity of a key synthetic enzyme, galactinol synthase (EC 2.4.1.123). However, the results with both the soybean MIPS mutation, where reduced Ins production results in reduced raffinosaccharides (LR33; Hitz *et al.*, 2002) and the barley mutations, where reduced conversion of Ins to Ins P_6 increases free Ins and increases raffinose, clearly indicate that substrate supply is at least as importance as enzymatic regulation.

The second enzyme in the Ins synthesis pathway, IMP, is important both to Ins synthesis and to recycling of Ins from Ins phosphates generated during

signal-transduction. IMP is inhibited by lithium, and inhibition studies have shown that suppression of IMP greatly impacts signal-transduction (Berridge and Irvine, 1989). In terms of Ins synthesis, studies such as those discussed above have focused on MIPS as the key regulatory site. In this context, IMP is often viewed as a constitutively expressed enzyme. Of relevance to this discussion, molecular genetics studies have shown that in all plant and non-plant organisms studied to date, IMP is encoded by a multigene family (Gillaspy *et al.*, 1995; Styer *et al.*, 2004). In plant systems detailed studies of IMP have used tomato (*Lycopersicon esculentum*) as the model system (Gillaspy *et al.*, 1995), and these studies have shown that at least two tomato IMP genes are differentially expressed (Styer *et al.*, 2004). The barley genome contains only copy of an IMP-encoding sequence (J. Fu, M. Guttieri, E. Souza, V. Raboy, unpublished results). Therefore it is incorrect to suggest that the multigene copy number of IMP is fundamentally important to differential expression and function in all higher eukaryote.

The molecular genetics studies of both IMP and MIPS-encoding sequences in various plant species provides for this review a first case study for questions pertaining to the biological role of gene copy number. First we must briefly review the general question of gene duplication. Genetics and genomics studies have shown that gene duplication is perhaps the rule rather than the exception, ranging from small duplications of single genes or parts of genes to large duplications of chromosomal sequence, to genomic doubling events and polyploidy. Both genomic doubling events and polyploidy are common in higher plants, whereas only genomic doubling is common in animals. In terms of sequences themselves, and not taking into account epigenetics, following duplication there are perhaps four alternative fates for each of a given ancestral gene's multiple copies (Force *et al.*, 1999; Lynch and Katju, 2004). First, in "nonfunctionalization," a copy might simply provide redundant function, and following accumulation of deleterious "loss-of-function" function mutations, become a non-functional "pseudo-gene." Second, in "neo-functionalization," a duplication may provide the genetic material necessary for the evolution of entirely new functions. Third, in "subfunctionalization," following the "duplication-degeneration-complementation" model, each of the two or more copies of an ancestral gene might assume subsets of that gene's original multiple functions. This last fate would include heritable events that lead to differences in tissue-specificity of gene expression. A fourth possibility might be relevant in plant biology, where polyploidy is so common. Perhaps a copy may provide redundant function, but that redundant function itself might add to an organism's robustness, and therefore be selected for. This fourth possibility has not been given a name following the "nonfunctionalization/neofunctionalization/subfunctionalization" model. Perhaps "superfunctionalization" would be appropriate.

In the case of MIPS, the multiple copy number observed in maize and soybean led to the hypothesis that a seed-specific MIPS is required in large-seeded cultivated species in order to provide sufficient substrate for additional nutrient

accumulation (Hegeman *et al.*, 2001). Smaller-seeded species like barley and rice do have only one copy of MIPS. However, it is not readily clear that the relatively small-seeded barley and rice are less fit in general terms than is the relatively closely related maize. Consideration of IMP gene copy number provides a second example of this argument. Tomato and Arabidopsis have three copies of IMP and barley has only one, but barley functions perfectly well in terms of fitness, the ability to make seeds and to do signal transduction. An alternative view is that the benefit of gene duplication in terms of fitness is genome-wide and not gene-specific. In this view, the evolutionary benefits of genome-duplication are in neo-functionalization and super-functionalization, and both non-functionalization and sub-functionalization are essentially neutral in terms of fitness. Barley represents an excellent model system for studies of Ins synthesis since in both the case of MIPS and IMP the complex regulation of the expression of an original, ancestral single-copy gene provides all the functions necessary for the organism's fitness. If there are two closely related species where in one species one copy of gene satisfies all needs, whereas in the sister species multiple copies exist and some have subsets of expression, or "tissue-specific" expression, and if both species clearly are robust, then the evolution of multiple copies and tissue-specific expression is clearly seen as neutral in terms of selection value.

4. INOSITOL TO INS P_6

A brief review of the pathways from Ins to Ins P_6, necessarily limited in details concerning any given enzyme or gene, will provide a framework for discussion of biology and function. In a pathway proposed by Biswas *et al.* (1978a), obtained from studies of Ins P_6 synthesis is mung bean seeds, the product of MIPS, Ins(3)P_1, is directly converted to Ins(1,3,4,5,6)P_5 via sequential phosphorylation catalyzed by a "phosphoinositol kinase" of which two electrophoretic forms were identified. The conversion of Ins(1,3,4,5,6)P_5 to Ins P_6 is then catalyzed by a phytic acid-ADP phosphotransferase (Biswas *et al.*, 1978b). This enzyme is now commonly referred to as Ins(1,3,4,5,6)P_5 2-kinase (Phillippy *et al.*, 1994). Phillippy *et al.* (1994) showed that in the presence of high Ins P_6 and high ADP, a soybean Ins(1,3,4,5,6)P_5 2-kinase could regenerate ATP. This provides biochemical evidence in support of the original hypothesis of Morton and Raison (1963) that Ins P_6 serves as a phosphate donor of sufficient transfer potential for the regeneration of ATP in seeds. This is a question of relevance to seed biology. During the initial stage of germination sufficient membrane integrity has not been established for ATP regeneration that requires generation of the proton-motive force. However, mature seed tissues do contain high concentrations of Ins P_6. The hypothesis that Ins P_6 might function as a high-energy phosphate bond substrate for regeneration of ATP was initially dismissed. More recent studies of pyrophosphate containing Ins phosphates (see

below) indicate that this early hypothesis was possibly more accurate than first thought.

A pathway to Ins P_6 that begins with Ins as initial substrate and proceeds through site-specific sequential phosphorylation steps of defined, soluble Ins phosphates (Figure 2 middle left), was described in studies of the cellular slime mold *Dictyostelium discoideum* (Stephens and Irvine, 1990), and of the monocot *Spirodela polyrhiza* (Brearley and Hanke, 1996a). The first step in these pathways is the phosphorylation of Ins at the "3" position via an Ins 3-kinase activity (EC 2.7.1.6.4; English *et al.*, 1966; Loewus *et al.*, 1982). Ultimately of course the source of Ins has to be via MIPS and IMP activity. Next, Ins(3)P_1 is converted to Ins(1,3,4,5,6)P_5 via sequential phosphorylation. The *Dictyostelium* and *Spirodela* pathways are similar in that they share the common intermediate Ins(3,4,6)P_3, but differ in their Ins *bis*phosphate intermediates (Ins(3,6)P_2 versus Ins(3,4)P_2) and Ins tetra*kis*phosphate intermediates (Ins(1,2,4,6)P_4 versus Ins(3,4,5,6)P_4). Finally, Ins(1,3,4,5,6)P_5 is converted to Ins P_6 via an Ins(1,3,4,5,6)P_6 2-kinase (Figure 3). There is little genetic or molecular evidence of an Ins 3-kinase, that converts Ins to Ins(3)P_1. There is one study (Drayer *et al.*, 1994; discussed below) of Ins phosphates in a phospholipase C-null *Dictyostelium* line that provides indirect genetic proof that a pathway proceeding entirely via soluble Ins phosphates and the intermediate Ins(3,4,6)P_3 actually exists.

The above pathway to Ins P_6 that proceeds entirely via soluble Ins phosphates has been described as the phospholipase C (PLC)-independent pathway, to distinguish it from the pathway to Ins P_6 in yeast described below (Stevenson-Paulik *et al.*, 2002). This PLC-independent pathway can be summarized as follows: Ins $\rightarrow$ Ins(3)P_1, catalyzed by Ins 3-kinase *or* glucose 6-P to Ins(3)P_1 catalyzed by MIPS; Ins(3)P_1 $\rightarrow$ Ins(3,4)P_2 or Ins(3,6)P_2 $\rightarrow$ Ins(3,4,6)P_3 $\rightarrow$ Ins(3,4,5,6)P_4 or Ins(1,3,4,6)P_4 $\rightarrow$ Ins(1,3,4,5,6)P_5, catalyzed by Biswas' "phosphoinositol kinases" or via a series of as yet not well described Ins monophosphate, *bis*phosphate and polyphosphate kinases; Ins(1,3,4,5,6)P_5 $\rightarrow$ Ins P_6 via a 2-kinase. The alternative routes between Ins(3)P_1 and InsP_5 in the slime mold versus plant pathways provide a first example of the importance or lack thereof of differences in Ins phosphate positional esters; i.e., Ins(3,4)P_2 versus Ins(3,6)P_2, or Ins(3,4,5,6)P_4 versus Ins(1,3,4,6)P_4. Generally in this field, every observed difference in positional esters is considered of biological significance. Is there any real biological significance to these particular differences?

There is growing genetic evidence that in many organisms Ins P_6 synthesis proceeds via pathways that involve the PtdIns phosphate intermediates PtdIns(4)P_1 and PtdIns(4,5)P_2, followed by PtdIns-specific phospholipase C activity and release of Ins(1,4,5)P_3 (Figure 2 middle right). Up to this point this represents the canonical signal-transduction pathway to Ins(1,4,5)P_3 (Berridge and Irvine, 1989). For this compound to function as a specific and transient signal, it must be rapidly metabolized. This can be accomplished by further phosphorylation, or by dephosphorylation, the later catalyzed by 1-phosphatase and

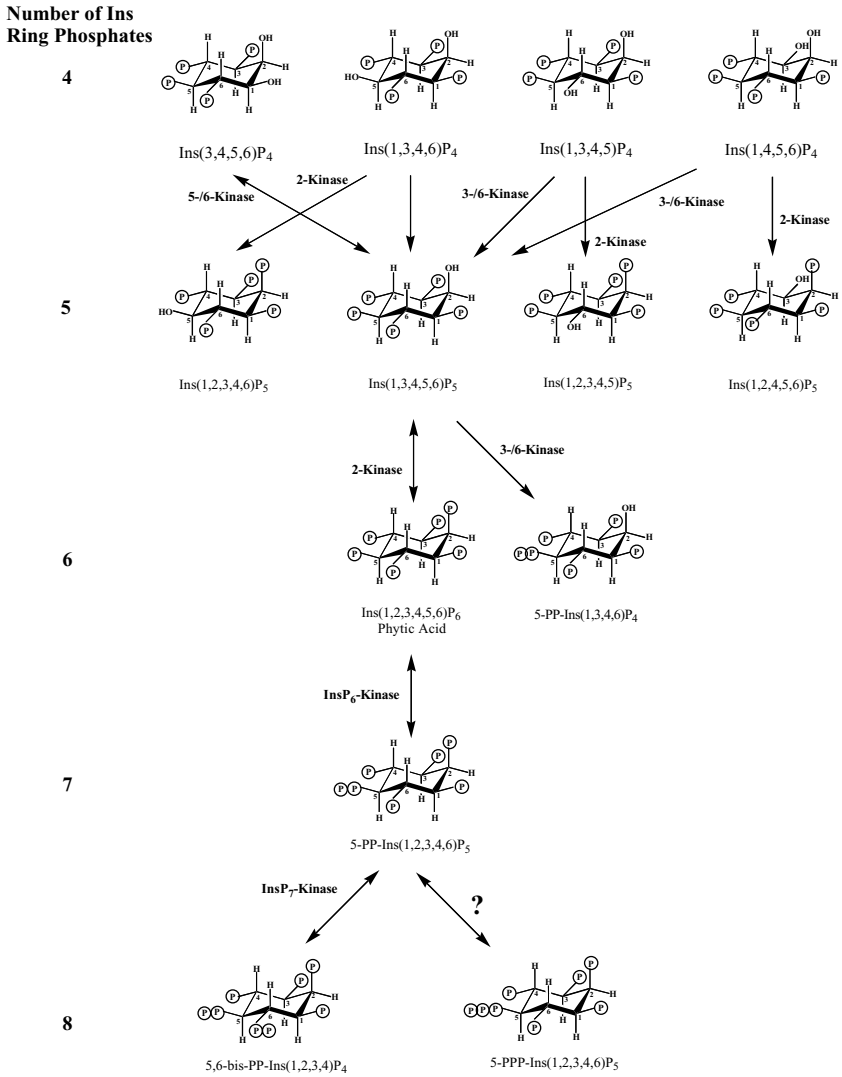

Figure 3. Biosynthetic pathways from *myo*-inositol (Ins) tetra*kis*phosphates to Ins(1,2,3,4,5,6)P_6 ($InsP_6$ or "phytic acid") and the pyrophosphate-containing Ins phosphates. The six carbons of the Ins ring are numbered according to the "D-numbering convention." Questionmarks indicate synthetic steps that have not been confirmed in chemical, molecular or genetic analyzes. In addition, the tri-phosphate-containing 5-PPP-Ins(1,2,3,4,6)P_5 is purely speculative. P = PH_2O_4.

5-phosphatases. There are a growing number of studies that provide genetic proofs that this second messenger pathway functions in plants in ways very similar to its function in other higher eukaryotes. A screen for *Arabidopsis* genes induced by the plant hormone ABA or by stress identified the *FIERY1* (*FRY1*) gene (Xiong *et al.*, 2001). This gene encodes an Ins polyphosphate

1-phosphatase. Mutation of this gene results in the accumulation of Ins(1,4,5)P_3 and in super-induction of ABA- and stress response. Mutations in the *Arabidopsis cotyledon vascular pattern2* (*cvp2*) gene perturb plant development, altering venation patterns (Carland and Nelson, 2004). The *cvp2* gene encodes an Ins polyphosphate 5-phosphatase. The *Arabidopsis* genome contains 15 putative Ins polyphosphate 5-phosphatases (Ercetin and Gillaspy, 2002). Thus there is ample substrate for the evolution of differential substrate specificity, and tissue/developmental/functional specificity of gene expression in signal termination in this plant genome.

In addition to its clear role in signal transduction in plants, Ins(1,4,5)P_3 also may be an important intermediate in pathways to Ins P_6. Depending on the tissue, cellular site or process, this Ins P_6 might function simply as a pool in Ins/PtdIns phosphate pathways, as an effector molecule itself, or as a storage metabolite. In a pathway to Ins P_6 expressed in the nucleus of yeast (York *et al.*, 1999), PtdIns(4,5)P_2 is hydrolyzed to yield Ins(1,4,5)P_3, which is then phosphorylated directly to Ins(1,3,4,5,6)P_5 by an Ins(1,4,5)P_3 3-/6-kinase. This enzyme is also referred in the literature as an IPMK and, in the yeast is encoded by the *IPK2* gene. To avoid confusion we will refer to this enzyme solely as Ins(1,4,5)P_3 3-/6-kinase. Stevenson-Paulik *et al.* (2002) isolated two *Arabidopsis thaliana* genes related to the yeast Ins(1,4,5)P_3 3-/6-kinase, termed AtIpk2α and AtIpk2β An independent study demonstrated that the second of these enzymes is primarily found in the plant nucleus, but is also detected at lower levels in the cytoplasm (Xia *et al.*, 2003). In yeast there is also genetic evidence (York *et al.*, 1999) that Ins(1,3,4,5,6)P_5 is the penultimate Ins phosphate in the pathway to Ins P_6 (Figure 3), and that its conversion is catalyzed by a 2-kinase similar to that described by Biswas *et al.* (1978a,b). The yeast Ins(1,3,4,5,6) 2-kinase is encoded by the *IPK1* gene (York *et al.*, 1999).

The essentials of the major pathway to Ins P_6 in yeast nuclei can be summarized as follows: Ins → PtdIns, catalyzed by PtdIns synthase; PtdIns →→PtdIns(4,5)P_2, catalyzed sequentially by two specific kinases; PtdIns(4,5)P_2→Ins(1,4,5)P_3, catalyzed by a PtdIns-specific phospholipase C; Ins(1,4,5)P_3→→Ins(1,3,4,5,6)P_5, catalyzed by an Ins(1,4,5)P_3 3-/6-kinase; Ins(1,3,4,5,6)P_5 → Ins P_6, catalyzed by an Ins(1,3,4,5,6)P_5 2-kinase. This is now viewed as the canonical PLC-dependent pathway. Recent genetics studies have shown that this pathway is also critical to Ins P_6 synthesis in *Drosophila* and rat cells (Fujii and York, 2004; Seeds *et al.*, 2004). In contrast, the sole genetic evidence in any organism for a PLC-independent pathway like that described above in *Dictyostelium* and *Spirodela*, one that proceeds solely via soluble Ins phosphates, consists of one elegant study using *Dictyostelium* (Drayer *et al.*, 1994). In this study the presence and levels of the whole series of Ins phosphates typical of a wild-type *Dictyostelium*, including Ins(1,4,5)P_3 and Ins P_6, where shown to be essentially identical in a PLC-null line. Thus some pathway to Ins(1,4,5)P_3 and Ins P_6 *independent of* PLC must exist in this organism.

A PLC-dependent pathway representing an alternative to the yeast PLC-dependent pathway has been described in studies of human cells (Chang *et al.*,

2002; Verbsky *et al.*, 2004; Wilson and Majerus, 1996). These studies described a pathway where Ins(1,4,5)P_3 is first converted to Ins(1,3,4,5)P_4 via an Ins(1,4,5)P_3 3-kinase. This enzyme's activity is very specific for the substrate Ins(1,4,5)P_3. Ins(1,3,4,5)P_4 is then converted to Ins(1,3,4)P_3 via a 5-phosphatase. The 3-kinases and 5-phosphatases are critical to signal transduction pathways in that their activity represents signal termination events, necessary for the transience of the second messenger Ins(1,4,5)P_3. Ins(1,3,4)P_3 is then phosphorylated to Ins(1,3,4,5,6)P_5 via what was first defined as an Ins(1,3,4)P_3 5-/6-kinase (Wilson and Majerus, 1996, 1997). At present there are no genetic proofs that an Ins(1,4,5)P_3 3-/6-kinase of the type identified in Arabidopsis by Stevenson-Paulik *et al.* (2002) is in fact important for the bulk of seed Ins P_6 synthesis or accumulation. That is, mutations in genes encoding an Ins(1,4,5)P_3 3-/6-kinase have not yet been shown to impact seed InsP_6 accumulation, as they have been shown to do in yeast. However, there is genetic evidence that an Ins(1,3,4)P_3 5-/6-kinase has some role in seed Ins P_6 accumulation. The maize *lpa*2 gene encodes an Ins(1,3,4)P_3 5-/6-kinase (Shi *et al.*, 2003). Homozygosity for recessive alleles of maize *lpa*2 reduce seed Ins P_6 by 30% (Raboy *et al.*, 2000). One class of barley "low-phytate" mutants, the "A-Type," accumulate Ins(1,3,4,5)P_4, and it has been proposed that these are mutations in an Ins(1,3,4)P_3 5-6-kinase (Hatzack *et al.*, 2001).

While plant genomes contain sequences encoding both Ins(1,4,5)P_3 3-/6-kinases and Ins(1,3,4)P_3 5-/6-kinases, the yeast genome apparently contains only an Ins(1,4,5)P_3 3-/6-kinase. However, careful analyzes of Ins phosphates in a yeast line null for Ins(1,4,5)P_3 3-/6-kinase indicate that they still accumulate Ins(1,3,4,5)P_4 and low levels of Ins P_6 (Saiardi *et al.*, 2000b). Therefore there must be a second, minor (in terms of yeast) route to Ins P_6 in this organism. The observation that a block in one particular branch of the Ins phosphate pathways reveals an alternative branch, is reminiscent of the findings of Acharya *et al.* (1998). In this study, a *Drosophila* null for Inositol polyphosphate 1-phosphatase (IPP), was shown to be incapable of metabolizing Ins(1,4)P_2, a step critically important to signal transduction via termination of the Ins(1,4,5)P_3 transient signal. However, *Drosophila* IPP nulls "demonstrate compensatory upregulation of an alternative branch in the inositol-phosphate metabolic tree, thus providing a means of ensuring continued availability of inositol" (Acharya *et al.*, 1998). In this alternative branch Ins(1,4,5)P_3 is first converted to Ins(1,3,4)P_3, which is then broken down via activity of a 4-phosphatase, a 3-phosphates, and ultimately the IMP described above.

Perhaps the alternative pathway to Ins(1,3,4,5)P_4 in the yeast Ins(1,4,5)P_3 3-/6-kinase null results from low levels of activity of other enzymes towards various Ins or PtdIns phosphate substrates found in yeast, in a parallel to the results of Acharya *et al.* (1998) concerning the *Drosophila* IPP null. For example, perhaps the accumulation of Ins(1,3,4,5)P_4 results from phospholipase C action on PtdIns(3,4,5)P_3. While no such phospholipase C activity has been documented in any organism, this hypothesis provides a way to discuss a

very interesting recent study of PtdIns(3,4,5)P_3 in the fission yeast, *Schizosaccharomyces pombe* (Mitra *et al.*, 2004).

PtdIns(3,4,5)P_3 is a lipid critically important to signal transduction in mammalian cells, and is synthesized from PtdIns(4,5)P_2 primarily via the activity of "class I" phosphoinositide 3-kinases (Cantley, 2002). Thus the pathway to PtdIns(3,4,5)P_3 would proceed as follows: PtdIns $\rightarrow$ PtdIns(4)P_1 $\rightarrow$ PtdIns(4,5)P_2 $\rightarrow$ PtdIns(3,4,5)P_3, the last step catalyzed by the "class I" phosphoinositide 3-kinase. Until recently the lipid PtdIns(3,4,5)P_3 was not observed in yeast cells, and the yeast genome lacks a "class I" phosphoinositide 3-kinase gene. Just as cells need a mechanism to both generate and efficiently breakdown Ins(1,4,5)P_3 if it is to serve as a transient second messenger, mammalian cells express a phosphatase, encoded by the PTEN ("phosphatase and tensin homologue deleted on chromosome 10"), that dephosphorylates PtdIns(3,4,5)P_3, and that is critical to its role in the regulation of cellular development. The fission yeast genome was found to contain one sequence with homology to PTEN, and its deletion resulted in accumulation of PtdIns(3,4,5)P_3. This prompted a re-examination of the pathway to PtdIns(3,4,5)P_3 in the fission yeast. Wild-type fission yeast cells make PtdIns(3,4,5)P_3 via an alternative pathway to that observed in mammalian cells, one that does not utilize "class 1" phosphoinositide 3-kinase. Instead, fission yeast first convert PtdIns to PtdIns(3)P_1, via the activity of a "class III" PtdIns 3-kinase. PtdIns(3)P_1 is then sequentially phosphorylated to yield PtdIns(3,4,5)P_3, whose metabolism via PTEN activity is so rapid that there is no detectable steady state levels. Since the pathway from PtdIns(3)P_1 to PtdIns(3,4,5)P_3 existed in yeast prior to the evolution of "class I" PtdIns 3-kinases in higher eukaryotes, in terms of evolution it has a more ancient function.

The study of Mitra *et al.* (2004) illustrates that the lack of observation of a given compound does not represent proof that that compound has no role in a given organism or tissue. Prior lack of detection of PtdIns(3,4,5)P_3 in yeast simply reflected the fact that this compound is rapidly metabolized, and therefore doesn't accumulate to any detectable steady-state levels. The "class III" PtdIns 3-kinase was first identified as the protein encoded by yeast *vps34* gene, a mutant of which was first isolated in a screen for "vacuolar protein sorting" mutants. The *A. thaliana* genome contains a homolog of this gene, *AtVps34* (Welters *et al.*, 1994). Reduced expression of the *A. thaliana* homolog, achieved via transformation with and expression of an "anti-sense" construct, resulted in severely inhibited growth and development. Clearly, the PtdIns 3-kinase function this gene encodes is important to plant growth and development.

It is interesting that yeast accumulates the linear polyphosphate form of storage phosphate, also found in prokaryotes, but plant cells do not. This suggests one possible explanation for the lack of Ins(1,3,4)P_3 5-/6-kinases in yeast. Perhaps the pathway to Ins P_6 via the Ins(1,4,5)P_3 3-/6-kinase route is primarily nuclear and found in all eukaryotes, whereas the pathway to InsP_6 via the

Ins(1,3,4)P_3 5-/6-kinase route is cytoplasmic, found in those eukaryotes that synthesize Ins P_6 as a non-nuclear storage or inert cellular deposit, and only lost in yeast since this organism uses an alternative pathway to store or sequester excess cellular P.

A recent structural study of the Ins(1,4,5)P_3 3-kinase illustrated that a unique lobe contains four helixes that embrace each of the phosphates in Ins(1,4,5)P_3, explaining both why this enzyme is so substrate specific, and why it cannot phosphorylate PtdIns(4,5)P_2, the "membrane-resident analog of Ins(1,4,5)P_3" (González *et al.*, 2004). Genes encoding this enzyme, along with those encoding the Ins(1,4,5)P_3 3-/6-kinases and the Ins P_6 kinases discussed below, all belong to one gene family descendent from an ancestral Ins phosphate kinase-encoding sequence (Schell *et al.*, 1999). The study of Schell *et al.* (1999) is relevant to this discussion for two reasons. A rabbit cDNA was found to stimulate inorganic P uptake upon injection into a *Xenopus* oocyte, and was termed *Pi Uptake Stimulator* (*PiUS*). This cDNA was found to encode a sequence with homology to Ins(1,4,5)P_3 3-kinase, but in fact the enzyme it encoded had InsP_6 kinase activity. This indicates a link between cellular phosphate nutrition and Ins phosphate metabolism. This link has subsequently been further emphasized by the studies of Almaguer *et al.* (2003, 2004) concerning glycerophosphoinositols, and Steger *et al.* (2003) and El Alami *et al.* (2003), that link Ins phosphates and *Pho* gene expression, as discussed above.

Second, Schell *et al.* (1999) conclude that the Ins(1,4,5) 3-kinase is in evolutionary terms the youngest member of this family of Ins phosphate kinases, its evolution representing a late addition that followed the evolution of Ins(1,4,5)P_3 as a second messenger. An alternative interpretation might be that that the evolution of the function of Ins(1,4,5)P_3 as a second messenger followed the earlier evolution of functions having to do with basic cellular structure and nutrition. This parallels the discussion of Mitra *et al.* (2004) concerning the evolution of the pathway to PtdIns(1,4,5)P_3 in yeast. In that case too what was thought of as the classic signal transduction pathway evolved subsequent to other, more ancient pathways involving these molecules.

The Ins(1,3,4)P_3 5-/6-kinase-encoding genes belong to a second separate and distinct family of Ins phosphate kinases (Cheek *et al.*, 2002). While plant genomes contain Ins(1,4,5)P_3 3-/6-kinases and Ins P_6 kinases, they do not contain members of the gene family encoding the Ins(1,4,5)P_3 3-kinase. How can this be reconciled with the fact that there is solid genetic evidence that Ins P_6 accumulation in seeds requires at least in part a pathway involving Ins(1,3,4)P_3 5-/6-kinase, a pathway that in human cells is thought to require Ins(1,4,5)P_3 3-kinase in order to produce substrate for the Ins(1,3,4)P_3 5-/6-kinase?

There is at least one plausible explanation for the fact that plant genomes don't contain Ins(1,4,5)P_3 3-kinase but require Ins(1,3,4)P_3 5-6-kinase for Ins P_6 production, at least in part. While the Ins(1,4,5)P_3 3-kinase only recognizes Ins(1,4,5)P_3 as substrate, in contrast, both the Ins(1,3,4)P_3 5-/6-kinase and the

Ins(1,4,5)P_3 3-/6-kinase can phosphorylate multiple Ins phosphates. The Ins(1,4,5)P_3 3-/6-kinase has been referred to as a "multikinase," but the Ins(1,3,4)P_3 5-/6-kinase also has "multikinase" and phosphatase activities. For example, the Ins(1,4,5)P_3 3-/6-kinase also can have 5-kinase activity (Chang *et al.*, 2002; Stevenson-Paulik *et al.*, 2002), and can also catalyze the conversion of Ins(1,3,4,5,6)P_5 to a pyrophosphate-containing "5-diphosphoinositol Ins(1,3,4,5)P_4," a "non-phytic acid" InsP_6 (Figure 3; Saiardi *et al.*, 2001a). Similarly, the Ins(1,3,4)P_3 3-/6-kinase also has 1-kinase activity (Yang and Shears, 2000), and can function as a reversible kinase/phosphatase. These two types of Ins polyphosphate mutlikinases can first be distinguished on the basis that one class recognizes Ins(1,4,5)P_3 as substrate (the Ins(1,4,5)P_3 3-/6-kinases) and the second recognizes Ins(1,3,4)P_3 (the Ins(1,3,4)P_3 5-/6-kinases). However, each kind of kinase actually recognizes a unique set of Ins phosphates that ultimately have structural similarities to these two different Ins *tris*phosphates (reviewed in Shears, 2004; Saiardi *et al.*, 2000b, 2001a; Yang and Shears, 2000). It has been hypothesized that the individual Ins phosphates comprising each set contain functionally similar "recognition motifs" consisting of a specific distribution of phosphate and hydroxyl moieties around the Ins ring. One example of this that is relevant to this discussion is that if one rotates and flips Ins(1,3,4)P_3, the distribution of phosphates and one hydroxyl group appear similar to that of Ins(3,4,6)P_3 (Fig. 4). Thus Ins(1,3,4)P_3 5-/6-kinase can convert Ins(1,3,4)P_3 to Ins(1,3,4,6)P_4, but might also be an Ins(3,4,6)P_3 1-kinase, converting it to Ins(1,3,4,6)P_4. Taking the above into consideration, it is possible that one, or perhaps both of these kinases working together, might be able to convert Ins(3,4,6)P_3 to Ins(1,3,4,5,6)P_5. Thus a pathway to Ins P_6 that proceeds entirely via soluble Ins phosphates might in fact utilize enzymes thought of as being part of the PtdIns-intermediate pathway to Ins P_6 (Raboy, 2003; Stevenson-Paulik *et al.*, 2002). These enzymes might in fact represent the two isoforms of "phosphoinositol kinase" first described by Biswas *et al.* (1978a,b). However, it is important to emphasize that to date no single enzyme has been identified that is analogous to Biswas' phosphoinositol kinase, in that it can convert Ins(3)P_1 to Ins(1,3,4,5,6)P_5.

Except for the lack of detailed studies in plant systems, Ins phosphates more highly phosphorylated than Ins P_6, containing pyrophosphate moieties, such as 5-diphosphoinostol(1,2,3,4,6)P_5 ("PP-InsP_5," an Ins P_7) and bis-diphosphoinositol(1,2,3,4)P_4 (bis-PP-InsP_4, an Ins P_8; Figure 3 bottom), have been documented to occur relatively widely in eukaryotic cells (Laussmann *et al.*, 2000; Safrany *et al.*, 1999; Stephens *et al.*, 1993). Enzymes that can generate Ins pyrophosphates include Ins P_6 kinases that produce PP-InsP_5 (Saiardi *et al.*, 1999, 2000a, 2001b), and PP-InsP_5 kinases that produce bis-PP-InsP_4 (Huang *et al.*, 1998; Figure 3 bottom). Ins P_6 kinases appear to be members of the Ins(1,4,5)P_3 3-/6-kinase family that also includes Ins(1,4,5)P_3 3-kinase. There has to date been very little progress in the study of these pyrophosphate-containing compounds in plant systems, with few reports and little detailed

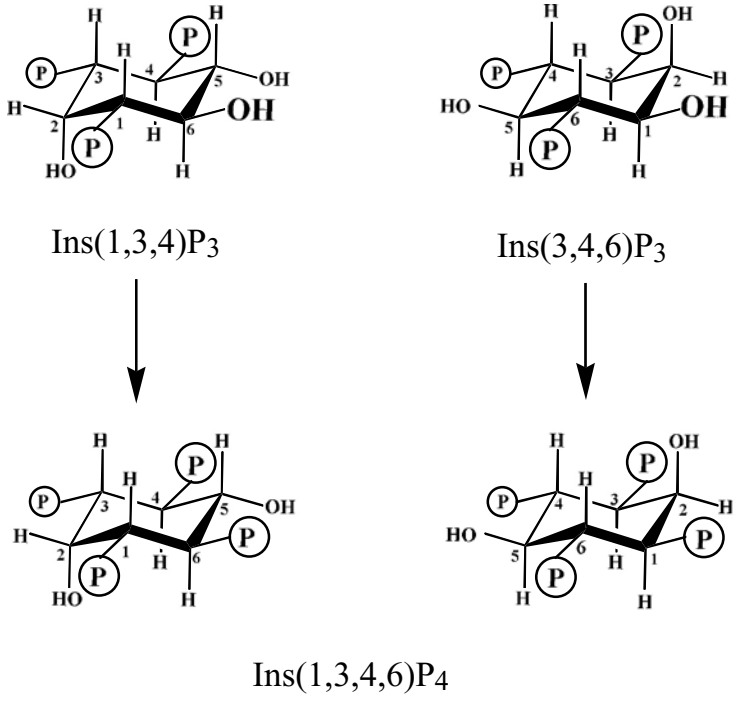

Figure 4. Two proposed modes of action for the *myo*-inositol(1,3,4)P_3 "5-/6-kinase" (Shears, 2004). This enzyme was originally defined as an Ins(1,3,4)P_3 5-/6-kinase, and the 6-kinase step is shown to the left. If one rotates and flips Ins(3,4,6)P_3, as shown to the right, the distribution of phosphates and hydroxyl groups, representing recognition sites for the enzyme, appears similar to that of Ins(1,3,4)P_3. This enzymes putative 1-kinase activity using Ins(3,4,6)P_3 as substrate is shown to the right. P = PH_2O_4.

evidence documenting their presence (Brearley and Hanke, 1996b; Dorsch *et al.*, 2003; Flores and Smart, 2000). There is at present no genetics or molecular genetics of pyrophosphate-containing Ins phosphates in plant systems of any kind.

The PP-InsP_5 kinase of Huang *et al.* (1998) can also act in the reverse direction, using bis-PP-InsP_4 as a phosphate donor in the regeneration of ATP from ADP, "an indication of the high phosphoryl group transfer potential of bis-PP-InsP_4." This is very reminiscent of the original hypothesis of Morton and Raison (1963) that InsP_6 could serve as a donor for the regeneration of ATP, and the subsequent work of Biswas *et al.* (1978a,b) and Phillippy *et al.* (1994) demonstrating that what became known as the Ins(1,3,4,5,6)P_5 2-kinase could, under the right substrate concentrations (high Ins P_6, high ADP), catalyze the reverse reaction and generate ATP from ADP. A second possible function for the pyrophosphate-containing Ins phosphates is that they might bind as effectors that bind to various proteins, acting as a molecular switch in signal transduction (reviewed in Shears, 2004). One example of this is the role Ins phosphates may play as

effectors in the *PHO* pathway in yeast. El Alami *et al.* (2003) demonstrated that activity of the yeast InsP_6 kinase encoded by the *KCS1* gene is required for repression of *PHO* genes. A third possibility is that pyrophosphate-containing Ins phosphates might indeed serve in protein activation but not directly as effectors that bind to protein. Rather, it has recently been shown that they can non-enzymatically phosphorylate, and thus activate, eukaryotic proteins (Saiardi *et al.*, 2004). A fourth possibility pertains to the classical function of pyrophosphate breakdown. The tri-phosphate-containing 5-triphosphoinositol(1,2,3,4,6)P_5 (another potential Ins P_8 illustrated in Figure 1 bottom right; Shears, 2004) is purely speculative, and has not been documented to exist in any species. It is shown here because its hydrolysis might directly yield pyrophosphate and Ins P_6, providing a model for Ins P_6 transport across a membrane, where pyrophosphate breakdown is used as a driving force (discussed below).

Mammalian "diphosphoinositol polyphosphate phosphohydrolases" ("DIPPs," Figure 1 bottom left) have been identified that cleave Ins pyrophosphates back to Ins P_6 (Caffrey *et al.*, 2000; Hidaka *et al.*, 2002; Hua *et al.*, 2003; Safrany *et al.*, 1998). However Ins P_6 kinases can act in the reverse direction, utilizing Ins pyrophosphates as phosphate donor to regenerate ATP from ADP (Huang *et al.*, 1998), whereas DIPPS act only in Ins pyrophosphate breakdown.

5. LOCALIZATION AND DEPOSITION OF INS P_6 IN THE SEED

The bulk of Ins P_6 that accumulates in seeds is deposited as a mixed salt in discreet inclusions referred to as globoids (Lott, 1984; Raboy, 1997). In the cereal grains Ins P_6 is deposited primarily as a mixed a salt of K and Mg. Globoids are found in one class of storage microvacuoles referred to as Protein Storage Vacuoles (PSVs). PSVs might primarily contain storage proteins, or mixed salts of Ins P_6, or both. In many plant species Ins P_6 deposits occur in specific tissues of the seed. Of particular relevance here is that in the cereal grains, most Ins P_6 deposition occurs within the aleurone layer, the outer layer of the endosperm, and within the germ, consisting of the embryo and scutellum. In normal cereal grains, the central starchy endosperm contains little or no phosphate or Ins P_6 at maturity. Interestingly, even though the cotyledonary tissues of the legume yellow lupine seed are not as differentiated as are the germ and aleurone layer of cereal grains, Ins P_6 deposits still are concentrated in several outer layers of cells (Sobolev *et al.*, 1976). Even though these outer parenchyma cells are otherwise indistinguishable from the inner parenchyma of the cotyledon, they do form a tissue analogous to the cereal aleurone layer, in that they accumulate Ins P_6.

Ogawa *et al.* (1979) demonstrated that early in the development of the rice grain, phosphate, K and Mg are evenly distributed throughout the tissues of the seed. As development progress phosphate becomes more concentrated in the

germ and aleurone layer, first accompanied by Mg and later by K. It was hypothesized that is differential timing of deposition of Mg and K simply reflects the changing physiological needs of the developing seed. Thus in seeds, Ins P_6 deposition is intimately linked with mineral storage and deposition. The mechanism by which Ins P_6 synthesis is localized within the developing seed may be central to how mineral deposits are localized. Many other mineral cations can be found as salts of Ins P_6, including Fe, Ca, Mn, and Zn (Lott, 1984). In a study of mineral deposition in developing *A. thaliana* seeds, Otegui *et al.* (2002) showed that while PSVs accumulate K/Mg Ins P_6 salts that are retained till maturity, transient deposits of Mn and Zn salts of Ins P_6 occur during different stages of seed development and are subsequently remobilized. Thus in seeds Ins P_6 appears to function as a counter-ion to minerals and heavy metals, functioning both in transient sequestration and long-term deposition, and the regulation of its synthesis may determine patterns of localization.

Many questions remain as to how Ins P_6 accumulation is localized in seed tissues, how its synthesis is compartmentalized within a given cell, and how deposits are compartmentalized in globoids. One study indicated that Ins P_6 deposits are first observed in the cytoplasm, possibly in association with the endoplasmic reticulum, but that these deposits are remobilized for ultimate deposition with in globoids within PSVs (Greenwood and Bewley, 1984). This study would indicate that Ins P_6 is synthesized in the cytoplasm, and transported for deposition in the globoid. While the focus in this discussion is the plant seed, Ins P_6 deposition is not restricted to the seed in plants, nor to higher plants. Other plant tissues that accumulate nutrient stores, or enter a resting stage and subsequently germinate, such as roots, tubers, turions, and pollen, accumulate Ins P_6 (reviewed in Raboy, 1997). While there is no evidence for deposition of Ins P_6 salts as discrete inclusions in human or yeast cells, recent studies have shown that the parasitic cestode *Echinococcus granulosus* synthesizes Ins P_6, then secretes it into its hydatid cyst wall, depositing it as discreet inclusions consisting of a Ca salt, similar to Ins P_6 mixed salt deposits in plant cells as globoids (Irigoin *et al.*, 2002, 2004).

One mechanism for the localization of Ins P_6 synthesis in a given tissue is via the localized coordinated expression of genes encoding biosynthetic enzymes. Thus Yoshida *et al.* (1999) demonstrated that MIPS expression is localized to the site of Ins P_6 within the rice seed. Shi *et al.* (2003) demonstrated that expression of the maize *lpa*2 gene which encodes an Ins(1,3,4)P_3 5-/6-kinase appears specific to the developing germ tissue. Also, the barley *lpa*1-1 mutation impacts Ins P_6 accumulation in the barley aleurone layer, but has not effect on germ tissue, thus expression of the gene it encodes is tissue specific. While Ins P_6 synthesis probably occurs in most cells of the plant, its accumulation and deposition also represents one component of embryogenesis, and as such is regulated by coordinated gene expression. The *A. thaliana Pickle* (*PKL*) gene encodes a chromatin remodeling factor that normally functions in vegetative tissues to suppress the expression of genes involved in embryogenesis (Ogas

et al., 1999). In *pkl* mutants derepression of embryogenesis-related genes results in the expression of embryonic traits in roots, producing "pickle roots." Rider *et al.* (2004) recently demonstrated that this includes increased accumulation of Ins P_6, as compared with normal roots.

If tissue-specific expression of genes contributes to the localization of Ins P_6 synthesis in plant seeds tissues, how is this localized within the cell, and how does deposition of mixed Ins P_6 salts within globoids occur? Figure 5 provides a model for the synthesis of Ins P_6, and its transport with minerals into the PSVs. There is some argument as to whether or not globoids are membrane-bound. Most recently Jiang *et al.* (2001) have provided evidence that globoids are contained within a membrane-bound compartment that itself is contained within the PSV, making the PSV a "compound organelle." For the purposes of this discussion, only a single membrane-bound compartment will be considered. First, water must be transported into the PSV or globoid-containing compartment. This is accomplished via transport through "aquaporins," one of the several isoforms of "Tonoplast Intrinsic Proteins" (TIPs). TIPs are encoded by a large gene family. Different TIPs define functionally distinct vacuoles, and one or more TIPs are probably specific to the globoid containing PSV (Jauh *et al.*, 1999; Takahashi *et al.*, 2004). Second, the membrane delimiting the PSV

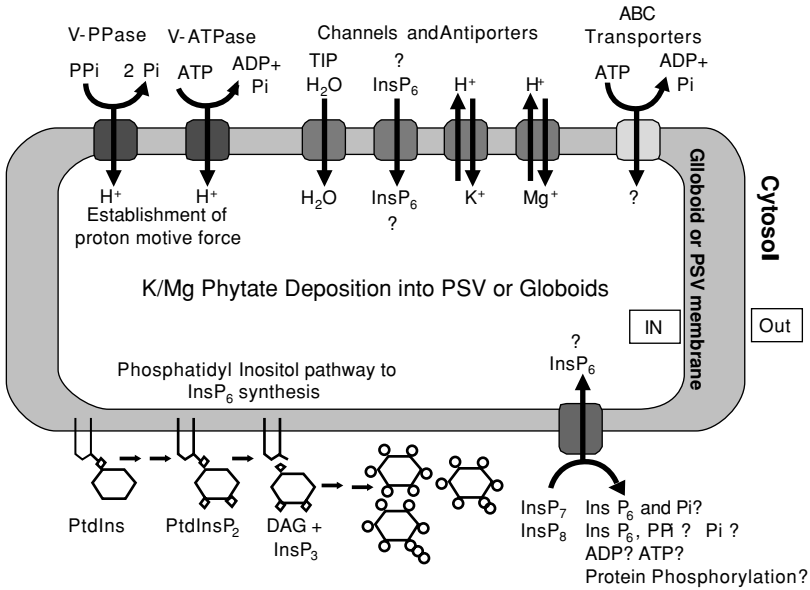

Figure 5. Components and processes of a membrane-bound organelle, representing either a Protein Storage Vacuole (PSV) or a membrane-bound globoid found within a compound PSV. V-PPase = vacuolar inorganic pyrophosphatase. V-ATPase = vacuolar ATPase. TIP = Tonoplast Intrinsic Protein. PtdIns = phosphatidylinositol. DAG = diacylglycerol. Questionmarks indicate purely speculative aspects of the diagram.

(or globoid) is known to contain the vacuolar pyrophosphatase (V-PPase; Jiang *et al.* 2001), which breaks down inorganic pyrophosphatase and in so doing pumps protons into the internal vacuolar space (Maeshima, 2000). Similarly, the vacuolar ATPase breaks down ATP and thereby pumps protons into the vacuolar internal space. Thus a concentration gradient of protons is established that can, via channels and antiporters, drive transport of various solutes from the cytoplasm into the PSV. These solutes must include the counterions K and Mg, and possibly other minerals. Additionally, ABC transporters, which directly use the energy provided by ATP breakdown to transport a variety of solutes, might play some role (Jasinski *et al.*, 2003; Martinoia *et al.*, 2000).

A genetics study of *Arabidopsis* lines that differ quantitatively in the levels of phosphate and Ins P_6 in vegetative and seed tissues identified a Quantitative Trait Locus (QTL) that accounts for a significant amount of the variation observed (Bentsink *et al.*, 2003). Contained within the 99-kb chromosomal segment represented by this QTL were 13 ORFs, one of which encoded a putative V-ATPase. Bentsink *et al.* (2003) hypothesized that the variation in phosphate and Ins P_6 levels observed among the *Arabidopsis* lines in their study was in large part due to variation in phosphate transport caused by heritable differences the V-ATPase, providing experimental evidence for this aspect of the model described in Figure 5.

A pathway to Ins P_6 that proceeds via PtdIns phosphates suggests an mechanism for localization to a specific membrane (Figure 5). However, the question remains as to how Ins P_6 is transported into the PSV or the globoid? Ins polyphosphates containing pyrophosphate moieties may play a key role in this transport. This role could be metabolic. Synthesis of an Ins P_7 or Ins P_8 might first occur in the cytoplasm. Cleavage of the pyrophosphate moiety might then directly drive transport of Ins P_6 into the internal compartment, in a fashion analogous to the function of V-PPases. However, Ins pyrophosphates could also function directly via protein phosphorylation (Saiardi *et al.*, 2004). In this alternative, the Ins pyrophosphate directly phosphorylates the channel protein, resulting in its activation/opening and transport of Ins P_6 to the internal compartment, in a fashion analogous in some ways to Ins (1,4,5)P_3 binding proteins.

If the localization of InsP_6 synthesis is critical to patterns of mineral deposition in the developing seed, then one might hypothesize that the pattern of mineral deposition would be altered in *lpa* mutants. For example, in cereal grains the bulk of Ins P_6 and most minerals is found in the germ and aleurone. The central starchy endosperm normally has very low levels of these seed components. A simple hypothesis would then be that in *lpa* mutants the block in Ins P_6 synthesis would also block the localization of mineral deposition in the germ and aleurone layer, increasing the levels of these minerals in the central endosperm. In a first analysis (Liu *et al.*, 2004) of mineral deposition in the rice *lpa*1-1 mutant (Larson *et al.*, 2000) seeds were dissected into two fractions: a germ fraction including the embryo and scutellum, versus a "rest-of-grain" fraction including the endosperm and aleurone layer. The concerntrations of

phosphorus and minerals in these two fractions were similar in normal versus *lpa*1-1 seed. However, this experimental approach cannot detect differences between the central endosperm and aleurone layer, since both were contained in the "rest-of-grain" fraction. A second approach could provide such a distinction. In this second approach, normal and *lpa* grain are milled to varying degrees. Milling produces milled products either enriched in central endosperm, such as white rice, or enriched in aleurone and germ, such as the bran fraction. Analysis of milling fractions in normal versus *lpa*1-1 rice did indicate that the concentrations of K and Mg, but not other minerals such as Fe and Zn, were in fact higher in *lpa*1-1 milled rice (white rice), as compared with white rice produced from the non-mutant control (Bryant *et al.*, 2005). This provides evidence that the localization of mineral deposits in seeds, particularly with reference to K and Mg, is in some part dependent on Ins P_6 synthesis. Since milled rice and wheat products, like white rice and refined wheat flour, are so critical to human nutrition world-wide, enhancing the mineral concentration in milled products could provide an important tool for the improvement of nutritional quality. The increase in K and Mg in white rice produced from the *lpa*1-1 mutant, as compared with that produced from normal rice, where moderate. A similar study of the first wheat *lpa*1-1 mutant (Guittieri *et al.*, 2004) found no substantial changes in mineral distribution in wheat milled products. However, the reduction in seed Ins P_6 in both the first rice and wheat *lpa* mutants are moderate (about 45%). Therefore, additional analyzes of mineral distribution in wheat or rice *lpa* mutants that have more substantial reductions in seed Ins P_6, such as those like the barley M955 mutation, along with analyzes of the set of barley mutants,(Dorsch *et al.*, 2003) would provide a test for the role of Ins P_6 synthesis and mineral deposition in seeds.

6. CONCLUSION

The closer one looks, the biology of Ins, phosphate, PtdIns phosphates, and Ins phosphates, do appear more similar than different across widely divergent organisms. It is possible that distinct pathways exist to Ins P_6 in nuclei versus cytoplasm, but there really isn't strong evidence that divergent eukaryotes differ greatly in their use of these pathways. The differences observed might represents exceptions, not rules. It is possible that the perception of differences results from studies that proceed using paradigms for certain functions and pathways. For example in yeast, the regulation of Ins synthesis was first viewed as critical to lipid metabolism, but now it is becoming clear that the regulation of the yeast *INO1*gene is linked to phosphate nutrition as well. Perhaps in plant systems it will turn out that the regulation of Ins synthesis has importance to lipid metabolism, just as it does in yeast.

The field of Ins phosphate/PtdIns phosphate metabolism and biology has benefited greatly, in fact is largely defined, by the ability to distinguish different

positional isomers of these various phosphate esters. However, this paradigm might obscure at times the irrelevance of some differences. Similarly, the signal-transduction paradigm might obscure at times the perhaps more central roles of these metabolites in basal cellular biology.

Finally, new studies often illuminate the importance of pathways and metabolites that simply were not initially observed in first-generation studies. A great example of this is the study of Martin *et al.* (2000) which showed that the Ins phosphate pathways in the amoeba *Entamoeba histolytic* in fact use *neo*-inositol and not *myo*-inositol as the backbone for Ins phosphate synthesis. This suggests that a reanalysis of basic assumptions, leading to a new synthesis of the nature and role of these pathways, might be achieved in the coming years.

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