Origin and Seed Phenotype of Maize *low phytic acid 1-1* and *low phytic acid 2-1*[1]

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Phytic acid (*myo*-inositol-1, 2, 3, 4, 5, 6-hexa*kis*phosphate or Ins P_6) typically represents approximately 75% to 80% of maize (*Zea mays*) seed total P. Here we describe the origin, inheritance, and seed phenotype of two non-lethal maize *low phytic acid* mutants, *lpa1-1* and *lpa2-1*. The loci map to two sites on chromosome 1S. Seed phytic acid P is reduced in these mutants by 50% to 66% but seed total P is unaltered. The decrease in phytic acid P in mature *lpa1-1* seeds is accompanied by a corresponding increase in inorganic phosphate (P_i). In mature *lpa2-1* seed it is accompanied by increases in P_i and at least three other *myo*-inositol (Ins) phosphates (and/or their respective enantiomers): D-Ins(1,2,4,5,6) P_5; D-Ins (1,4,5,6) P_4; and D-Ins(1,2,6) P_3. In both cases the sum of seed P_i and Ins phosphates (including phytic acid) is constant and similar to that observed in normal seeds. In both mutants P chemistry appears to be perturbed throughout seed development. Homozygosity for either mutant results in a seed dry weight loss, ranging from 4% to 23%. These results indicate that phytic acid metabolism during seed development is not solely responsible for P homeostasis and indicate that the phytic acid concentration typical of a normal maize seed is not essential to seed function.

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate or Ins P_6, Fig. 1A) is the most abundant P-containing compound in mature seeds, typically representing from 65% to 80% of the mature seed's total P (Cosgrove, 1980; Raboy, 1997). In the mature maize (*Zea mays*) seed, most (>80%) of the phytic acid is found in the germ with the remainder in the aleurone layer (O'Dell et al., 1972). In normal nonmutant seeds, phytic acid P typically represents >95% of total, acid-extractable *myo*-inositol (Ins) phosphates. Substantial quantitative variation in seed phytic acid P has been observed among genotypes, lines, or cultivars of several crop species. However, in these earlier studies the relationship between seed total P and phytic acid P was not observed to vary greatly, with the correlation between seed phytic acid P and seed total P typically ≥95% (Raboy, 1990).

In the context of plant and seed biology, phytic acid has been viewed primarily as a P and mineral storage compound or as an important metabolite in P homeostasis (Strother, 1980; Lott, 1984; Raboy, 1997). Regulation of cellular inorganic phosphate (P_i) concentration may play an important role in starch synthesis and accumulation and in the function of other metabolic pathways (Strother, 1980). Recent studies have shown that phytic acid may be ubiquitous in eukaryotic cells and that phytic acid and certain Ins penta*kis*phosphates typically represent the most abundant Ins phosphates in cells (Sasakawa et al., 1995; Safrany et al., 1999).

The biosynthetic pathway to phytic acid can be summarized as consisting of two parts: Ins supply and subsequent Ins polyphosphate synthesis (Fig. 1C). The sole synthetic source of the Ins ring (Fig. 1B) is the enzyme Ins(3) P_1 synthase (MIPS), that converts Glc-6-P to Ins(3) P_1 (Fig. 1C, step 1; Loewus and Murthy, 2000). Proximal MIPS activity in the developing seed may provide Ins as Ins(3) P_1 (Yoshida et al., 1999), which then may be converted directly to phytic acid via sequential phosphorylation by two or more kinases (Biswas et al., 1978; Stephens and Irvine, 1990; Fig. 1C, step 4). The Ins backbone for phytic acid may also derive in part from MIPS activity at distal vegetative sites, followed by Ins translocation to the developing seed (Sasaki and Loewus, 1990). The first Ins phosphorylation step would then be catalyzed by the enzyme Ins kinase, which also produces Ins(3) P_1 (English et al., 1966; Loewus et al., 1982; Fig. 1C, step 3). A pathway to phytic acid that begins with Ins as initial substrate and Ins kinase activity and proceeds through sequential phosphorylation steps via defined intermediates, was first described in studies of the cellular slime mold *Dictyostelium discoideum* (Stephens and Irvine, 1990), and subsequently in studies of the monocot *Spirodela*

[1] This work was supported in part by the Cooperative Research and Development Agreement (grant no. 58–3K95–3–166) between Pioneer Hi-Bred International and the U.S. Department of Agriculture-Agricultural Research Service.

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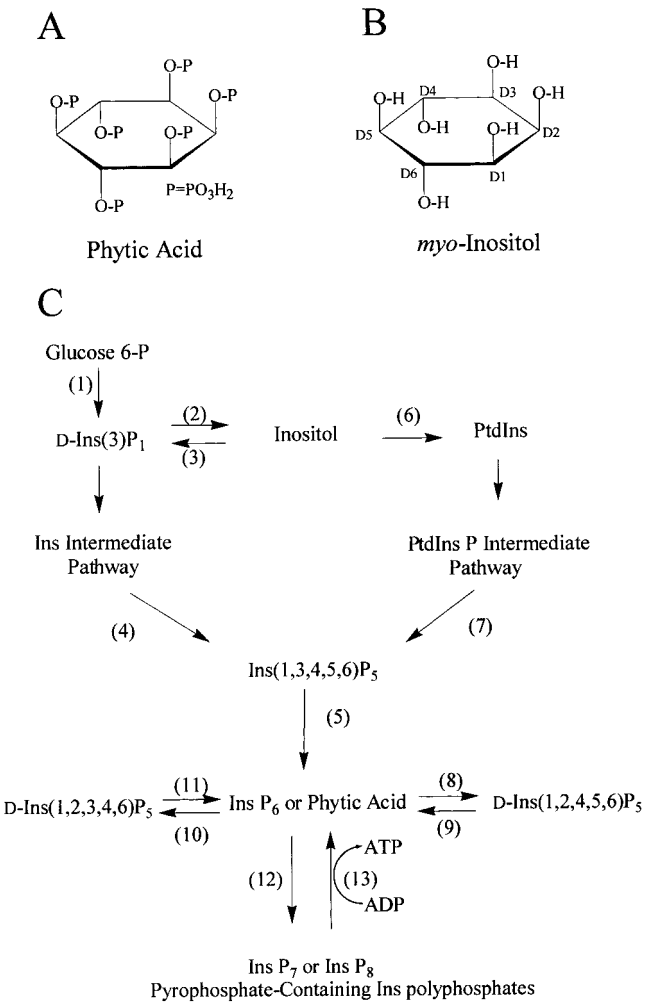

Figure 1. Biosynthetic pathways to phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate or Ins P_6) in the eukaryotic cell. A, Structure of phytic acid. B, Structure of Ins. The numbering of the carbon atoms follows the "D-Convention" (Loewus and Murthy, 2000). C, Biochemical pathways: (1), D-Ins(3)-P_1 (or L-Ins[1]-P_1) synthase; (2), D-Ins 3-phosphatase (or L-Ins 1-phosphatase); (3), D-Ins 3-kinase (or L-Ins 1-kinase); (4), Ins P- or polyP kinases; (5), Ins (1,3,4,5,6) P_5 2-kinase or phytic acid-ADP phosphotransferase; (6), PtdIns synthase; (7), PtdIns and PtdIns P kinases, followed by PtdIns P-specific phospholipase C, and Ins P kinases; (8), D-Ins(1,2,3,4,5,6) P_6 3-phosphatase; (9) D-Ins(1,2,4,5,6) P_5 3-kinase; (10), D-Ins(1,2,3,4,5,6) P_6 5-phosphatase; (11), D-Ins(1,2,3,4,6) P_5 5-kinase; (12), pyrophosphate-forming Ins P_6 kinases; (13), pyrophosphate-containing Ins PolyP-ADP phosphotransferases.

polyrhiza (Brearley and Hanke, 1996a, 1996b). The *D. discoideum* pathway proceeded through the intermediates Ins(3) P_1, Ins(3,6) P_2, Ins(3,4,6) P_3, Ins(1,3,4,6) P_4, and Ins(1,3,4,5,6) P_5. The *S. polyrhiza* pathway proceeded through the intermediates Ins(3) P_1, Ins(3,4) P_2, Ins(3,4,6) P_3, Ins(3,4,5,6) P_4, and Ins(1,3,4,5,6) P_5. These pathways are similar in their first and last intermediates, and in that these Ins phosphates are not known to function as second messengers. In none of the above studies has the relative contribution, in spatial or temporal terms, of MIPS or Ins kinase activity been determined unequivocally.

Phytic acid synthesis may also proceed in part via pathways typically associated with second messenger metabolism that involve phosphatidylinositol (PtdIns) phosphate intermediates and Ins(1,4,5) P_3 (Fig. 1C, steps 6 and 7; Van der Kayy et al., 1995; York et al., 1999). Also, Ins phosphates more highly phosphorylated than phytic acid, such as Ins P_7 and Ins P_8, have been documented to occur widely in eukaryotic cells (Fig. 1C, steps 12 and 13; Mayr et al., 1992; Menniti et al., 1993; Stephens et al., 1993; Brearley and Hanke, 1996c; Safrany et al., 1999). These compounds contain pyrophosphate moieties and may be involved in ATP regeneration. Phytic acid was originally proposed to play a role in ATP regeneration by Morton and Raison (1963). Therefore in a current view, phytic acid is seen not simply as a P-storage product or end-product for Ins phosphorylation, but as a pool for both P and Ins phosphates, the latter function of importance to signaling and ATP formation (Voglmaíer et al., 1996; Safrany et al., 1999). Recently a role for Ins P_6 in mRNA export in yeast was demonstrated (York et al., 1999).

These and other studies (Biswas et al., 1978b; Phillippy et al., 1994; Brearley and Hanke, 1996b) have led to a consensus that, regardless of precursor pathway, Ins(1,3,4,5,6) P_5 represents the penultimate Ins phosphate in the primary synthetic pathway to phytic acid in the eukaryotic cell. In *D. discoideum*, two additional Ins penta*kis*phosphates were observed to accumulate, Ins(1,2,4,5,6) P_5 and Ins(1,2,3,4,6) P_5 (Stephens et al., 1991). Although all three compounds serve as substrate for Ins P_5 kinase(s), conversion of these latter compounds to phytic acid was slower than that observed for D-Ins(1,3,4,5,6) P_5, and they accumulate to higher steady-state levels. These two compounds appeared only to interconvert with phytic acid. Both compounds were also observed in the soybean (*Glycine max*; Phillippy and Bland, 1988), in *S. polyrhiza* (Brearley and Hanke, 1996a), and in the barley (*Hordeum vulgare*) aleurone layer (Brearley and Hanke, 1996c).

We sought non-lethal mutants that would greatly alter the basic P and Ins phosphate phenotype of normal seeds and decouple the close relationship between seed total P and phytic acid P. We reasoned that such mutants would represent mutations proximal to phytic acid synthesis in the developing seed and would be valuable in studies of phytic acid biology. The first two non-lethal mutants of this type we found were maize *low phytic acid 1-1* (*lpa1-1*) and *lpa2-1* (Raboy and Gerbasi, 1996). Recently similar mutants have also been isolated in barley (Larson et al., 1998; Rasmussen and Hatzack, 1998). Here we describe the origin and inheritance of maize *lpa1-1* and *lpa2-1*, characterize their seed P and Ins phosphate phenotypes, and report an association between

reduced seed phytic acid and reduced seed dry weight.

RESULTS

Origin, High-Voltage Paper Electrophoresis (HVPE) Phenotype, and Chromosomal Map Position of *lpa1-1* and *lpa2-1*

The *lpa1-1* mutant was first observed segregating in a single M_2 progeny obtained following the self-pollination of the M_1 plant, 90046-13. No phenotypically similar mutant was observed in any of other M_2 descendants of M_1 90046, nor in the M_2 descendants of other M_1s comprising this first screened population. Therefore this mutation probably occurred in a single ethyl methanesulfanate-treated pollen grain, as expected. The HVPE phenotype of this mutant (Fig. 2A, lane 2) is an approximately 66% reduction in seed phytic acid P as compared with sibling non-mutant seeds (Fig. 2A, lane 1). This reduction in phytic acid P is accompanied by what appears to be a molar-equivalent (in terms of P) increase in P_i. No unusual accumulations of Ins phosphates other than phytic acid are observed. Also, the mutant phenotype of seeds produced by a plant homozygous for *lpa1-1* (Fig. 2A, lanes 3–5) is similar if not identical to the mutant phenotype of homozygous *lpa1-1* seeds obtained following the self-pollination of a heterozygote. This indicates that the *lpa* genotype or phenotype of the parent plant does not greatly affect the *lpa1-1* seed phenotype.

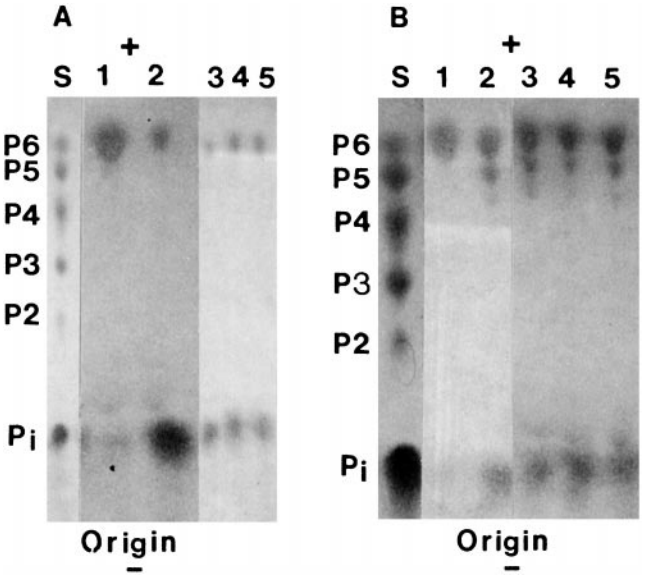

Figure 2. HVPE of inositol phosphates and P_i in *lpa1-1* and *lpa2-1* seed. A, HVPE phenotype of *lpa1-1*: lane S, standards: P6, phytic acid or Ins hexa*kis*phosphate; P2 through P5 are a mixture of Ins bis- through penta*kis*phosphates produced via the partial hydrolysis of phytic acid; lanes 1 and 2, HVPE tests of sibling normal (+/+ or +/*lpa1-1*, lane 1) and homozygous mutant (*lpa1-1*/*lpa1-1*, lane 2) kernels sampled from an F_2 ear produced by the self-pollination of an F_1 heterozygote (+/*lpa1-1*); lanes 3 through 5, HVPE tests of three kernels sampled from an ear produced by the self-pollination of an F_2 *lpa1-1* homozygote. B, HVPE phenotype of *lpa2-1*: lane S, standards as in A; lanes 1 and 2, HVPE tests of sibling normal (+/+ or +/*lpa2-1*, lane 1) and homozygous mutant (*lpa2-1*/*lpa2-1*, lane 2) kernels sampled from an F_2 ear produced by the self-pollination of an F_1 heterozygote (+/*lpa2-1*); lanes 3 through 5, HVPE tests of three kernels sampled from an ear produced by the self-pollination of an F_2 *lpa2-1* homozygote.

The HVPE phenotype of *lpa2-1* is what appears to be a 50% reduction in seed phytic acid P (Fig. 2B, lane 2) as compared with sibling non-mutant seeds (Fig. 2B, lane 1). This reduction in phytic acid P is accompanied by an increase in P_i and novel accumulations of two P-containing compounds with mobilities similar to Ins P_4 and Ins P_5, the latter being the more abundant of the two. This mutant phenotype was first observed in seeds obtained from not one, as would be expected, but three related M_2 progenies; 90041-1, 90041-4, and 90041-12. The seed that produced these progenies were siblings from a single M_1 ear, M1 90041. Mutants phenotypically similar to *lpa2-1* were not observed to segregate in any other M_2 ears of this population. This indicates that the mutation occurred spontaneously in one of the two parent plants used to produce 90041, prior to chemical mutagenesis. If it had occurred at an earlier point in the inheritance of this population, we would have observed it segregating in additional M_2 progenies, descended from other M_1s. All subsequent studies of *lpa2-1* were conducted using materials developed from M_2 90041-4. As with *lpa1-1*, the mutant phenotype of seeds obtained from a homozygote (Fig. 2B, lanes 3–5) is similar or identical to that observed in mutant seeds obtained from the self-pollination of a heterozygote (Fig. 2B, lane 2). Therefore the mutant seed phenotype is a seed-specific effect.

HVPE tests of seeds produced by the cross-pollination of *lpa1-1* and *lpa2-1* homozygotes indicated that these seeds contained non-mutant levels of phytic acid P and P_i and no unusual accumulations of Ins phosphates other than phytic acid, demonstrating that these two mutants complement each other and therefore are non-allelic (data not shown). This was confirmed in the following chromosomal-mapping experiments (Fig. 3). We obtained crosses of *lpa1-1* homozygotes by 13 different simple and compound B-A translocations, representing portions of 15 different chromosome arms. The TB-1Sb translocation stock, which contains approximately 75% of chromosome 1S arm distal to the centromere (Fig. 3A), was the only translocation that uncovered the *lpa1-1* phenotype at a significant frequency (11 of 40 seeds obtained from the cross displayed the mutant phenotype). TB1Sb-2L4464, a compound translocation that uncovers approximately 50% of the same chromosome arm, but not the distal-most portion of the 1S arm (Fig. 3A), did not uncover the *lpa1-1* phenotype. This indicates that *lpa1-1* maps to the distal region of 1S. In the case of *lpa2-1*, we obtained crosses

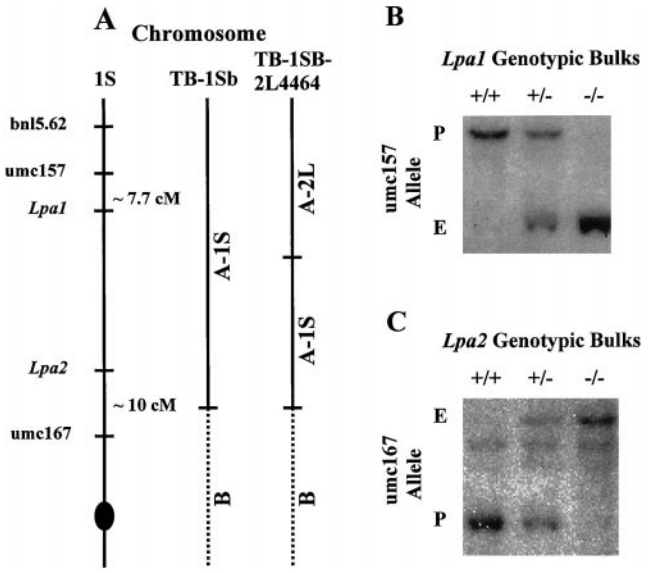

Figure 3. Chromosomal mapping of maize *lpa1* and *lpa2*. A, Approximate map positions of *lpa* loci and markers on chromosome 1S and their relation to two chromosome 1S B-A translocations. Approximate distance (cM) of *lpa1* to umc157 and *lpa2* to umc167 is shown. For B-A translocations TB-1Sb and TB-1SB-2L4464, B (dashed line) indicates B chromosome component and A-1S or A-2L (solid lines) indicate relative position and composition (to chromosome 1S sequence) of indicated A chromosome component. B and C, RFLP mapping of *lpa* loci using bulked segregant analyses. A genotypic bulk DNA was prepared to represent the three *lpa1* or *lpa2* F_2-mapping population segregant classes: +/+, homozygous normal (or *Lpa*/*Lpa*); +/−, heterozygous (+/*lpa* or *Lpa*/*lpa*); −/−, homozygous mutant (*lpa*/*lpa*). DNAs isolated from each of the individuals representing each class were combined so that each individual contributed equally to the bulk. Bulk DNA was digested with *Eco*RV, fractionated, and probed with the indicated RFLP marker. P and E are the parental Pioneer Hi-Bred inbred and Early-ACR RFLP alleles, respectively.

by 19 simple and compound translocations, representing significant portions of 19 chromosome arms (all but 8S). As in *lpa1-1*, TB-1Sb uncovered *lpa2-1* (23 of 90 seeds obtained from the cross displayed the mutant phenotype). However, TB1Sb-2L4464 also uncovered the mutant (18 of 63 seeds obtained from the cross displayed the mutant phenotype), indicating that *lpa2-1* is located on the proximal half of chromosome 1S.

These approximate chromosome arm positions for *lpa1-1* and *lpa2-1* were confirmed with RFLP mapping (Fig. 3, B and C). Bulk-segregant analysis of the 50 segregating F_2s first identified linkage of *lpa1-1* to the RFLP marker umc157 (Fig. 3B), which maps to the distal portion of chromosome 1S (Davis et al., 1999). A readily scorable *Eco*RV polymorphism was detected. Based on observed differences in signal, the parental linkage "E;−" and "P;+" was observed in approximately 90% of the chromosomes assayed, with the "E;+" and "P;−" crossover types observed in approximately 10% of the chromosomes assayed, indicating linkage of approximately 10 centiMorgans (cM). A follow-up study of the individual F_2s, using umc157 and a second marker that maps to the distal region of chromosome 1S, bnl5.62, confirmed the bulk-segregant result and further defined *lpa1−1* map position. Two "E;+" and two "P;−" recombinants between *lpa1−1* and umc157 were found in the 28 homozygous F_2 individuals, and 16 recombinants between *lpa1−1* and bnl5.62 were found in these 28 F_2s. These data place *lpa1−1* approximately 7.7 cM proximal to umc157 (Fig. 3A). Bulk-segregant analysis detected linkage of *lpa2−1* to umc167 (Fig. 3C), which maps to the centromere-proximal portion of chromosome 1S (Davis et al., 1999), with the RFLP marker at a position proximal to the TB1-Sb breakpoint of chromosome 1S. The relative amount of signal observed in the "E" and "P" alleles in the three F_2 genotypic bulks was similar to that observed in the *lpa1-1* bulk segregant test (approximately 90% parental linkage in the chromosomes assayed). These data place *lpa2-1* approximately 10 cM distal to umc167 on chromosome 1S (Fig. 3A).

Quantitative Analyses of Seed P Fractions

The ferric-precipitation method yields an accurate and reproducible assay of phytic acid P in non-

Table I. *Seed dry wt and P fractions in non-mutant and lpa genotypes*

Mature seed of the indicated genotypes were harvested from field-grown plants and assayed for seed total P, total inositol P, and P_i. These fractions are expressed as P concentrations (atomic wt = 31) to facilitate comparisons. The data represent the mean of duplicate analyses of two individuals of each genotype on a dry wt basis.

Genotype[a]	Seed Dry Wt	Total P	Total Inositol P		P_i		Total Inositol P + P_i	
	mg seed^{-1}	*mg g*$^{-1}$	*mg g*$^{-1}$	*% total P*	*mg g*$^{-1}$	*% total P*	*mg g*$^{-1}$	*% total P*
+/+	282	4.5	3.4	76	0.3	7	3.7	82
+/*lpa1-1*	208	4.3	3.5	77	0.5	11	3.9	87
+/*lpa2-1*	265	4.3	3.4	79	0.3	7	3.7	80
lpa1-1/*lpa1-1*	238	4.7	1.1	23	3.1	66	4.2	89
lpa2-1/*lpa2-1*	232	4.6	2.6	57	1.3	28	3.9	85
SE	26	0.48	0.23	–	0.28	–	0.36	–

[a] The genotypes indicated are as follows: +/+, sibling homozygous non-mutant line; +/*lpal-1* and +/*lpa2-1*, heterozygotes produced by pollinating a non-mutant female by a homozygous mutant male; *lpa1-1*/*lpa1-1* and *lpa2-1*/*lpa2-1*, sibling homozygous mutants in the M_4 generation.

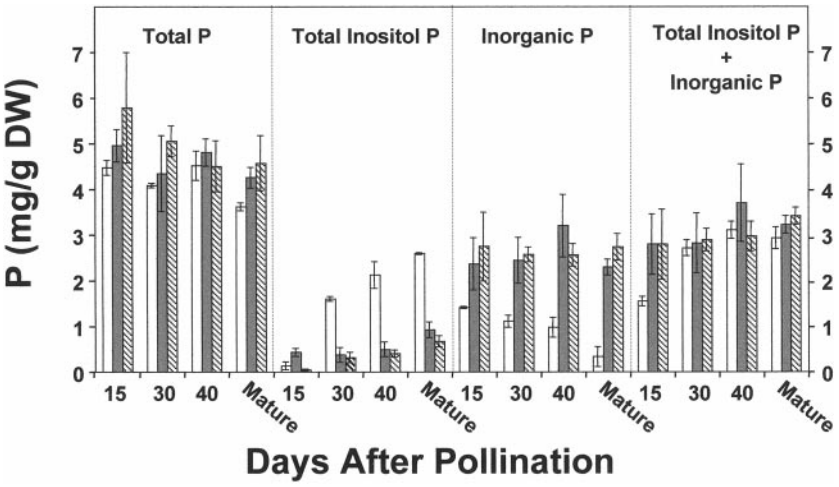

Figure 4. Seed phosphorus fractions in non-mutant (white bars), *lpa1-1* (gray bars), and *lpa2-1* (hatched bars) homozygotes during development. Seed of the three genotypes were harvested from field-grown plants at three dates during development (15, 30, and 40 DAP) and at maturity, and assayed for seed total P, total inositol P, and P_i. These fractions are expressed as P concentrations (atomic weight = 31) to facilitate comparisons. The data represent the mean of duplicate analyses of three individuals of each genotype at each date and are expressed on a dry weight basis.

mutant and *lpa1-1* seeds where phytic acid P represents >95% of total Ins phosphate (Fig. 2A). However, HVPE indicated that *lpa2-1* seeds may contain more substantial amounts (>5% of total Ins phosphate) of Ins phosphates other than phytic acid (Fig. 2B). These would be precipitated in the ferric salt along with phytic acid P and incorrectly measured as "phytic acid P." Therefore we will refer to the value for the P-fraction obtained using the ferric-precipitation method as "total Ins phosphate." This assay indicated that in mature *lpa1-1* seeds, total Ins phosphate is reduced approximately two-thirds, compared with non-mutant seeds (Table I). This is accompanied by a molar-equivalent (in terms of P) increase in P_i, with no net change in seed total P. This represents approximately a 5- to 10-fold greater level of P_i as compared with levels typical of mature, non-mutant seeds. The total Ins phosphate in mature *lpa2-1* seeds is reduced by approximately one-third, as compared with non-mutant seeds (Table I). As in *lpa1-1* seeds, this reduction is accompanied by a molar-equivalent (in terms of P) increase in P_i, with no net change in seed total P. The level of P_i in mature *lpa2-1* seeds represents approximately a 3- to 4-fold increase over that observed in mature non-mutant seeds. Thus in both *lpa1-1* and *lpa2-1* seeds the sum of total Ins phosphate and P_i is constant and similar to that of non-mutant seeds.

Heterozygosity for either mutant had little observable effect on mature seed total P, phytic acid P, and in the case of *lpa2-1*, P_i (Table I). P_i appeared to be increased approximately 2-fold in *lpa1-1* heterozygotes as compared with normal seeds. This increase in P_i was confirmed in an additional analysis of *lpa1-1* heterozygotes obtained by the reciprocal pollination of mutant and non-mutant homozygotes (data not shown) and has also been observed in numerous studies of *lpa1-1* inheritance. Thus, whereas studies to date indicate that the *lpa2-1* mutant allele is recessive to non-mutant, the *lpa1-1* mutant allele clearly is not strictly recessive. This first quantitative analysis also indicated a trend for reduced seed dry weight in *lpa* genotypes as compared with non-mutant (Table I).

Analyses of P fractions during the development of normal, *lpa1-1*, or *lpa2-1* seed revealed that at any given point in development the three genotypes had similar levels of seed total P (Fig. 4). Seed total P concentrations remained relatively constant throughout the development of each genotype (4–5 mg total P g^{-1}), indicating that P uptake closely paralleled dry weight accumulation (Table II). By 30 d after pollination (DAP) reductions in seed dry weight were observed in both mutants as compared with the non-mutant control, typically ranging from 10% to 20%. In normal seeds total Ins phosphate concentration increased, and P_i concentration decreased, throughout development, maintaining a relatively constant sum of total Ins phosphate and P_i. In contrast, total Ins phosphate accumulation was perturbed throughout seed development in both mutants such that clear differences between mutant and non-mutant were observed by 30 DAP (Fig. 4). The reductions in total Ins phosphate concentration observed in *lpa1-1* and *lpa2-1* during development, as compared with normal seed, were in both cases closely matched by increases in P_i. Thus the sum of total Ins phosphate

Table II. *Seed dry wt in non-mutant (+/+), lpa1-1, and lpa2-1 homozygotes during development*

Seeds were harvested at three dates during development (15, 30, and 40 d after pollination) and lyophilized. Mature seed was harvested and oven-dried. Dry wts were recorded for duplicate samples of three individuals representing each genotype at each date.

Genotype	Days after Pollination			
	15	30	40	Mature
	mg dry wt seed^{-1}			
+/+	23	130	161	262
lpa1-1/lpa1-1	34	78	144	177
lpa2-1/lpa2-1	19	104	144	198
SE	1	6	6	14

and P_i remained relatively constant throughout development of normal and mutant seed, representing approximately 75% of seed total P concentration (Fig. 4).

HPLC analysis confirmed that in *lpa1-1* seeds the reduction in total Ins phosphate is primarily accounted for by a reduction in phytic acid P (Fig. 5). HPLC analysis of normal seeds reproducibly detects a small peak with a mobility similar to an Ins P_5 (Fig. 5B), representing 0.12 mg P g^{-1} or 4% of total Ins phosphate. This peak is reduced to the extent that it is not detectable in HPLC assays of *lpa1-1* seeds (Fig. 5C). HPLC also confirmed that no unusual accumulations of other Ins phosphates are observed in *lpa1-1* seeds. Similar findings were reported in an independent analysis of the same non-mutant and *lpa1-1* materials tested here (Mendoza et al., 1998). HPLC analysis (Fig. 5D) also confirmed the Ins phosphate phenotype of *lpa2-1* seeds observed with HVPE: phytic acid P is reduced approximately 50% as compared with normal seeds, and represents approximately 75% of *lpa2-1*'s reduced levels of total Ins phosphate. The remaining 25% consists primarily of what appears to be an Ins P_5, representing 0.45 mg P g^{-1}, or 22% of total Ins phosphate, and trace levels of the less abundant Ins P_4.

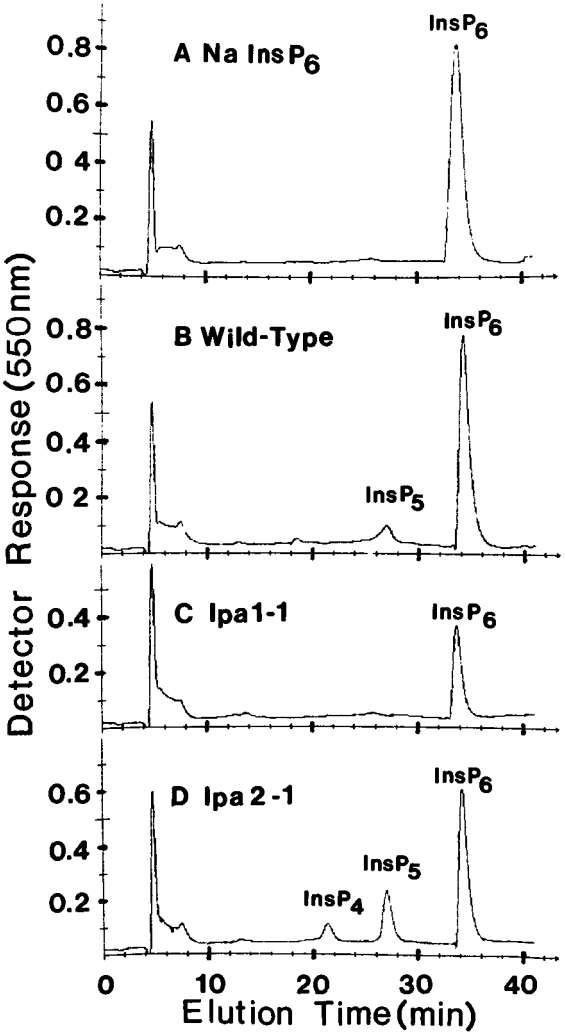

Figure 5. HPLC of acid-soluble Ins phosphates in non-mutant and *lpa* seed. A, Na Ins P_6 or phytic acid standard. Shown is a typical result obtained from the elution of 99.5 nmol of phytic acid. B through D, HPLC tests of extracts prepared from homozygous non-mutant (or *Lpa*) seed (B), homozygous *lpa1-1* seed (C), and homozygous *lpa2-1* seed (D). To allow for direct comparison, equal amounts of flour and equal aliquot sizes were tested. Ins P_4, Ins P_5, and Ins P_6 are Ins tetra*kis*-, penta*kis*-, and hex*kis*phosphates, respectively. These identities were obtained and confirmed via comparisons with known standards in HPLC and HVPE, comparison with results of quantitative analyses following ferric-precipitation, and with subsequent NMR.

Purification and Structural Identification of Ins Phosphates in *lpa2-1* Seeds

The two putative novel Ins phosphates that accumulate in *lpa2-1* seeds to an extent sufficient for reproducible detection with the HVPE and HPLC methods used here were obtained as individual, purified free acids (data not shown). In addition, one-third less abundant P-containing compound was obtained from the same bulk ferric-precipitate. ^{1}H-NMR revealed that the most abundant novel Ins phosphate in *lpa2-1* seeds is an isomer of Ins P_5 (Fig. 6A). The relative up-field (approximately δ 3.5 ppm) position of a doublet of doublets (J = 10 and 3.0 Hz) compared to the other resonances was clearly evident and this indicates that dephosphorylation had occurred at the H-3, or the enantiomeric H-1, position. Enantiomeric protons cannot be distinguished by NMR spectroscopy so the structure is D-Ins(1,2,4,5,6) P_5 and/or D-Ins(2,3,4,5,6) P_5. Additional information obtained by ^{31}P-decoupling and J-resolved NMR experiments provided confirmation of the structure (data not shown).

^{1}H-NMR revealed that the second most abundant Ins phosphate in *lpa2-1* seeds is an Ins P_4 (Fig. 6B). The appearance of a triplet (J = 2.9 Hz) at approximately δ 4.16 in addition to the resonance from H-3 (or H-1) on non-phosphorylated carbon (mentioned above) indicated that the additional dephosphorylation had occurred at H-2. Additional experiments (homonuclear decoupling and J-resolved experiments) were conducted and the structure consistent with all the NMR data was D-Ins(1,4,5,6) P_4 and/or its enantiomer D-Ins(3,4,5,6) P_4. The third and least abundant Ins phosphate obtained from our purification of *lpa2-1* seed Ins phosphates was identified as an Ins P_3, D-Ins(1,2,6) P_3, and/or its enantiomer D-Ins(2,3,4) P_3 (Fig. 6C). The ^{1}H-NMR of this compound showed three sets of "up-field" resonances (relative to other resonances) thus suggesting three protons geminal to hydroxyl groups. The presence of triplets at the δ 3.4 (J = 9 Hz) and at δ 3.66 (J = 9.5 Hz) are due to H-5 and H-4 (or H-6) respectively, and a doublet of doublets (J = 10 and 3.0 Hz) is due to H-3

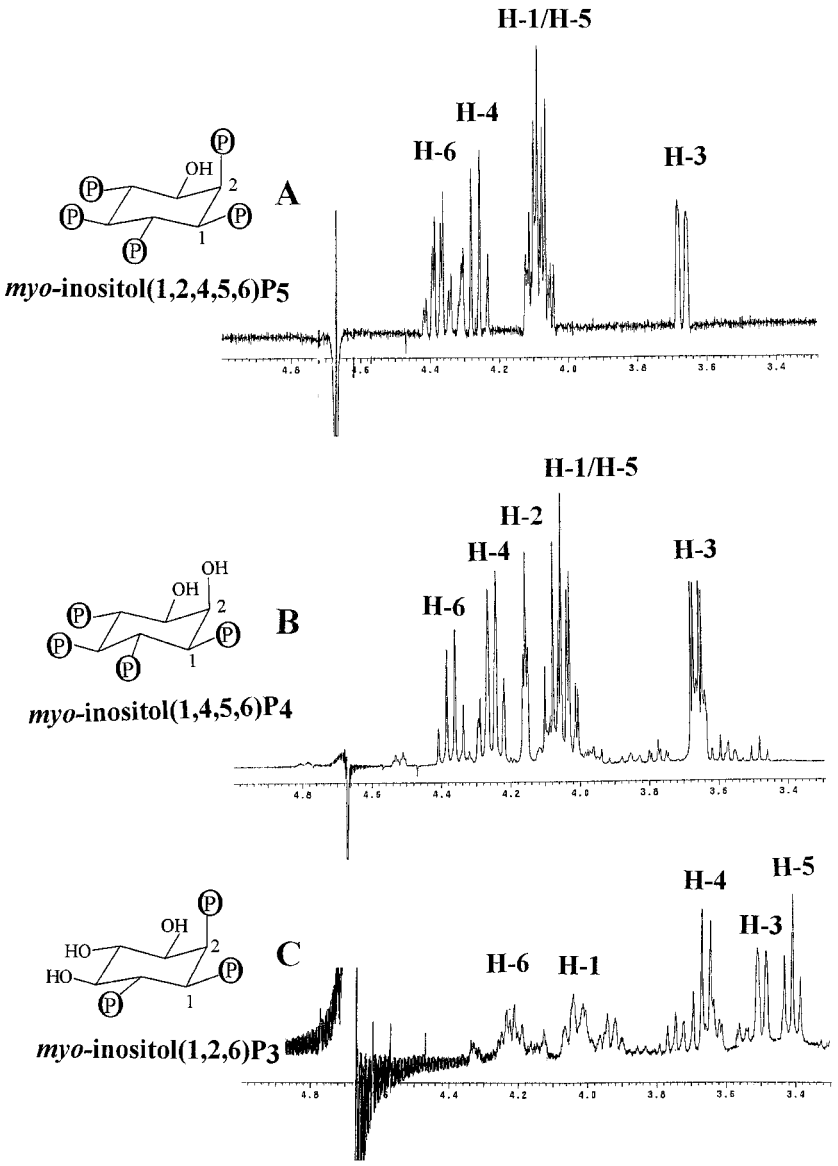

Figure 6. Determination of structure of Ins tris-, tetra*kis*-, and penta*kis*phosphates that accumulate in homozygous *lpa2-1* seed. Putative Ins phosphates were purified to homogeneity, and one-dimensional-NMR spectra were obtained. In descending order the most abundant Ins Ps were found to be D-Ins(1,2,4,5,6) P_5 or its enantiomer D-Ins(2,3,4,5,6) P_5, D-Ins(1,4,5,6) P_4 or its enantiomer D-Ins(3,4,5,6) P_4, and D-Ins(1,2,6) P_3 or its enantiomer D-Ins(2,3,6) P_3.

(or H-1). The structure consistent with these resonances, the rest of the NMR spectrum, and additional J-resolved and two-dimensional-DQCOSY experiments was D-Ins(1,2,6) P_3, or its enantiomer D-Ins(2,3,4) P_3. The presence of small concentrations of additional Ins phosphates are evident in the spectra (Fig. 6C), however the concentrations were insufficient for unequivocal identification.

Correspondence between Reduced Phytic Acid, Increased P_i, and Reduced Seed Weight

Since normal mature maize seeds contain consistently low levels (0.3–0.5 mg g^{-1}) of P_i, the high-P_i (HIP) phenotype of *lpa* seeds (Table I, Figs. 2 and 4) should provide a quick and inherently sensitive assay for *lpa* genotype. A survey of maize *defective kernel* (*dek*) mutants revealed that mutations that perturb germ or aleurone development, the tissues that accumulate phytic acid in maize and other cereals, result in substantial reductions in phytic acid P, and these are always accompanied by equivalent increases in P_i (Raboy et al., 1990). However all such *dek* mutants are lethal as homozygotes. If care is taken to inspect for the presence of normal germ and aleurone tissues, the HIP phenotype (Fig. 7) should accurately and consistently predict homozygosity for *lpa1-1* or *lpa2-1*. The following inheritance experiments tested the correspondence between the "low phytic acid," "high P_i," and reduced seed weight phenotypes of *lpa* seeds. F_1 heterozygotes were either self-pollinated to produce F_2s, or used both as males and females in pollinations with the appropriate homozygous mutant testers. In the case of *lpa1-1*, all seeds from a total of six F_2 ears and 12 test-cross ears were individually inspected, weighed, and tested for P_i (using the assay

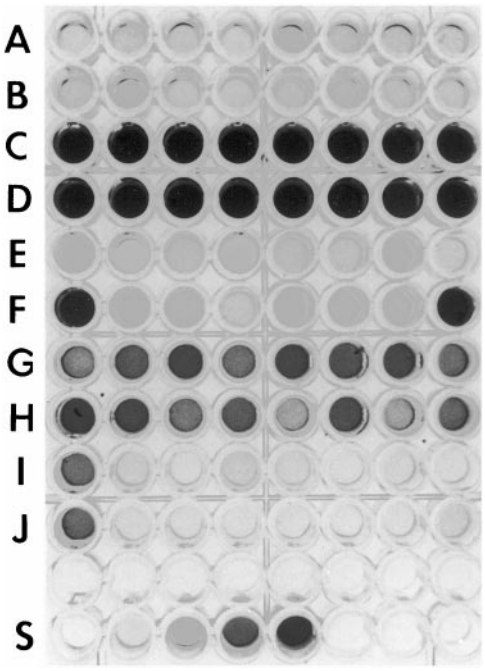

Figure 7. The HIP phenotype of *lpa* seeds. Twenty seeds from a given ear were individually crushed, extracted, and assayed for P_i using a microtitre plate-based colorimetric assay. To allow for direct comparison, all seeds were extracted in 10 volumes on a single-seed basis, and equal aliquot volumes were tested. A and B, Twenty seeds from a non-mutant (*Lpa*) homozygote; C and D, 20 seeds from a *lpa1-1* homozygote; E and F, 20 sibling F_2 seeds sampled from an ear obtained following the self-pollination of an F_1 +/*lpa1-1* (or *Lpa1*/*lpa1-1*) heterozygote; G and H, 20 seeds from a *lpa2-1* homozygote; I and J, 20 sibling F_2 seeds sampled from an ear obtained following the pollination of an F_1 +/*lpa2-1* (or *Lpa2*/*lpa2-1*) heterozygote. S, Standards; five standards contained 0.0, 0.15, 0.46, 0.93, and 1.39 μg of P.

illustrated in Fig. 7). Approximately 5% of the seed extracts were also tested with HVPE to confirm correspondence between "low phytic acid" and "high P_i." In the case of *lpa2-1*, all seeds from a total of six F_2s and five test-cross ears were similarly analyzed for P_i, and in addition all seed extracts were also tested with HVPE for the distinctive *lpa2-1* HVPE phenotype. Of the six *lpa2-1* F_2s, only three showed segregation for a consistent and stable *lpa2-1* HVPE phenotype that could be reliably scored, and these were included in the analysis below. The remaining three showed no clear segregation for an *lpa2-1*-like HVPE phenotype that could be reliably scored, even though tests showed that the sibling M3 *lpa2-1* parents used to make the F_1s appeared homozygous for the *lpa2-1* allele. Since inheritance of *lpa2-1*, or expression of its HVPE phenotype, could not be detected in these three F_2 progenies, they could not be included in the subsequent analyses. The cause of this reduced penetrance or instability of inheritance is not known, and such instability was not observed with *lpa1-1*.

There was a strict correspondence between reduced seed phytic acid and increased P_I in all seeds tested. In every ear tested, the mean dry weight of the *lpa* mutant class of seeds was reduced as compared with its sibling non-mutant seed class (Table III). This reduction in seed dry weight approached being twice as great in the case of *lpa1-1*, ranging from 8% to 23%, as compared with *lpa2-1*, where the reductions ranged from 4% to 16%. The results also confirm the monogeneic inheritance of both *lpa1-1* and *lpa2-1* (Table III).

DISCUSSION

These results indicate that *lpa1-1* and *lpa2-1* represent reduced-function or loss-of-function alleles at two loci on chromosome 1S in maize. It is unlikely that either mutant represents a gain-of-function mutation such as a novel increase in phytase activity. Such gain of function mutations are rare events typically found once in 10^5 individuals in a mutated population, rather than once in 10^3 individuals as observed here, typical of loss-of-function mutations. Also, gain of function mutations usually are additive or dominant, whereas both *lpa1-1* and *lpa2-1* appear recessive or nearly so. When homozygous these mutants are viable and result in substantial reductions in phytic acid P accumulation during seed development but have little or no effect on seed total P. Therefore, the reduction in seed phytic acid P is not due to reduced uptake or translocation of P to the developing seed. The alteration of a biochemical or genetic function in *lpa1-1* and *lpa2-1* seed is sufficient to condition the mutant seed phenotype, independent of parent plant genotype. Homozygosity for these alleles may also alter some function throughout the plant, but if so it does not appear to contribute to the seed phenotype. We have isolated a number of additional alleles at these two loci. Studies of these additional alleles will determine if homozygosity for one or more conditions a plant or seed phenotype more extremely than that observed in the initial alleles.

In *lpa1-1*, seed reductions in all soluble Ins phosphate species typically observed in normal seeds contribute to total Ins phosphate reduction. In *lpa2-1* seed total Ins phosphate is reduced as compared with normal seed, but this reduction is accompanied by increases in novel Ins phosphates not observed to accumulate in normal seeds. Based on these phenotypes and the observation that these Ins phosphate reductions occur in the presence of normal levels of total P, we hypothesize that *lpa1-1* is a mutation in the first part of the phytic acid synthesis pathway, Ins supply, and *lpa2-1* is a mutation in the later part, Ins phosphate metabolism. The maize genome contains a number of MIPS-homologous sequences (possibly as many as seven), and one maps in the proximity of *lpa1-1* on chromosome 1S (Fig. 3; Larson and Raboy, 1999). Studies are under way to determine if *lpa1-1* is in fact a lesion in the chromosome 1S MIPS or in some other function in this part of the pathway.

The correspondence between the reduction in phytic acid and increase in D-Ins(1,2,4,5,6) P_5 (and/or

Table III. *Segregation of lpa1-1 and lpa2-1 in F_2 and test-cross progenies and its association with seed dry wt reduction*

Every seed from the ears representing each type of genetic test were inspected for normal germs, individually weighed, and tested for the mutant phenotype associated with homozygosity for either *lpa1-1* or *lpa2-1*.

Mutant	Genetic Test	Ear	Non-Mutant Seeds		Mutant Seeds		Chi-Square[a]	Dry Wt Reduction
			No.	Mean dry wt	No.	Mean dry wt		
				mg ± SD		mg ± SD		%
lpa1-1	F_2	1	142	305 ± 29	32	259 ± 24	4.15*	15
lpa1-1	F_2	2	142	315 ± 31	36	290 ± 20	2.17	8
lpa1-1	F_2	3	139	261 ± 25	40	239 ± 25	0.67	8
lpa1-1	F_2	4	135	256 ± 27	31	234 ± 22	3.54	9
lpa1-1	F_2	5	165	339 ± 26	45	307 ± 21	1.43	9
lpa1-1	F_2	6	109	277 ± 29	21	241 ± 28	5.45*	13
lpa1-1	TC-F[b]	1	162	223 ± 26	166	193 ± 23	0.04	13
lpa1-1	TC-F	2	112	277 ± 34	92	248 ± 27	1.96	10
lpa1-1	TC-F	3	127	250 ± 34	121	228 ± 27	0.15	9
lpa1-1	TC-F	4	112	274 ± 25	99	233 ± 25	0.80	15
lpa1-1	TC-F	5	72	346 ± 38	77	299 ± 28	0.17	14
lpa1-1	TC-F	6	69	332 ± 30	88	287 ± 35	2.30	13
lpa1-1	TC-M[c]	1	131	219 ± 34	102	192 ± 32	3.61	12
lpa1-1	TC-M	2	98	201 ± 19	72	166 ± 24	4.31*	17
lpa1-1	TC-M	3	139	205 ± 31	100	174 ± 26	6.36*	15
lpa1-1	TC-M	4	51	262 ± 30	47	202 ± 25	0.16	23
lpa1-1	TC-M	5	78	278 ± 25	98	233 ± 31	2.27	16
lpa1-1	TC-M	6	80	234 ± 32	78	195 ± 27	0.02	17
lpa2-1	F_2	1	238	256 ± 33	77	247 ± 30	0.05	4
lpa2-1	F_2	2	186	323 ± 27	42	295 ± 28	5.26*	9
lpa2-1	F_2	3	303	184 ± 26	93	176 ± 23	0.48	4
lpa2-1	TC-F	1	241	249 ± 30	251	235 ± 33	0.23	6
lpa2-1	TC-F	2	83	340 ± 25	101	324 ± 30	1.76	5
lpa2-1	TC-M	1	84	287 ± 29	89	240 ± 33	0.14	16
lpa2-1	TC-M	2	108	223 ± 22	101	207 ± 21	0.23	7
lpa2-1	TC-M	3	36	276 ± 35	33	251 ± 32	0.13	9

[a] The asterisk signifies that the deviation from the expected ratio is significant at the $P = 0.05$ level of probability. [b] TC-F, F_1 heterozygote used as a female and used tester as male. [c] TC-M, F_1 heterozygote used as a male and tester used as a female.

its enantiomer) observed in *lpa2-1* seeds indicates that this later compound plays some significant role in phytic acid metabolism in the maize seed. Maize *lpa2-1* may be a lesion in a gene encoding a D-Ins(1,2,4,5,6) P_5 3-kinase. Such a lesion might also account for the accompanying accumulations of D-Ins(1,4,5,6) P_4 and D-Ins(1,2,6) P_3 (and/or their respective enantiomers) in *lpa2-1* seed. The presence of these apparent breakdown products of D-Ins(1,3,4,5,6) P_5 indicates that this later compound does not simply interconvert with phytic acid but can be further metabolized in the developing maize seed.

Previous studies uniformly show that the most likely synthetic pathway to phytic acid begins with the synthesis of D-Ins(3) P_1 and ends with the conversion of D-Ins(1,3,4,5,6) P_5 to phytic acid (Biswas et al., 1978a; Stephens and Irvine, 1990; Phillippy et al., 1994; Van der Kayy et al., 1995; Brearley and Hanke, 1996b). Therefore, an alternative is that D-Ins(1,2,4,5,6) P_5 accumulates in *lpa2-1* seed indirectly as a result of a lesion in gene encoding something other than a 3-kinase, such as a gene encoding a D-Ins(1,3,4,5,6) P_5 2-kinase. A study of fruitfly (*Drosophila melanogaster*) Ins polyP 1-phosphatase (*ipp)* mutants demonstrated that flies homozygous for an *ipp* allele cannot metabolize Ins(1,4) P_2, a critical component of the Ins(1,4,5) P_3 signaling pathway (Majerus, 1992), yet several cellular processes dependent on Ins P_3 signaling pathways functioned normally (Acharya et al., 1998). Apparently *ipp* homozygotes adjust in vivo via compensatory up-regulation of an alternative Ins P_3 pathway involving Ins(1,3,4) P_3. A study of *D. discoideum* PtdIns P-specific phospholipase C nulls, incapable of synthesizing Ins(1,4,5) P_3 via the PtdIns intermediate pathway (Fig. 1C, step 7), revealed that Ins(1,4,5) P_3 pools and the signaling processes using it were maintained via a PtdIns P-independent, alternative pathway involving breakdown of Ins P_6 (Van Dijken et al., 1995). These studies illustrate the metabolic adjustment and balancing that the Ins polyP and PtdIns P pathways are capable of in vivo. In the case of maize *lpa2-1*, the novel accumulation of D-Ins(1,2,4,5,6) P_5 and/or its enantiomer may occur in compensation for a block in Ins P_6 synthesis. Perhaps D-Ins(1,2,4,5,6) P_5 along with D-Ins(1,2,3,4,6) P_5 and phytic acid together represent a "buffer pathway," functioning as a complex pool for Ins phosphate (Fig. 1C).

As one approach to the nutritional and environmental problems attributed to seed-derived dietary phytic acid (Erdman, 1981; Cromwell and Coffey,

1991), efforts are under way to breed "low phytic acid" crops using *lpa* mutants. The initial efforts to breed elite maize "low phytic acid" inbreds and hybrids used *lpa1-1* and simple backcrossing methods (Ertl et al., 1998). The HIP phenotype of this mutant provided a quick, inexpensive, and accurate test for its inheritance, greatly facilitating introgression of the trait into numerous breeding lines. Fourteen "near-isogeneic" hybrid pairs were produced, each consisting of sibling non-mutant and *lpa1-1* variants. Field studies of these found little or no effect of homozygosity for the *lpa1-1* allele on germination, or on stalk strength, grain moisture at harvest, and flowering date. However, yield reductions were observed in eight of the 14 hybrid pairs. When meaned across the 14 pairs, a yield reduction of 5.5% was observed.

This yield loss in *lpa1-1* hybrids may be due in part to the inheritance of deleterious factors inherited from the "Early-ACR" parent, closely linked in coupling to *lpa1-1* ("linkage drag"). However, in the present study the seed dry weight loss was observed for both mutants between sibling seed classes on individual ears and within the Early-ACR genetic background. Linkage drag is therefore probably not the major cause of this seed-specific effect. Blocks in either Ins supply (*lpa1-1*), or Ins phosphate metabolism (*lpa2-1*), may contribute in part to this dry weight loss. The seed dry weight loss may also in part be a direct outcome of the increase in P_i concentration that results from each mutant's block in phytic acid synthesis. For example, the rate-limiting step in starch synthesis in the cereal seed is catalyzed by the enzyme ADP-Glc pyrophosphorylase, and this enzyme is allosterically inhibited by P_i (Plaxton and Preiss, 1987). This hypothesis is supported by the fact that the dry-weight loss was inversely proportional to the increase in P_i in *lpa1-1* and *lpa2-1* seed. The level of yield reduction observed in the study of *lpa1-1* hybrids and its variability closely reflects the seed dry weight reduction associated with homozygosity for *lpa1-1* observed in the present study. It is therefore also most likely a direct outcome of the genetic lesion and its mutant phenotype. Studies to address this phenomenon and breeding efforts to overcome it are currently under way.

Previous studies have observed substantial variation in seed total P concentration among different non-mutant lines of a given species grown in the same environment (for review, see Raboy, 1997). Variation in seed total P concentration can also result from varying levels of nutrient P supply to the developing plant. During the development of normal seeds total P content (net total P) typically increases in a linear fashion (Raboy, 1997). In each of these three cases phytic acid P accumulation varies in turn to maintain a relatively constant non-phytic acid P, or "cellular P," level (defined as all P necessary for basic cellular metabolism). In this context phytic acid P is seen as excess P or storage P (all seed P over and above that needed for cellular metabolism). However the present studies of *lpa1-1* and *lpa2-1* indicate that it is not solely phytic acid P but the sum of phytic acid P and P_i that represents excess or storage P. It is this sum that remains relatively constant across the genotypes and developmental stages studied here. It remains to be determined how the relative contributions of phytic acid P and P_i to their sum might vary in response to variation in the supply of P to the developing seed of these genotypes.

That both mutants result in seed dry weight loss suggests that phytic acid metabolism is at least in part a component of P homeostasis during seed development. However, both mutants are viable as homozygotes and at least in the case of *lpa1-1* have little effect on seed function other than a relatively minor loss in dry weight accumulation. Therefore if P homeostasis is critical to seed function, some second mechanism not involving phytic acid metabolism, such as a combination of localization and compartmentalization of P, must play the major role. In light of the other possible functions for phytic acid metabolism, such as an Ins phosphate pool important for signaling pathways and possibly ATP regeneration (Menniti et al., 1993; Van der Kayy et al., 1995) or as an anti-oxidant (Graf et al., 1987), it is surprising that *lpa1-1* and *lpa2-1* seeds are in fact viable and are essentially normal in phenotype other than in their seed P chemistry. Perhaps the major function for phytic acid accumulation in seeds is as an efficient P-storage metabolite. Under cultivation, long-term sequestering of P in seeds may not be essential. However, efficient storage of P may be essential in the natural environment where plants evolved, where seeds must survive in soils for extended periods. The impact of the change in seed storage P chemistry (phytic acid P to P_i) in *lpa* mutants, on this long-term P storage function, remains to be determined.

MATERIALS AND METHODS

Plant Materials

A population of ethyl methanesulfanate-induced mutants was generated using the pollen-treatment method (Neuffer and Coe, 1978). The main maize (*Zea mays*) stock used for these studies, a synthetic population referred to as "Early-ACR," was kindly provided by Dr. M.G. Neuffer (University of Missouri, Columbia). In addition, an F_2 obtained from the cross of the public inbred lines A632 and Mo17 was also used as a pollen parent for some of the mutagenesis treatments. Treated pollen was applied to silks of 54 untreated Early-ACR plants, producing M_1 seeds heterozygous for induced mutations. These were planted and self-pollinated to produce 872 M_2 progenies each consisting of sibling seeds on a single M_2 ear. We screened for M_2s segregating for seeds with reduced phytic acid P or unusual increases of other Ins phosphates or P_i, as compared with that typical of non-mutant seeds. Five or more

seeds that appeared phenotypically normal or non-mutant to the unaided eye were sampled from each M_2 ear, individually crushed with a hammer blow, and incubated overnight at 4°C in 0.4 M HCl (10 μL per mg seed weight). The extracts were then briefly vortexed and allowed to settle for a minimum of one-half h. Aliquots were fractionated using a HVPE assay for acid-extractable P-containing compounds (Raboy et al., 1990). Standards were Na phytate (Sigma, St. Louis) and a mixture of Ins phosphates and P_i produced by the chemical hydrolysis of phytic acid (Raboy et al., 1990).

Remnant seed from M_2s containing putative mutants were planted in a field nursery. The resulting plants were self-pollinated to produce M3 ears and cross-pollinated onto non-mutant Early-ACR lines to produce F_1 ears. To provide materials for quantitative analyses and to test allelic relationships, M3 and F3 homozygotes were identified, seeds were planted, and the resulting plants were self- or sib-pollinated and intercrossed. For analyses of P fractions during seed development, immature ears representing each genotype were harvested at 15, 30, and 40 DAP, frozen in liquid N_2, and stored at −80°C. Ears harvested at maturity were dried at 40°C for 48 h and stored at 4°C. To provide materials for inheritance studies, F_1 heterozygote seeds were planted in field nurseries, and the resulting plants were either self-pollinated to produce F_2 progenies or used in test-crosses to respective mutant homozygote testers.

Chromosomal-Mapping Experiments

B-A translocation stocks were used to map the first two mutants, *lpa1-1* and *lpa2-1*, to chromosome arm (Beckett, 1978). B-A translocations undergo non-disjunction during the developing microspore's second mitotic division, producing male gametes containing two sperm nuclei. One of the sperm nuclei is hyperploid (containing two copies of the A chromosome segment contained in the translocation) and one is hypoploid (containing no copies of the translocated segment; Beckett, 1978). Preferential fertilization by the hyperploid sperm typically occurs in approximately 66% of zygotes. Therefore, if the frequency of non-disjunction approaches 100%, the frequency of fertilization by a hypoploid sperm will approach 33%. The bulk of seed phytic acid P is localized in the diploid embryo. Fertilization of an egg produced by an *lpa* homozygote by a sperm hypoploid for the corresponding chromosome segment will result in a germ hemizygous for the mutant allele, "uncovering" the mutant phenotype. This indicates that the mutant locus was contained on the A chromosome fragment contained in the translocation. We crossed *lpa1-1* and *lpa2-1* homozygotes by a collection of B-A translocations, and analyzed the resulting seeds for their respective mutant phenotypes.

For these and other genetic analyses, we followed the inheritance of *lpa1-1* or *lpa2-1* via testing for the HIP phenotype associated with homozygosity for either mutant. Single seeds were weighed, crushed, and extracted overnight in 10 (v/w) 0.4 M HCl at 4°C and 10 μL of extract were assayed for P_i using the method of Chen et al. (1956), modified to be conducted in microtitre plates. To each microtitre plate well were added 10 μL of extract, 90 μL distilled, deionized water, and 100 μL of colorimetric reagent consisting of a 1:1:1:2 mixture of 10% (w/v) ascorbic acid:6 N H_2SO_4:2.5% (w/v) ammonium molybdate:distilled, deionized water. Each microtitre plate also contained five wells prepared to contain the following P standards: 0.0 μg P; 0.15 μg P; 0.46 μg P; 0.93 μg P; 1.39 μg P. Following development for 2 h at ambient temperature, results were obtained either via visual inspection of the plates or quantified via use of a microtitre-plate spectrophotometer. Depending on the study, selected extracts were also tested with HVPE to confirm the correspondence of HIP with the HVPE phenotype of either *lpa1-1* or *lpa2-1*.

The mutants were then mapped in segregating F_2-mapping populations using RFLPs. F_2 seed were obtained from a cross of a homozygous *lpa1-1* plant (Early-ACR or "E" background) and the inbred PHP38 (Pioneer or "P" background) and from a cross of a homozygous *lpa2-1* plant ("E" background) and the inbred PHN46 ("P" background), and planted in a field nursery. The inbred lines and initial crosses were kindly provided by Pioneer Hi-Bred International (Des Moines, IA). DNAs were prepared from leaf samples obtained from each individual in the F_2 populations (Dellaporta et al., 1983). F_2 plants were then self-pollinated to produce F3 progeny ears. These F3 progenies were then tested to determine parent F_2 plant *lpa* genotype: homozygous normal (+/+); heterozygous (+/*lpa* or +/−); homozygous mutant (*lpa*/*lpa*, or −/−). A bulk-segregant analysis was first conducted to identify linkage to RFLP markers (Michelmore et al., 1991). Three "bulk" DNAs were prepared to represent each of the three *lpa* F_2 genotypic classes by combining aliquots of DNA from all of the individuals representing a given class. These bulk DNAs were digested with restriction endonucleases, fractionated on agarose gels (Southern, 1975), and probed with RFLP markers kindly provided by the Maize RFLP Lab (Dr. Edward Coe, University of Missouri, Columbia). If a scorable polymorphism at a given RFLP locus exists between the "E" and "P" parental backgrounds, producing "E" and "P" alleles, and if this RFLP locus is linked to an *lpa* locus, then as the proximity of linkage increases an increase in signal in the "E" allele relative to the "P" allele will be observed in the −/− bulk, the reverse will be observed in the +/+ class, and similar levels of signal in "E" and "P" alleles will be observed in the heterozygous +/− class. If there is no linkage between an RFLP locus and the *lpa* locus then similar amounts of signal in both the "E" and "P" alleles will be observed in tests of the three class bulks. In the case of *lpa1-1*, F_2 DNAs representing the individuals comprising the two homozygous segregant classes were individually subjected to analysis. The data obtained were analyzed with MAPMAKER 3 (Lander et al., 1987).

Quantitative Analyses of Seed P and Inositol P Fractions

Samples of immature seeds were lyophilized. Samples of mature seeds were dried for 48 h at 60°C. These were then

milled to pass through a 2-mm screen and stored in a desiccator until analysis. Seed total P was determined following wet-ashing of aliquots of tissue (typically 150 mg) and colorimetric assay of digest P (Chen et al., 1956). The ferric-precipitation method was used to determine total, acid-soluble Ins phosphates (Raboy et al., 1990). Aliquots of tissue (typically 0.5–1.0 gm) were extracted in 0.4 M HCl:0.7 M Na_2SO_4. Acid-soluble Ins phosphates were obtained as a ferric precipitate, wet-ashed, and assayed for P as in the total P analysis. Phytic acid or Ins phosphates are expressed as their P (atomic weight = 31) content to facilitate comparisons between seed P fractions. Seed P_i was determined colorimetrically following extraction of tissue samples (typically 0.5 g in non-mutant seeds and 0.15 g in mutant seeds) in 12.5% (w/v) trichloroacetic acid:25 mM $MgCl_2$.

Anion-exchange HPLC analyses of seed Ins phosphates were performed using a modification of the method as described (Phillippy and Bland, 1988; Rounds and Nielsen, 1993). Samples of seeds were dried and milled as described above, and extracted in 40 volumes 0.4 M HCl overnight. Following centrifugation (10,000*g*, 10 min), supernatants were filtered through number 1 filter paper (Whatman, Clifton, NJ), and passed through HV 0.45-μm filters (Millipore, Bedford, MA). Two hundred-microliter aliquots were then fractionated on an IonPac AS7 anion-exchange column (Dionex, Sunnyvale, CA), equipped with an IonPac AG7 guard column (Dionex), which had been equilibrated with 10 mM methyl piperazine, pH 4.0 (buffer A). The Ins phosphates were then eluted with the following gradient system at a flow rate of 0.5 mL min^{-1}: 0 to 1 min 100% (v/v) buffer A; 1 to 26 min a concave gradient from 0% to 15% 1 M $NaNO_3$, pH 4.0 (buffer B); 21 to 41 min a linear gradient from 15% to 100% (v/v) buffer B. The column elutent was mixed with colorimetric reagent (0.015% [w/v] $FeCl_3$:0.15% [w/v] sulfosalicylic acid) at a flow rate of 0.5 mL min^{-1}, using a PEEK tee and a Lazar pulseless pump (Alltech, Deerfield, IL), and the mixture passed through a 290-cm reaction coil prior to peak detection via A_{550}. Ins phosphate in a sample peak was calculated using the following standard curve, obtained via the analysis of four Na Ins P_6 standards containing 24.9, 49.7, 74.6, and 99.5 nM Na Ins P_6; nM Ins P = 1.66×10^{-5} (peak area) − 3.85; $R^2 = 0.99$.

Purification of Inositol Phosphates in *lpa2-1* Seeds and Structural Identification Using NMR

The objective was to purify to homogeneity the most abundant Ins phosphates, other than phytic acid P, found in maize *lpa2−1* seed, and then to determine their structures using NMR. One hundred grams of seed homozygous for *lpa2-1* was ground with a coffee grinder and extracted in 1 L of 0.4 M HCl overnight. Extracts were centrifuged (10,000*g*, 10 min), and Ins phosphates were obtained as a ferric precipitate with a modification of the method as described above. Ferric Ins phosphate precipitates were converted to soluble Na Ins phosphate salts by treatment with NaOH, and the insoluble ferric hydroxide was removed via centrifugation. To obtain individual Ins phosphates in pure form, the supernatants containing the Na Ins phosphates were neutralized with HCl and loaded onto preparative Dowex (Sigma) 1X2-400 anion-exchange columns (packed volume 5 mL). These were eluted with a 400-mL 0.0 to 0.4 M HCl linear gradient or a 400-mL 0.4 M HCl isocratic gradient and collected in 5-mL fractions. Fractions containing Ins phosphates were identified following acid digestion of fraction aliquots, and colorimetric assay for P in the digests. Ins phosphates in peak fractions were precipitated as barium salts, and then converted to free acids via passage through AG 50W-X8 cation exchange columns. The purity of a given sample was confirmed with HVPE and HPLC (data not shown) and subsequently NMR. Aliquots of these free acids were then dehydrated in a Speed-Vac Concentrator (Savant Instruments, Holbrook, NY).

The structures of these Ins phosphates were determined by a combination of one- and two-dimensional NMR spectroscopy. NMR characteristics that are particularly useful for structure determination of Ins phosphates have been previously described (Barrientos et al., 1994; Johnson et al., 1995; Barrientos and Murthy, 1996). NMR spectra were recorded on a 400-MHz Unity Inova-400 spectrometer (Varian, Palo Alto, CA). The dehydrated samples (0.002–0.2 g) were dissolved in D_2O (0.8 mL), and the pH adjusted to 5.0 by addition of NaOH (1 M) or perdeuterated acetic acid, as necessary. The pH values reported in this paper are readings of the glass electrode, uncorrected for deuterium effects. One-dimensional ^{1}H-NMR spectra were obtained at 399.943 MHz. ^{1}H-Chemical shifts were referenced to the residual proton absorption of the solvent, D_2O (δ 4.67). The acquisition conditions were as follows: spectral windows 6,738 Hz, pulse width 90° tipping angle. Typically, 16 to 32 scans with recycle delays of 4 to 6 s between acquisitions were collected. The residual H_2O resonance was suppressed by a 1.5-s selective presaturation pulse. ^{31}P-decoupled spectra were decoupled continuously with Waltz decoupling. TOCSY, DQCOSY, and J-resolved spectra were obtained as described previously (Barrientos et al., 1994; Johnson et al., 1995; Barrientos and Murthy, 1996).

ACKNOWLEDGMENTS

The authors thank Cathy Waterman, Brian Quigly, Teresa Galli, Sean Sandborgh, Mathew Jackson, and Valerie Wagner for assistance in the laboratory and Allen Cook for assistance in the field. The authors also thank Luther Talbert and John Sherwood for critical reading of the manuscript.

Received January 3, 2000; accepted May 30, 2000.

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