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Seed phosphorus and inositol phosphate phenotype of barley *low phytic acid* genotypes

John A. Dorsch[a], Allen Cook[a], Kevin A. Young[a], Joseph M. Anderson[b], Andrew T. Bauman[c], Carla J. Volkmann[c], Pushpalatha P.N. Murthy[c], Victor Raboy[a,*]

[a]*USDA-ARS, 1691 South 2700 West, Aberdeen, ID 83210, USA*
[b]*USDA-ARS, 1150 Lilly Hall, Purdue University, West Lafayette, IN 47907, USA*
[c]*Department of Chemistry, Michigan Technological University, Houghton, MI 49931, USA*

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Abstract

myo-Inositol-1,2,3,4,5,6-hexa*kis*phosphate (Ins P_6 or "phytic acid") typically represents ~75% of the total phosphorus and >80% of soluble *myo*-inositol (Ins) phosphates in seeds. The seed phosphorus and Ins phosphate phenotypes of four non-lethal barley (*Hordeum vulgare* L.) *low phytic acid* mutations are described. In seeds homozygous for M 635 and M 955 reductions in Ins P_6, ~75 and >90% respectively, are accompanied by reductions in other Ins phosphates and molar-equivalent increases in Pi. This phenotype suggests a block in supply of substrate Ins. In seeds homozygous for barley *low phytic acid* 1-1 (*lpa*1-1), a 45% decrease in Ins P_6 is mostly matched by an increase in Pi but also accompanied by small increases in Ins(1,2,3,4,6)P_5. In seeds homozygous for barley *lpa*2-1, reductions in seed Ins P_6 are accompanied by increases in both Pi and in several Ins phosphates, a phenotype that suggests a lesion in Ins phosphate metabolism, rather than Ins supply. The increased Ins phosphates in barley *lpa*2-1 seed are: Ins(1,2,3,4,6)P_5; Ins(1,2,4,6)P_4 and/or its enantiomer Ins(2,3,4,6)P_4; Ins(1,2,3,4)P_4 and/or its enantiomer Ins(1,2,3,6)P_4; Ins(1,2,6)P_3 and/or its enantiomer Ins(2,3,4)P_3; Ins(1,5,6)P_3 and/or its enantiomer Ins(3,4,5)P_3 (the methods used here cannot distinguish between enantiomers). This primarily "5-OH" series of Ins phosphates differs from the "1-/3-OH" series observed at elevated levels in seed of the maize *lpa*2 genotype, but previous chromosomal mapping data indicated that the maize and barley *lpa*2 loci might be orthologs of a single ancestral gene. Therefore one hypothesis that might explain the differing *lpa*2 phenotypes is that their common ancestral gene encodes a multi-functional, Ins phosphate kinase with both "1-/-3-" and "5-kinase" activities. A putative pyrophosphate-containing Ins phosphate, possibly an Ins P_7, was also observed in the mature seed of all barley genotypes except *lpa*2-1. Barley M 955 indicates that at least for this species, the ability to accumulate Ins P_6 can be nearly abolished while retaining at least short-term (~1.0 years) viability.

Keywords: *Hordeum vulgare*; *Zea mays*; Poaceae; Barley; Maize; Seed phenotype; Inositol phosphates; *Low phytic acid*

1. Introduction

Among the *myo*-inositol (Ins; **1**) phosphate esters (2–20) *myo*-inositol(1,2,3,4,5,6)P_6 (Ins P_6 or phytic acid; **17**) is the most abundant Ins phosphate in nature (Cosgrove, 1980). It typically represents 65–85% of seed total phosphorus (Raboy, 1997). Ins P_6 (**17**) is ubiquitous in eukaryotic species and its metabolism might play a number of roles in the eukaryotic cell (Shears, 2001). These roles include phosphorus (P) and mineral storage and homeostasis in developing and germinating seeds and in other plant tissues and organs (reviewed in Raboy, 1997; Strother, 1980); messenger RNA export and DNA double-strand break repair (Hanakahi and West, 2002; York et al., 1999); as a major pool in Ins phosphate and phosphatidylinositol phosphate (PtdIns P) signaling and developmental pathways; and as an antioxidant (Graf et al., 1987).

One aspect of the role of Ins P_6 (**17**) as a major pool in Ins phosphate pathways is that it can serve as a substrate for the synthesis of pyrophosphate or "PP"-containing derivatives (Safrany et al., 1999). Examples of PP-containing Ins phosphates include: 5-PP-Ins(1,3,4,6)P_4 (**18**), a non-phytic acid-Ins P6; 5-PP-Ins(1,2,3,4,6)P_5 (**19**), an Ins P_7; and 5,6-*bis*-PP-Ins(1,2,3,4)P_4 (**20**), an Ins P_8. All Ins phosphates will be described here using the "D-numbering" convention for

* Corresponding author. Tel.: +1-208-397-4162x151; fax: +1-208-397-4165.
E-mail address: vraboy@uidaho.edu (V. Raboy).

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the identification of each of the six carbons in the Ins ring, as illustrated in Fig. 1 (Loewus and Murthy, 2000). The position of a given pyrophosphate moiety on the Ins ring has not been determined in every case, and the structures given in Fig. 1 are for illustrative purposes only.

PP-containing Ins phosphates might function in signal transduction/developmental pathways, in homologous recombination, or as P-donors in a novel pathway for ATP regeneration (Luo et al., 2002; Safrany et al., 1999). There has been little progress to date in studies of PP-Ins phosphate metabolism in plant cells, apart from the observation of Ins- and P-containing compounds more polar than Ins P_6 in cells of *Spirodela polyrhiza* L. during turion formation (Flores and Smart, 2000), and in barley (*Hordeum vulgare* L.) aleurone cells following imbibition (Brealey and Hanke, 1996). The potential role of PP-Ins phosphate as a high-energy phosphate source for ATP regeneration is particularly interesting for seed biology, in that it is reminiscent of the decades-old proposal that the "phytate-phosphoinositol system acts as a buffer of the ATP-ADP system" during wheat (*Triticum aestivum* L.) endosperm development, "maintaining the concentration of ATP at the optimum for synthetic functions" (Morton and Raison, 1963).

Pathways contributing to Ins P_6 (**17**) accumulation in developing seeds can be thought of as consisting of two

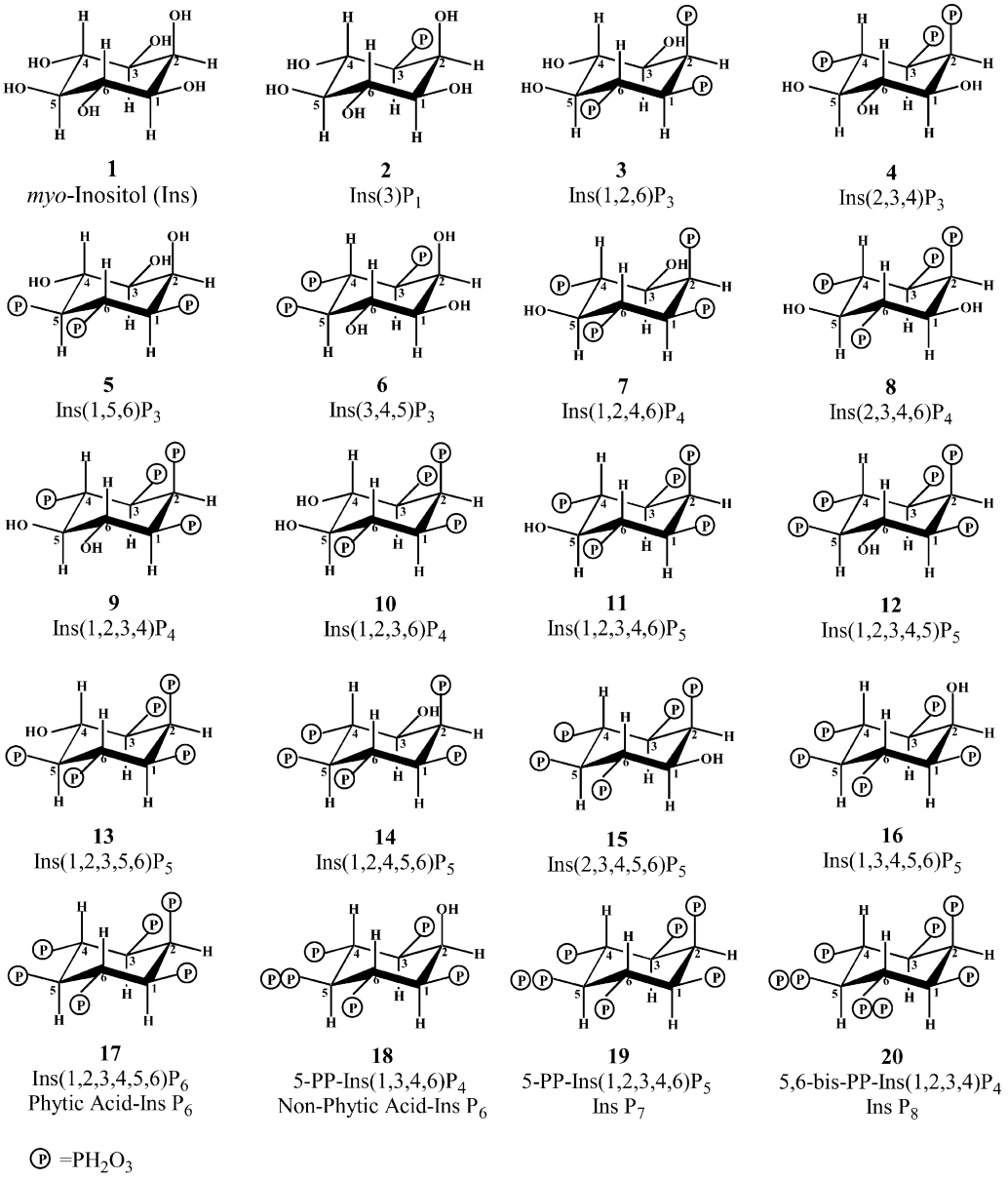

Fig. 1. Structures of *myo*-inositol or "Ins" (**1**) and various Ins phosphates (**2**–**20**) including Ins(1,2,3,4,5,6)P_6 or "phytic acid" (**17**). The numbering of the carbon atoms follows the "D-Convention" (Loewus and Murthy, 2000). "PP" indicates a pyrophosphate moiety. The three PP-containing Ins phosphates (**18**, **19**, and **20**) are included only for illustrative purposes. Their inclusion here does not indicate the detection of PP-containing Ins phosphates with these specific structures in the seed analyzed in this study.

parts; Ins (**1**) synthesis, and subsequent Ins phosphate metabolism. The basic structural pathways are probably similar in most eukaryotic cells. However, particular steps or their relative activity or physiological importance may differ among species, and large genetic differences can exist between relatively closely related genomes. For example, the sole synthetic source of Ins (**1**) is the conversion of glucose 6-P to Ins(3)P_1 (**2**), catalyzed by the enzyme Ins(3)P_1 synthase (MIPS; Loewus and Loewus, 1980; Loewus and Murthy, 2000). There are up to seven loci in the maize (*Zea mays* L.) genome that contain MIPS-homologous sequences (Larson and Raboy, 1999), but only one in barley (*Hordeum vulgare* L.; Larson and Raboy, 1999) and rice (*Oryza sativa* L.; Yoshida et al., 1999; Larson et al., 2000).

Mutations that perturb the normal accumulation of Ins P_6 (**17**) in seeds were first reported in maize (Raboy and Gerbasi, 1996), and subsequently in barley (Larson et al., 1998; Rasmussen and Hatzack, 1998), rice (Larson et al., 2000) and soybean [*Glycine max* (L.) Merr.; Hitz et al., 2002; Wilcox et al., 2000]. These *low phytic acid* or Low Phytate genotypes produce seed that have normal levels of total P but greatly reduced levels of Ins P_6 (**17**). The first two maize *low phytic acid* genotypes represented two distinct phenotypic classes (Raboy et al., 2000). In seed produced by maize *low phytic acid* 1-1 (*lpa*1-1, or the first recessive allele of the *lpa*1 locus), the reduction in Ins P_6 (**17**) is matched by a molar-equivalent increase in Pi. In seed produced by maize *low phytic acid* 2-1 (*lpa*2-1), the reduction in Ins P_6 is matched by increases both in Pi and Ins phosphates with five or fewer P esters. The most abundant Ins phosphate other than Ins P_6 in maize *lpa*2-1 seed is Ins(1,2,4,5,6)P_5 (**14**), a "3-OH" Ins penta*kis*phosphate, and/or its "1-OH" enantiomer, Ins(2,3,4,5,6)P_5 (**15**). The methods used to determine structures did not distinguish enantiomers. It was hypothesized (Raboy et al., 2000) that the early part of the pathway to Ins P_6 (**17**), Ins synthesis and supply, is perturbed in *lpa*1-1 seed, and that the later part of the pathway to Ins P_6 (**17**), Ins phosphate metabolism, is perturbed in *lpa*2-1. There are two alternative possibilities concerning maize *lpa*2: 1) it is a lesion directly impacting an Ins phosphate 1-/3-kinase activity; 2) it is a lesion in some other distal step in Ins P_6 (**17**) metabolism that indirectly results in the accumulation of Ins(1,2,4,5,6)P_5 (**14**) and/or Ins(2,3,4,5,6)P_5 (**15**), and their breakdown products. The isolation and characterization of seed P and Ins phosphate phenotypes of a selected set of barley *lpa* genotypes is described here, and compared with other previously reported barley low phytate genotypes and the phenotypically similar maize *lpa* genotypes.

2. Results

2.1. The phosphorus and Ins phosphate phenotype of non-mutant barley and maize seed

To define the seed P and Ins phosphate phenotype of the barley *lpa* genotypes, and to compare them with maize *lpa* genotypes, the non-mutant seed P and Ins phosphate phenotypes typical of the barley and maize genetic backgrounds used in this study must first be described. The approach to characterization of a seed P and Ins phosphate phenotype taken here consists of four steps: (1) quantitative analysis of seed P and total Ins phosphate; (2) a "phenotyping" HPLC assay; (3) a "10-X HPLC" assay; (4) NMR analyses of partially purified seed Ins phosphates obtained as free acids. The barley *lpa* genotypes studied here were isolated following chemical mutagenesis of the cultivar "Harrington". Quantitative analyses (Table 1) of non-mutant grain produced by this cultivar, when grown in a field nursery

Table 1
Seed dry weight, phosphorus and inositol phosphate fractions in wild-type and *low phytic acid* barley genotypes

Genotype[a]	Seed dry weight mg seed^{-1}	Total P mg g^{-1}	Total soluble inositol P[c]		Inorganic P		Total soluble inositol P + inorganic P	
			mg g^{-1}	% Total P	mg g^{-1}	% Total P	mg g^{-1}	% Total P
cv. Harrington wild-type	46	4.77	2.89	61	0.40	8	3.29	69
*lpa*1-1	44	3.69	1.19	32	1.21	33	2.39	65
M 635	42	5.04	0.74	15	2.47	49	3.21	64
M 955	42	4.98	ND[c]	–	3.31	66	3.31	66
*lpa*2-1	46	5.42	1.94	36	1.73	32	3.67	68
Standard error[b]	1	0.17	0.03	–	0.11	–	0.13	–

[a] Mature seed of the cultivar Harrington or seed homozygous for the indicated mutation were harvested from field-grown plants and assayed for seed total P, total soluble inositol P, and inorganic P (Pi). Total soluble inositol P was determined using the ferric-precipitation method. These fractions are expressed as P concentrations (atomic weight = 31) to facilitate comparisons. The data represent the mean of duplicate analyses of each of three sibling lines of each genotype and are expressed on a dry weight basis.

[b] Standard error = "Standard deviation of the means". Standard errors are calculated (Steel and Torrie, 1980) for each variable (column of means), and expressed in the same units as each variable (column), as follows; Standard error = Mean standard deviation/square root of n, where $n = 3$, the number of replicates represented by each mean.

[c] ND, not detectable. Inositol P levels in M 955 were below that reliably assayed with this method.

side-by-side with the *lpa* mutations described below, indicated that it contained 4.77 mg total P g^{-1}, of which 61% (2.89 mg g^{-1}) was total Ins phosphate (the sum of Ins phosphates obtained via ferric precipitation, including Ins P_6, **17**) and 8% (0.4 mg g^{-1}) Pi.

"Phenotyping-HPLC" is an approach designed to provide the most direct and reproducible phenotypic comparisons between genotypes (Fig. 2; Table 2). Seed samples were extracted and following identical filtration and dilution steps, immediately assayed with HPLC. No

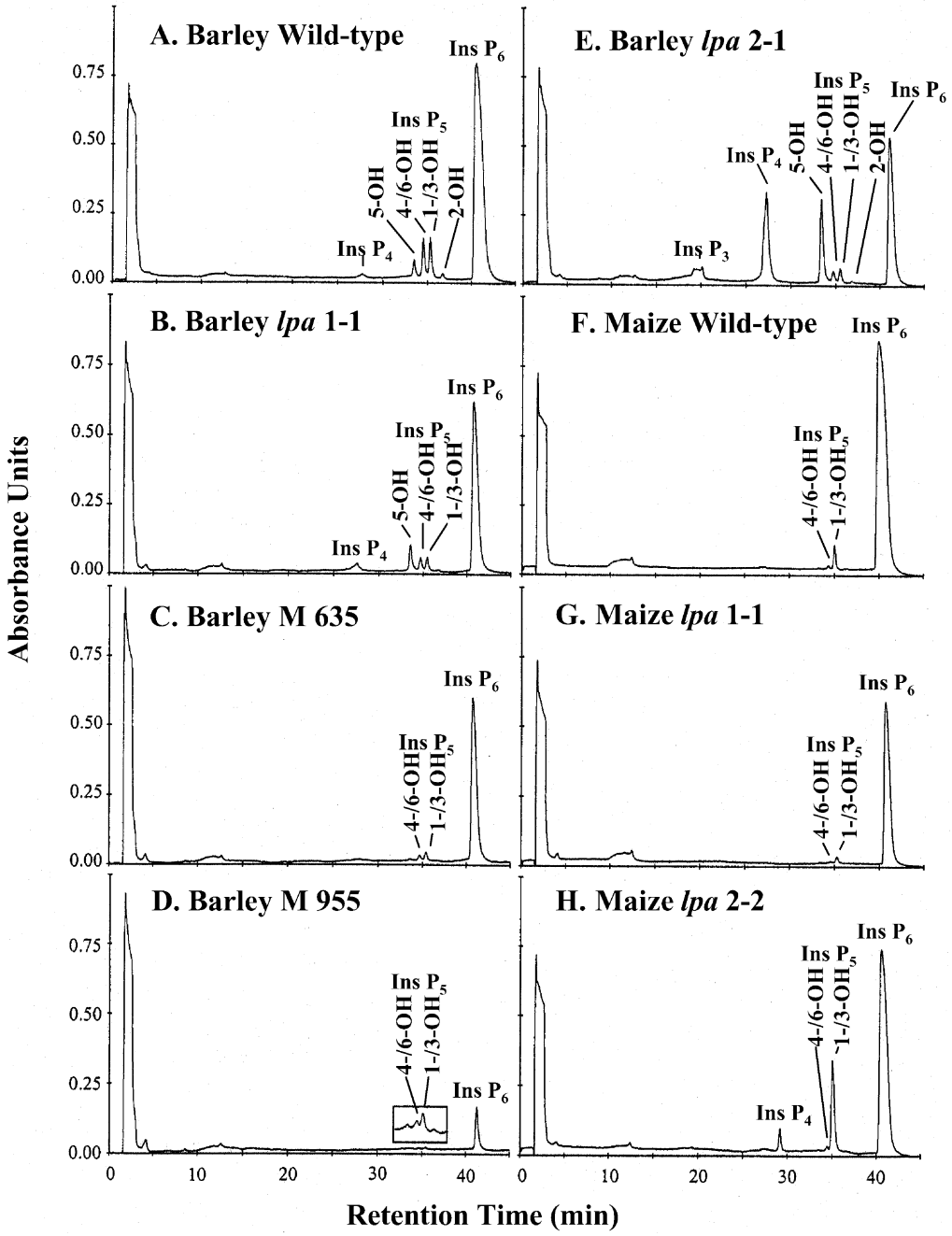

Fig. 2. "Phenotyping" High performance liquid chromatography (HPLC) of acid-soluble *myo*-inositol phosphates in "wild-type" (non-mutant) and *low phytic acid* (*lpa*) barley and maize seed. (A through E) barley wild-type and *lpa* genotypes. (A) Barley wild-type, (B) barley *lpa*1-1, (C) M 635, (D) M 955, (E) barley *lpa*2-1. (F through H), maize wild-type (non-mutant) and *lpa* genotypes. (F) maize wild-type, (G) maize *lpa*1-1, (H) maize *lpa*2-2. To facilitate direct comparisons all seed samples were milled, extracted (in 2.5 volumes per weight), diluted (1:5), and assayed (0.2 ml) identically. Ins P_3, Ins P_4, Ins P_5 and Ins P_6 are *myo*-inositol *tris*, tetra*kis*-, penta*kis*- and hexa*kis*phosphates, respectively. "5-OH", "4-/6-OH", "1-/3-OH", and "2-OH" indicates Ins(1,2,3,4,6)P_5 (**11**), Ins(1,2,3,5,6)P_5 (**13**) and/or Ins(1,2,3,4,5)P_5 (**12**) (since the HPLC and NMR methods used here do not distinguish between enantiomers), Ins(2,3,4,5,6) (**15**) and/or Ins(1,2,4,5,6)P_5 (**14**), and Ins(1,3,4,5,6)P_5 (**16**), respectively. These identities were obtained and confirmed via comparisons with known standards in HPLC and High Voltage Paper Electrophoresis, comparison with results of quantitative analyses following ferric-precipitation, and with subsequent NMR.

Table 2
Quantitation of inositol phosphates detected in HPLC (Fig. 2) of barley and maize seed homozygous for *low phytic acid* mutations

Genotype[a]	Ins P_3	Ins P_4	Ins(1,2,3,4,6)P_5	Ins(1,2,3,4,5)P_5 and/or Ins(1,2,3,5,6)P_5	Ins(1,2,4,5,6)P_5 and/or Ins(2,3,4,5,6)P_5	Ins(1,3,4,5,6)P_5	Ins P_6	Total soluble Ins P
	mg P gm^{-1} (% of total Ins phosphate)							mg P gm^{-1} (% of non-mutant control)
Non-mutant Barley cv. Harrington	ND	0.033 (1.4%)	0.064 (2.6%)	0.119 (4.9%)	0.129 (5.3%)	0.035 (1.4%)	2.040 (84.3%)	2.420 (100%)
Barley *lpa*1-1	ND	0.046 (3.4%)	0.091 (6.8%)	0.056 (4.2%)	0.055 (4.1%)	Trace	1.087 (81.1%)	1.340 (55%)
Barley M 635	ND	ND	Trace	0.043 (4.1%)	0.048 (4.5%)	ND	0.964 (91.4%)	1.055 (44%)
Barley M 955	ND	ND	Trace	0.006 (4.0%)	0.009 (6.0%)	ND	0.135 (90.0%)	0.150 (6%)
Barley *lpa*2-1	0.069 (4.1%)	0.361 (21.2%)	0.265 (15.6%)	0.056 (3.3%)	0.059 (3.5%)	0.028 (1.6%)	0.864 (50.8%)	1.702 (70%)
Non-mutant maize inbred A619	ND	ND	ND	0.028 (1.2%)	0.069 (2.9%)	ND	2.245 (95.9%)	2.342 (100%)
Maize *lpa*1-1	ND	ND	ND	0.026 (2.7%)	0.038 (3.7%)	ND	0.97 (93.8%)	1.041 (44%)
Maize *lpa*2-2	ND	0.05 (2.6%)	ND	0.029 (1.5%)	0.278 (14.1%)	ND	1.612 (81.8%)	1.971 (84%)

[a] Samples of seed produced by non-mutant barley (cv. Harrington) plants, barley plants homozygous for the indicated *low phytic acid* mutations in the Harrington genetic background, non-mutant maize (inbred A619) plants or plants homozygous for the indicated maize mutations in the A619 genetic background, were milled, and aliquots of flour were extracted and assayed with HPLC (Fig. 2). Inositol phosphates are expressed as their P content (atomic weight = 31) to facilitate comparisons. "Trace" indicates a small peak was observed in the HPLC run but represented $<1.0\%$ of total Ins phosphates, a level below that necessary for reliable quantitation with methods used here. ND = not detectable.

additional steps intermediate between extraction and analysis, each of which would add error to the measurement, were used. Phenotyping-HPLC analysis of Harrington seed extracts (Fig. 2A, Table 2) confirmed that in grain produced by this non-mutant barley, total Ins phosphate, as measured by the ferric-phytate precipitation method, primarily consisted of Ins P_6 (**17**), with other Ins phosphates representing 16% of total Ins phosphate. The soluble Ins phosphates other than Ins P_6 (**17**) appeared to primarily consist of four Ins penta*kis*phosphates, and low levels of one or more Ins tetra*kis*phosphates. Comparison with chromatographic standards indicated that the Ins penta*kis*phosphates were, from shortest to longest relative retention times: Ins(1,2,3,4,6)P_5 (**11**); Ins(1,2,3,4,5)P_5 (**12**) and/or Ins(1,2,3,5,6)P_5 (**13**) (since the HPLC and NMR methods used here do not distinguish between enantiomers); Ins(1,2,4,5,6)P_5 (**14**) and/or Ins(2,3,4,5,6)P_5 (**15**) ; and Ins(1,3,4,5,6)P_5 (**16**).

An HPLC assay approximately 10 times more sensitive than the "phenotyping assay" was next used (Fig. 3). For 10X-HPLC, seeds were first extracted and extracts filtered, then the Ins phosphates were concentrated via ferric precipitation, prior to assay. With this approach (Fig. 3A), in addition to the Ins penta*kis*phosphates (**11**, **12** and/or **13**, **14** and/or **15**, and **16**) and Ins P_6 (**17**), low levels of at least two Ins tetra*kis*phosphates, and low levels of a P-containing compound more polar than Ins P_6 ("Unknown 1", Fig. 3A), a putative pyrophosphate-containing Ins P_7 or Ins P_8 (such as **19** or **20**), were reproducibly observed in non-mutant seed extracts.

NMR analyses (Fig. 4) of Ins phosphates, obtained from extracts of non-mutant barley seeds as concentrated, semi-purified free acids, confirmed the presence of, Ins(1,2,3,4,6)P_5 (**11**), Ins(1,2,3,4,5)P_5 (**12**) and/or its enantiomer Ins(1,2,3,5,6)P_5 (**13**), and the presence of two Ins tetra*kis*phosphates. The presence of an Ins P_4 was indicated by the two comparatively upfield resonances at δ 3.66 (triplet, $J = 9.2$ Hz) and δ 3.8 (doublet of doublets, $J = 9.5$ and 2.2 Hz, Fig. 4). Chemical shifts of the other hydrogens, indicated in Fig. 4, were consistent with the presence of geminal phosphates. *J*-resolved and gDQCOSY spectra were consistent with the presence of Ins(1,2,4,6)P_4 (**7**) and/or its enantiomer Ins(2,3,4,6)P_4 (**8**). The two relatively upfield resonances at δ 3.5 (triplet, $J = 9.2$ Hz) and δ 3.78 (triplet, $J = 9.5$ and 2.2 Hz), and *J*-resolved spectra, indicates the presence of Ins (1,2,3,4)P_4 (**9**) and/or its enantiomer Ins(1,2,3,6)P_4 (**10**). Again, gDQCOSY spectrum was consistent with the structural assignment.

The non-mutant maize inbred that served as genetic background in these studies was "A619". The "pheno-

typing-HPLC'' assay (Fig. 2F, Table 2) of mature seed produced by non-mutant A619 revealed that Ins P_6 (**17**) represented 96% of the seed's total Ins phosphate. This assay also detected two Ins pentaphosphates: Ins(1,2,4,5,6)P_5 (**14**) and/or its enantiomer Ins(2,3,4,5,6)P_5 (**15**); Ins(1,2,3,4,5)P_5 (**12**) and/or its enantiomer Ins(1,2,3,5,6)P_5 (**13**). NMR analysis of a mixture of Ins phosphates purified from A619 seed indicated that Ins P_6 (**17**) was the most abundant Ins phosphate in the sample (Fig. 5). In addition, three Ins penta*kis*phosphates were detected. One of the resonances, a triplet ($J = 9.3$ Hz), is relatively upfield (about δ 3.66) suggesting that in this compound H-5 is geminal to a hydroxyl group. The fact that this compound shows connectivity to two other resonances (connectivity to H-2 is not evident) indicates that the compound is symmetrical and provides additional confirmation of the presence of Ins(1,2,3,4,6)P_5 (**11**). In another spin system, one of the resonances, a doublet of doublets ($J = 10.6$ and 2.5 Hz), is relatively upfield (about δ 3.84) suggesting H-3

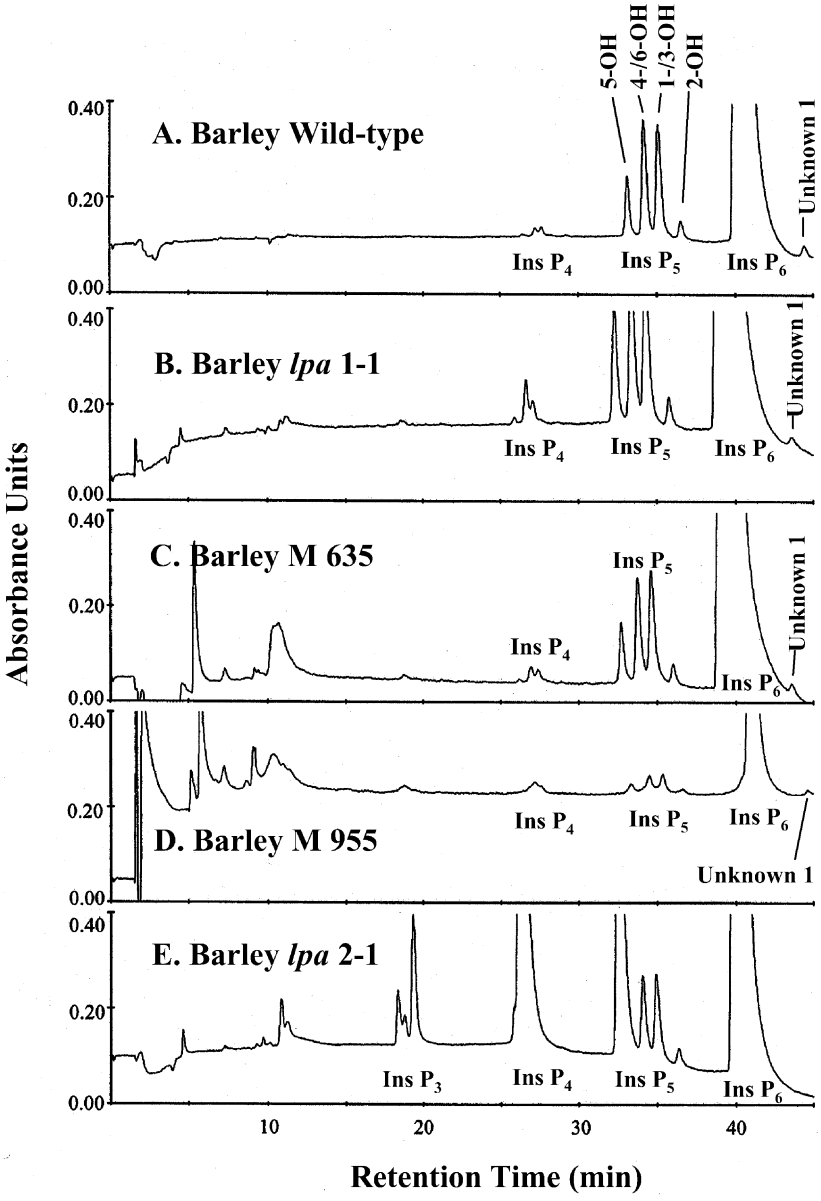

Fig. 3. ''10X'' High performance liquid chromatography (HPLC) of acid-soluble *myo*-inositol phosphates in wild-type (non-mutant) and *low phytic acid* (*lpa*) barley seed. (A) Barley wild-type, (B) barley *lpa*1-1, (C) M 635, (D) M 955, (E) barley *lpa*2-1. Extracts were handled as in ''Phenotyping HPLC'', except for an additional 1:10 concentration step prior to HPLC. Flour samples were extracted in 2.5 volumes per weight, Ins phosphates concentrated 1:10 using ferric precipitation, converted to a soluble Na salt, and diluted 1:5 prior to HPLC. Ins P_3, Ins P_4, Ins P_5 and Ins P_6 are *myo*-inositol *tris*, tetra*kis*-, penta*kis*- and hexa*kis*phosphates, respectively. ''5-OH'', ''4-/6-OH'', ''1-/3-OH'', and ''2-OH'' indicates Ins(1,2,3,4,6)P_5 (**11**), Ins(1,2,3,5,6)P_5 (**13**) and/or Ins(1,2,3,4,5)P_5 (**12**) (since the HPLC and NMR methods used here do not distinguish between enantiomers), Ins(2,3,4,5,6) (**15**) and/or Ins(1,2,4,5,6)P_5 (**14**), and Ins(1,3,4,5,6)P_5 (**16**), respectively. Unknown 1, a phosphate-containing compound more polar than Ins P_6 (**17**), and a putative pyrophosphate-containing Ins phosphate, possibly an Ins P_7.

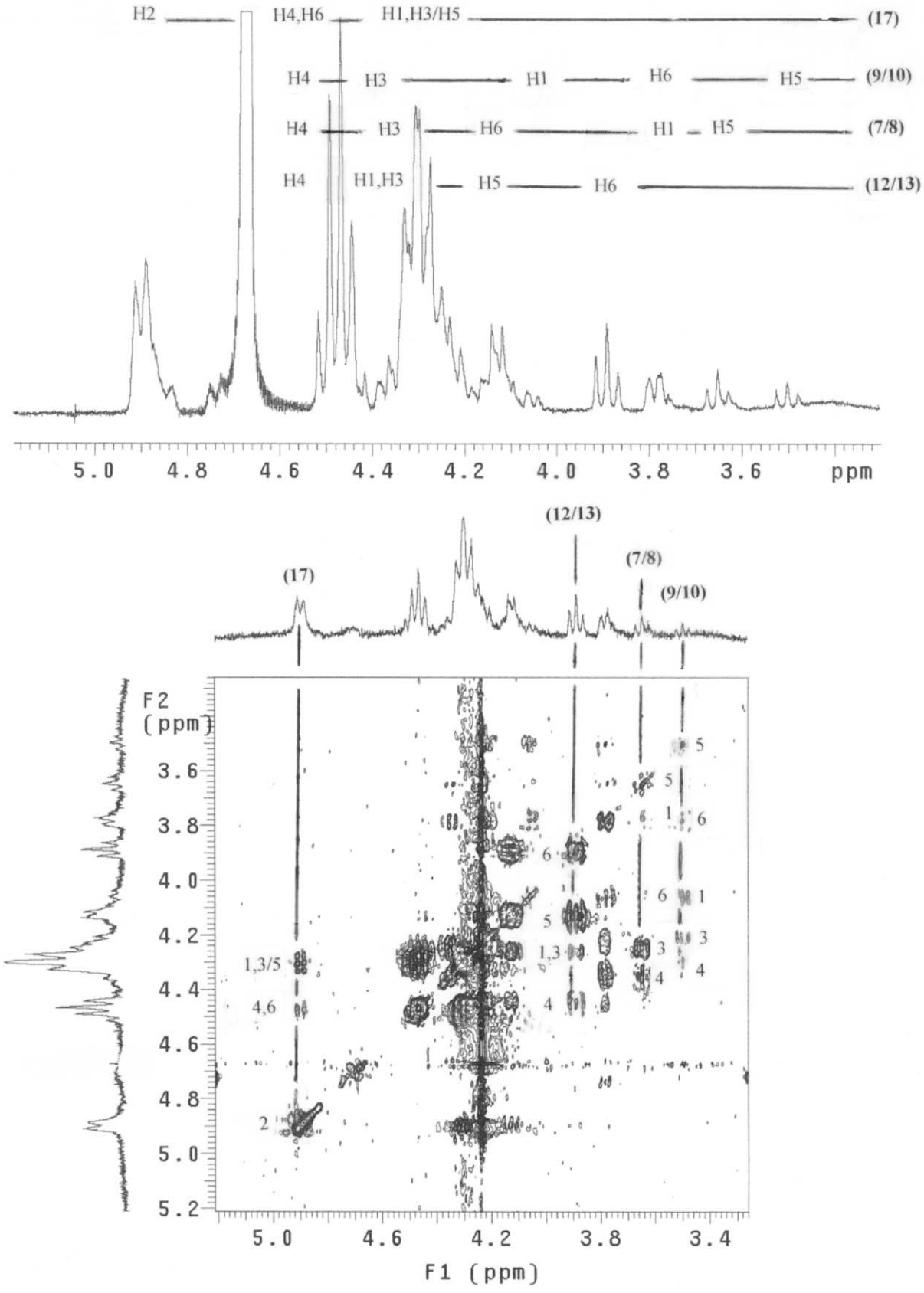

Fig. 4. Determination of structure of *myo*-inositol phosphates that accumulate in wild-type, non-mutant barley seed. Putative Ins phosphates were purified, and obtained as free acids. A number of NMR experiments were conducted; one- and two-dimensional NMR spectra are shown here. Proton assignments are indicated in the figure.

(or H-1) is geminal to a hydroxyl group. The other resonances, and results of gDQCOSY experiments, are also consistent with the presence of Ins(1,2,4,5,6)P_5 (**14**) and/or its enantiomer Ins(2,3,4,5,6)P_5 (**15**). Finally, a third spin system in Fig. 5 contains a triplet ($J = 9.7$ Hz) resonance that is comparatively upfield (about δ 3.82) and showed a connectivity to other resonances similar to that of (**12**/**13**) in Fig. 4, a spectrum consistent with the presence Ins(1,2,3,4,5)P_5 (**12**) and/or its enantiomer Ins(1,2,3,5,6)P_4 (**13**). The relative concentration of Ins P_6 (**17**): Ins(1,2,3,4,5)P_5 (**12**) and/or Ins(1,2,3,5,6)P_5 (**13**): Ins(1,2,4,5,6)P_5 (**14**) and/or Ins(2,3,4,5,6)P_5 (**15**),

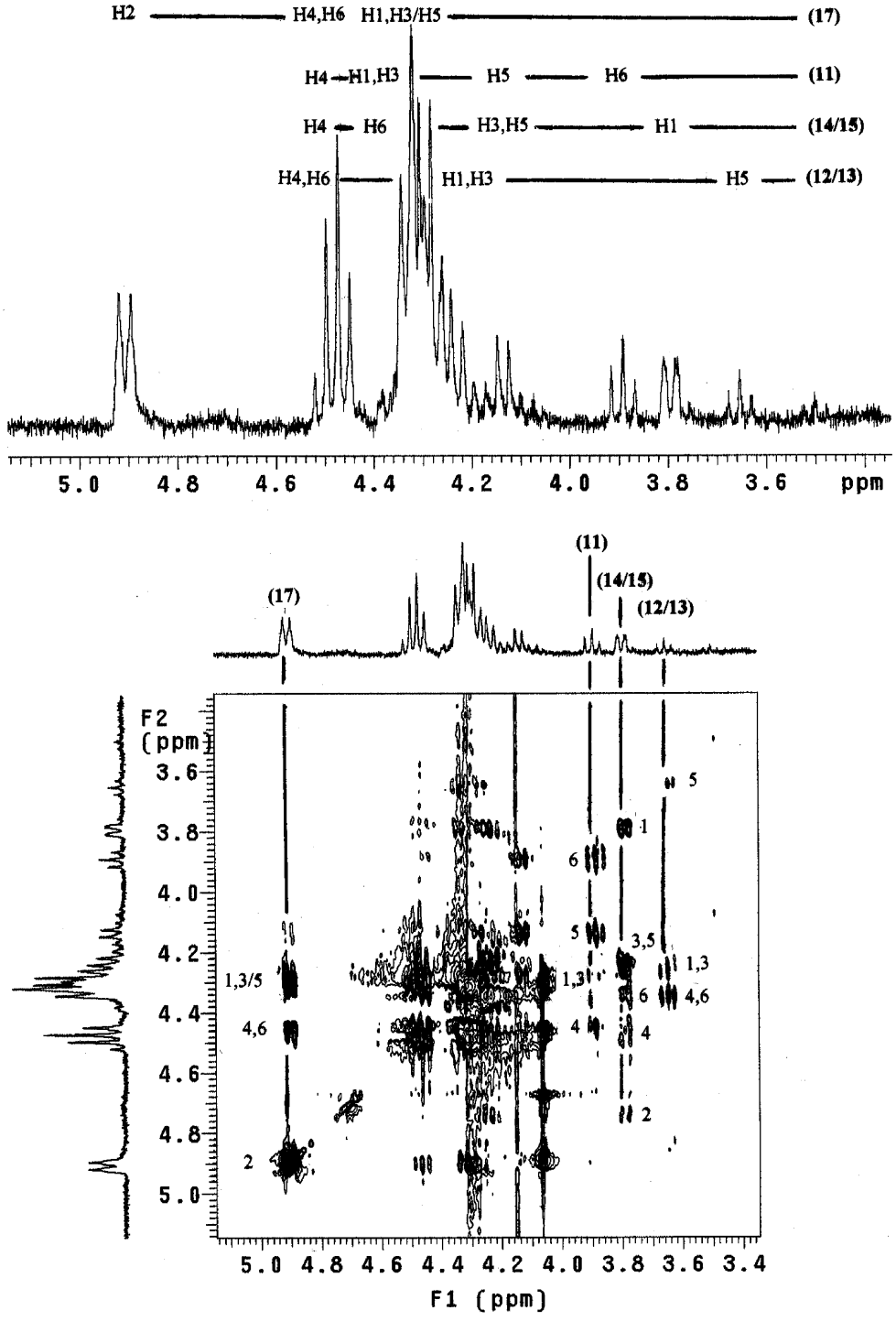

Fig. 5. Determination of structure of *myo*-inositol phosphates that accumulate in wild-type, non-mutant maize seed. Putative Ins phosphates were purified, and obtained as free acids. A number of NMR experiments were conducted; one- and two-dimensional NMR spectra are shown here. Proton assignments are indicated in the figure.

and Ins(1,2,3,4,6)P_5 (**11**) in this sample was: 1:0.28:0.21:0.18. Taken together, the HPLC and NMR results indicated that while Ins penta*kis*- and tetra*kis*-phosphates represented a larger fraction of seed total Ins phosphate in the non-mutant barley seed analyzed here than they did in the non-mutant maize seed, a largely similar pattern of Ins phosphates was observed in the mature, non-mutant seed of both species.

2.2. Isolation of barley lpa genotypes

A total of 2601 barley M_2s were screened for the *lpa* trait using a two-step screening process consisting first of an assay for the High Inorganic P (HIP) seed phenotype associated with homozygosity for an *lpa* mutation, followed by a High Voltage Paper Electrophoresis (HVPE) assay for altered levels of Ins phosphates and Ins P_6 (**17**) in seed of selected putative *lpa* genotypes (Larson et al., 2000; Raboy et al., 2000). Twenty-three independent, heritable *lpa* barley mutations were recovered as homozygotes. Four mutations representing a broad range of phenotypes based on the screening results were selected for initial study (Raboy et al., 2001). These were mutations found in the M_2 progenies No. 422, No. 635, No. 955 and No. 1070 (hereafter M 422, M 635, M 955, and M 1070).

Initial screening tests (HIP and HVPE) indicated that for M 422, M 635, and M 955, seed Ins P_6 (**17**) was reduced approximately 50, 75 and 95%, respectively, and these reductions were matched nearly entirely by increases in Pi (data not shown). In the case of M 955, HVPE tests of single seeds revealed a trace of Ins P_6 (**17**), thus Ins P_6 (**17**) appears greatly reduced but not absent from homozygous M 955 seeds (data not shown). For M 1070, a reduction in seed Ins P_6 (**17**) of about 50% appeared to be accompanied by increases in both Pi and Ins phosphates with five or fewer P esters. Based on these results, M 422 and M 1070 were identified as the first barley *lpa* genotypes possibly analogous to maize *lpa*1-1 and *lpa*2-1, respectively. They were demonstrated to be mutations of single genes that mapped to barley chromosomes 2H (M 422) and 7H (M1070) and were designated barley *low phytic acid* 1-1 (*lpa*1-1, indicating that it represents the first recessive allele of the barley *lpa*1 locus) and barley *lpa*2-1, respectively (Larson et al., 1998). Inheritance of M 635 and M 955 in field-grown F_2 progenies (Table 3) indicated that the mutant phenotype of these also were due to the inheritance of single-gene mutations. The allelic relationship or lack thereof of the M 635 and M 955 mutations to barley *lpa*1-1 and barley *lpa*2-1 is not yet known. M 635 and M 955 will continue to be referred to as such pending the results of ongoing allelism tests and/or chromosomal mapping.

Table 3
Inheritance of M 635 and M 955 *low phytic acid* mutations in segregating, field-grown F_2 progenies

Mutation	Generation	F_2 plant genotypes[a]			Chi-square[b]	
		+/+	+/*lpa*	*lpa*/*lpa*	Model	Value
M 635	BC_1 F_2	27	59	25	1:2:1	0.51
					3:1	0.36
M 955	F_2	22	53	19	1:2:1	1.73
					3:1	1.15

[a] The genotypes indicated are as follows: +/+, homozygous wild-type; +/*lpa*, heterozygous for the given *lpa* mutation; *lpa*/*lpa*, homozygous mutant.

[b] Inheritance models are: 1:2:1, 1/4 of a given progeny is +/+, 1/2 is +/*lpa*, and 1/4 is *lpa*/*lpa*; 3:1, 3/4 either +/+ or +/*lpa*, 1/4 *lpa*/*lpa*. Observed Chi-square values smaller than 5.991 for the 1:2:1 model (2 degrees of freedom, $P = 0.05$), or smaller than 3.841 for the 3:1 model (1 degree of freedom, $P = 0.05$), indicate an acceptable fit of the data to the model. Assuming a given model is correct, values greater than these thresholds would occur in less than 5% of progeny samplings by chance.

2.3. The inositol phosphate phenotype of selected barley lpa genotypes

When expressed as a percent of a given seed sample's total P, total soluble Ins phosphate in seeds homozygous for barley *lpa*1-1 (formerly M 422), M 635, and barley *lpa*2-1 (formerly M 1070) was reduced by 48, 75, and 41%, respectively, as compared with wild-type (Table 1). In the case of M 955, seed total Ins phosphate concentration is reduced to the extent that it is not reliably measured with the routine ferric precipitation method used here (Table 1). Expressing Ins phosphates as a percent of seed total P aids in comparisons between phosphate fractions, and in comparisons between different genotypes, lines or samples, since this normalizes data to variation in seed total P concentration. Some variation in seed total P may be a direct effect of the *lpa* mutation, or due to differences in environment or genetic background (independent of *lpa* loci) that impact the amount of seed total P. Seed produced by plants homozygous for each of the four barley *lpa* mutations, obtained from the same nursery as the non-mutant control, contained concentrations of total P ranging from 22% less than wild-type (in barley *lpa*1-1), to 14% more than wild-type (in barley *lpa*2-1, Table 1). In each *lpa* genotype the reduction in total Ins P was accompanied by a molar-equivalent increase in seed Pi concentration, so that the sum of total Ins P and Pi remained relatively constant, when expressed as a percent of seed total P (Table 1).

Phenotyping-HPLC analyses of barley *lpa*1-1 seed extracts (Fig. 2B; Table 2) indicated that the approximately 50% reduction in seed total Ins phosphate (compared to wild-type), observed with the ferric-precipitation method (Table 1), is due to reductions in the major Ins P_6 (**17**) peak, and to reductions in three of the

four Ins penta*kis*phosphates: Ins(1,2,3,4,5)P_5 (**12**) and/or Ins(1,2,3,5,6)P_5 (**13**); Ins(1,2,4,5,6)P_5 (**14**) and/or Ins(2,3,4,5,6)P_5 (**15**); and Ins(1,3,4,5,6)P_5 (**16**). However, there is a small but reproducibly detectable increase in an InsP_4 and Ins(1,2,3,4,6)P_5 (**11**), relative to the levels of the other Ins phosphates (Table 2). The 10X HPLC assay of barley *lpa*1-1 seed also indicated the continued presence of a phosphate-containing compound (''Unknown 1'', Fig. 3B) more polar than Ins P_6 (**17**), such as an Ins P_7 (**19**) or Ins P_8 (**20**).

Both phenotyping-HPLC (Fig. 2) and the 10-X assay (Fig. 3) confirmed that for barley M 635 (Figs. 2C and 3C) and M 955 (Figs. 2D and 3D), reductions in total soluble Ins P consists of proportional reductions in the major Ins P_6 (**17**) peak and the secondary Ins P_4 and Ins P_5 peaks observed in non-mutant seed (Figs. 2A and 3A). Thus for these two genotypes the net amount of total Ins phosphate is reduced, but the contribution of each Ins phosphate to total Ins phosphate is not greatly altered (Table 2). For example, the 10X-HPLC confirmed that while in M 955 seed both Ins P_6 (**17**) and Ins phosphates with five or fewer P esters are greatly reduced, at least trace amounts of Ins phosphates with five or fewer esters are still detectable and their amounts relative to each other and Ins P_6 (**17**) appears to be similar to that observed in seeds homozygous for M 635 or homozygous wild-type (Fig. 3D). The seed phenotype of M 635 and M 955 is analogous to maize *lpa*1-1 (Fig. 2G and Table 2) in that all soluble Ins phosphate peaks are reduced compared with wild-type (Fig. 2F and Table 2). These HPLC analyses also provide confirmation of the extent in total Ins phosphate reduction observed in *lpa* genotypes as indicated by the ferric-precipitation analysis (Table 1).

HPLC (Figs. 2E and 3E) and NMR analysis (Fig. 6) of barley *lpa*2-1 seed confirmed a phenotype in part similar to that of barley *lpa*1-1, but clearly different from M 635 and M 955. In barley *lpa*2-1 seed there is both a net reduction in Ins phosphate as compared with wild-type, and a substantial change in the relative contribution of Ins phosphates other than Ins P_6 (**17**). A change in the relative amounts of Ins phosphates in addition to a total Ins phosphate reduction represents a phenotype similar to that observed in maize *lpa*2 seed (Fig. 2H, Table 2). The sum of Ins P_3, Ins P_4 and Ins P_5 detected by HPLC analysis of barley *lpa*2-1 seed was 0.84 mg P g^{-1}, or 49% of total Ins phosphate in this seed (Fig. 2E and Table 2), whereas in wild-type barley Ins phosphates other than Ins P_6 (**17**) represented 16% of total Ins phosphate. HPLC analysis indicated that the altered distribution of Ins phosphates in barley *lpa*2-1 seed primarily consists of increases in one or more Ins tetra*kis*phosphates and Ins (1,2,3,4,6)P_5 (**11**). The more highly polar phosphate-containing compound (Unknown 1) was not detected in HPLC analysis of barley *lpa*2-1 seed extracts (Figs. 2E and 3E)

Six non-Ins P_6 Ins phosphates were identified via NMR of samples of purified free acids obtained from extracts of barley *lpa*2-1 seed (Fig. 6). 1D ^{1}H-NMR spectra (Fig. 6A and B) and 2D-TOCSY (not shown) experiments indicated that the most abundant Ins penta*kis*phosphate in barley *lpa*2-1 seeds is Ins(1,2,3,4,6)P_5 (**11**). The triplet ($J = 9.5$ Hz) splitting patterns at the relative upfield (approximately δ 3.6) position is clearly evident and this suggests that dephosphorylation has occurred at C-5 (proton assignments are indicated in Fig. 4; Barrientos et al., 1994). Spectra corresponding to Ins(1,2,3,4,6)P_5 (**11**) can be seen in Fig. 6B and C and the relative intensity suggests that Ins(1,2,3,4,6)P_5 (**11**) was a major component in these fractions.

In Fig. 6B, the triplet splitting pattern at approximately δ 3.82 ($J = 10$ Hz, H-6) indicates that this hydrogen is attached to a non-phosphorylated carbon at C-4/C-6. This NMR spectrum is consistent with the presence of two compounds, the major component being Ins(1,2,3,4,6)P_5 (**11**) and the minor component being Ins(1,2,3,4,5)P_5 (**12**) and/or Ins(1,2,3,5,6)P_5 (**13**) (proton assignments are indicated in Fig. 6B). Additional confirmation that the broad triplet centered at approximately δ 4.25 is due to overlapping resonances of H-1 and H-3 of both the Ins penta*kis*phosphates was obtained by J-resolved, DQCOSY, and TOCSY experiments (data not shown). The structure consistent with all these experiments is Ins(1,2,3,4,5)P_5 (**12**) and/or Ins(1,2,3,5,6)P_5 (**13**).

In Fig. 6C the two comparatively upfield resonances at δ 3.66 (triplet, $J = 9.2$ Hz) and δ 3.8 (doublet of doublets, $J = 9.5$ and 2.2 Hz), similar to spin system labeled (**7/8**) in Fig. 4, indicated the presence of Ins(1,2,4,6)P_4 (**7**) and/or Ins(2,3,4,6)P_4 (**8**). Chemical shifts of the other hydrogens, indicated in the fig. were consistent with the presence of geminal phosphates. The two additional relatively upfield resonances at about δ 3.5 (triplet, $J = 9.2$ Hz) and δ 3.78, similar to spin system labeled (**9/10**) in Fig. 4, indicate the presence of Ins (1,2,3,4)P_4 (**9**) and/or Ins(1,2,3,6)P_4 (**10**).

Two isomers of Ins P_3 were detected in NMR analysis of free acids obtained from barley *lpa*2-1 (Fig. 6D and E). 1D ^{1}H NMR spectra (Fig. 6D) and 2D-TOCSY (not shown) experiments revealed the presence of two compounds, Ins(1,2,3,4,6)P_5 (**11**) (compare with Fig. 6A) and an isomer of Ins P_3. The appearance of a triplet at approximately δ 3.48 ($J = 9.5$ Hz), a broad doublet centered at approximately δ 3.66 ($J = 10$ and 2 Hz) and a triplet at about δ 3.78 ($J = 9.5$ Hz) suggests that H-1/H-3, H-5 and H-4/H-6 are attached to hydroxyl-bearing carbons. J-resolved, DQCOSY and P-decoupled experiments were conducted and the structure consistent with all the data is Ins(1,2,6)P_3 (**3**) and/or Ins(2,3,4)P_3 (**4**). ^{1}H NMR of a second Ins *tris*phosphate (Fig. 6E) showed the presence of three resonances due to protons

attached to non-phosphorylated carbons, one centered at approximately δ 3.52 (doublet of doublets, $J = 10$ Hz and 2.5 Hz) due to H-1(or H-3), a second resonance at δ 3.69 (triplet, $J = 9.5$ Hz) due to H-6 (or H-4) and a third resonance at approximately δ 4.1 (triplet, $J = 2.6$ Hz) due to H-2 (Fig. 6E). Additional NMR spectroscopy experiments (J-resolved, Homonuclear decoupling, and DQCOSY) were conducted and the structure consistent with all the NMR data is Ins(1,5,6)P_3 (**5**) and/or Ins(3,4,5)P_3 (**6**).

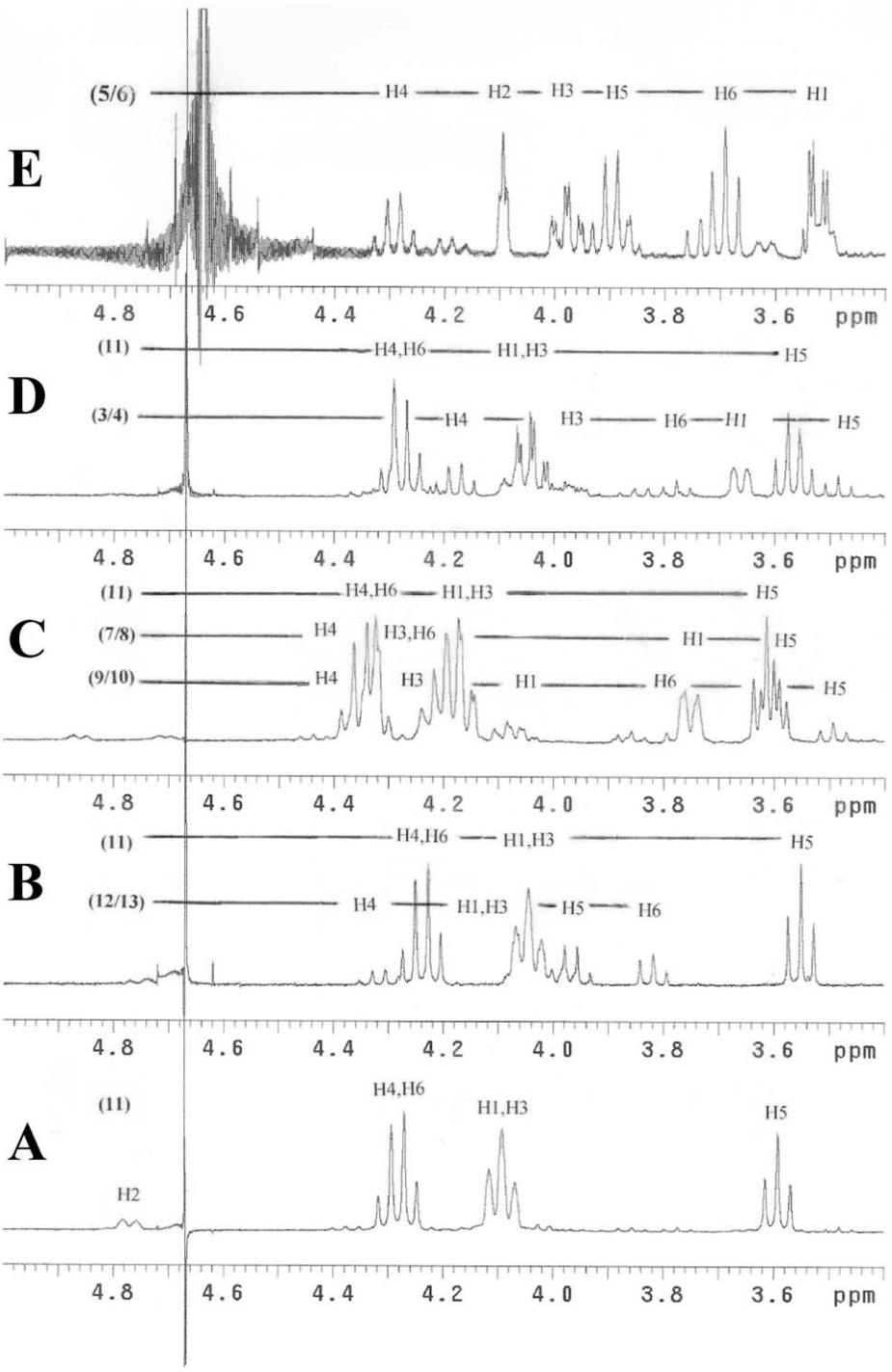

Fig. 6. Determination of structure of *myo*-inositol phosphates that accumulate in homozygous barley *low phytic acid* 2-1 (*lpa*2-1) seed. Putative Ins phosphates were purified, and obtained as free acids, either as highly pure individual free acids (A), or as mixtures. A number of NMR experiments were conducted; one-dimensional NMR spectra are shown here. Proton assignments are indicated in the figure. In (A), (B), (C) and (D), assignment of the major component, Ins(1,2,3,4,6)P_5 (**11**), is indicated in brackets and proton 2 is under the water peak and therefore not indicated.

3. Discussion

Based on the similarity in seed P and Ins phosphate phenotypes, we hypothesize that M 635 and M 955 are analogous to maize *lpa*1-1, and may be lesions in Ins (**1**) synthesis or supply in the developing seed. Similarly we hypothesize that barley *lpa*2-1 is analogous to maize *lpa*2-1 and is a lesion in subsequent Ins phosphate metabolism. Barley *lpa* 1-1 may also be an *lpa*2-like mutation, in that total seed Ins phosphate is reduced, and the relative contribution to total Ins phosphate of Ins phosphates with five or fewer P esters is altered, as compared with wild-type. However the increase in Ins(1,2,3,4,6)P_5 (**11**) in barley *lpa* 1-1, as compared with levels observed in wild-type seed, is relatively small when compared with the altered distribution of Ins phosphates in barley and maize *lpa* 2 mutations. Therefore further studies of barley *lpa* 1-1 are required before an hypothesis concerning the nature of its genetic lesion can be proposed.

The most straightforward explanation for the accumulation of a ''1-/3-OH'' penta*kis*phosphate in maize *lpa*2-1 seed and a ''5-OH'' penta*kis*phopshate in barley *lpa*2-1 seed is that they represent lesions in a 1-/3-kinase activity in maize and a 5-kinase activity in barley, like the one isolated from developing soybean seed (Phillippy, 1998). Accumulation of these Ins penta*kis*phosphates is then followed by smaller accumulations of their respective breakdown products, such as Ins(1,2,4,6)P_4 (**7**) and/or Ins(2,3,4,6)P_4 (**8**) in barley *lpa*2-1 and Ins(1,4,5,6)P_4 in maize *lpa*2-1. Studies of Ins phosphate metabolism in *Dictyostelium* had shown that while other Ins penta*kis*phosphates have short half-lives and do not accumulate, two Ins penta*kis*phosphates, Ins(1,2,4,5,6)P_5 (**14**) and Ins(1,2,3,4,6)P_5 (**11**), have slow turnover rates and accumulate to higher steady state levels (Stephens et al., 1991). It was therefore proposed (Raboy et al., 2000) that the accumulation of Ins(1,2,4,5,6)P_5 (**14**) in maize *lpa*2-1 reflected the fact that this compound functions, along with Ins(1,2,3,4,6)P_5 (**11**) and Ins P_6 (**17**), as part of a complex buffer or storage pathway for Ins phosphates in cells. The accumulation of Ins(1,2,3,4,6)P_5 (**11**) and its breakdown products in barley *lpa*2-1 seed supports this hypothesis. In the earlier *Dictyostelium* study (Stephens et al., 1991), the interconversion of Ins(1,2,4,5,6)P_5 (**14**) and Ins(1,2,3,4,6)P_5 (**11**) with Ins P_6 were seen as futile cycles, with no subsequent metabolism of the penta*kis*phosphates. In contrast, the results given here and in Raboy et al. (2000) indicate that these compounds may be further metabolized in seed tissues.

Mutants isolated in an independent study (Rasmussen and Hatzack, 1998) also fit into two distinct phenotypic classes that may be analogous to the *lpa*1 and *lpa*2 maize and barley genotypes described here. An ''A-type'' Low Phytate (LP) mutant was isolated that is similar to the maize and barley *lpa*2 genotypes in that its seed accumulate an Ins P_4, Ins(1,3,4,5)P_4, observed at only trace levels in wild-type, non-mutant kernels. It was proposed that this ''A-Type'' Low Phytate mutation may be an Ins(1,3,4)P_3 5/6 kinase mutation (Hatzack et al., 2001). Recent studies have shown that a mammalian Ins(1,3,4)P_3 5/6 kinase can also have Ins(3,4,5,6)P_4 1-kinase activity, and is therefore a multifunctional or multitasking enzyme (Yang and Shears, 2000). Since initial chromosomal mapping results suggest that the maize and barley *lpa* 2 mutations are in orthologous genes (Larson et al., 1998; Raboy et al., 2000), the most parsimonious hypothesis is that both are mutations in a single gene encoding a multifunctional Ins polyphosphate kinase that has 1-/3-OH, 5-OH and 6-OH kinase activities. Perturbation of such a single gene might result in the accumulation of 1-/3-OH penta*kis*phosphates in maize and 5-OH penta*kis*phosphate in barley.

A previous study of Ins phosphates in imbibed barley aleurone layers (Brearley and Hanke, 1996) identified a series of Ins phosphate compounds, including the Ins(1,2,3,4,6)P_5 (**11**) and Ins(1,2,3,4,5)P_5 (**12**), and/or its enantiomer Ins(1,2,3,5,6)P_5 (**13**), also observed here in mature barley *lpa*2-1 seed. The Ins phosphates identified in the earlier study of imbibing barley aleurones were all viewed as likely breakdown products of Ins P_6, catalyzed by endogenous phytase activity with stereospecificity similar to wheat-bran phytase. It is unlikely that the single-gene mutation in barley *lpa*2-1 or in maize *lpa*2-1 results in an increase in a single, specific phosphatase or phytase activity. Such a gain-of-function mutation is a much rarer event than a possible loss-of-function mutation that might explain this phenotype, such as a mutation in an Ins phosphate 5-kinase.

The hypothesis of Morton and Raison (1963) that Ins P_6 (**17**) metabolism functions in ATP regeneration necessary for storage metabolite synthesis during seed development needs to be revisited in light of this and other studies. Studies of a mung bean (*Phaseolus aureus* L.) ''InsP_6-ADP phosphotransferase'' (Biswas et al., 1978) and subsequent studies of a similar soybean Ins(1,3,4,5,6)P_5 2-kinase (Phillippy et al., 1994) indicated that these enzymes could use Ins P_6 (**17**) as a phosphate donor for the conversion of ADP to ATP. Studies of the soybean enzyme revealed that under sufficiently high concentration of Ins P_6 (**17**), as would be expected in aleurone cells at initial stages of germination, the reaction would proceed in the direction of ADP to ATP.

Recent studies have also demonstrated a role for the PP-containing Ins phosphates (**19**, **20**) in ATP regeneration (Sasakawa et al., 1995), and putative PP-containing Ins phosphates have been observed in both mature (Fig. 3) and germinating (Brearley and Hanke, 1996) barley seed tissues. The identification of putative

PP-Ins phosphates in plant tissues is based on the observation of P- or Ins phosphate-containing compounds chromatographically more polar than Ins P_6 (**17**) (Fig. 3; Brealey and Hanke, 1996; Flores and Smart, 2000). This was how such compounds were first discovered in the cellular slime mold *Dictyostelium discoideum* (Europe-Finner et al., 1989). The low levels of this putative PP-Ins phosphate, and of Ins(1,3,4,5,6)P_5 (**16**), in mature seeds may in fact reflect relatively rapid turnover rates or conversion to Ins P_6 (**17**) during development, not low rates of synthesis. One characteristic of the metabolism of PP-containing Ins phosphates observed in previous studies of non-plant systems is their rapid turnover rates (Safrany et al., 1999). However, PP-Ins phosphates do accumulate during spore formation in *Dictyostelium* and in a second slime mold *Polysphondylium pallidum*, a process similar to seed development in higher plants in that nutrient stores are accumulated and the organ enters a resting stage, and can represent 25–50% of spore Ins polyphosphate (Laussmann et al., 2000). Yet a "P storage" or "P metabolism" role for such compounds and Ins P_6 (**17**) is typically not considered in studies of non-plant systems.

The penultimate Ins phosphate in the primary synthetic pathway to Ins P_6 is believed to be Ins(1,3,4,5,6)P_5 (**16**), with the final phosphate addition catalyzed by a 2-OH kinase (Stephens and Irvine, 1990). Regardless of which genes are perturbed in specific *lpa* mutations, the Ins phosphate phenotypes of non-mutant maize and barley seed and their respective *lpa* genotypes supports the hypothesis that Ins P_6 metabolism is more complex than a simple, linear addition of phosphate esters. Rather, several Ins penta*kis*phosphates in addition to Ins(1,3,4,5,6)P_6 (**16**), and at least one more highly phosphorylated PP-Ins phosphate, together probably represent a complex buffer or pool that is an integral part of Ins phosphate metabolism and Ins P_6 accumulation.

The results of this study of barley *lpa* genotypes support and extend two main conclusions of the first study of maize *lpa* genotypes (Raboy et al., 2000). First, the viability of maize *lpa*1-1 homozygotes, with a 66% reduction in seed Ins P_6 (**17**), and maize *lpa*2-1 homozygotes, with a 50% reduction in seed Ins P_6 (**17**), provided genetic evidence that the ability to synthesize and accumulate Ins P_6 (**17**) levels typical of a non-mutant seed is not essential to basic seed function, at least for one cultivated species (maize) grown under typical cultural conditions. The viability of barley M 955 homozygotes indicates that, for barley at least, the ability of seed to accumulate Ins P_6 (**17**) can be reduced by 90% or greater while maintaining at least short-term ($\sim$1.0 year) viability. Careful studies have not yet been conducted to determine the long-term ($>$1.0 years) of any *low phytic acid* genotype in any species. The potential role for Ins P_6 (**17**) as an antioxidant (Graf et al., 1987) might be important to the maintenance of the long-term viability of seeds. The genetic resources represented by these *lpa* genotypes provides an experimental model useful for testing the potential role of Ins P_6 (**17**) as an antioxidant possibly important to long-term seed viability.

A second conclusion drawn in the initial studies of maize *lpa* genotypes (Raboy et al., 2000) is that Ins P_6 (**17**) metabolism during seed development probably plays only a minor role in P homeostasis. Some other mechanism, such as Pi compartmentalization, probably represents the major mechanism. Like the maize *lpa* genotypes, variable reductions in seed dry weight are also observed in the barley genotypes, with the greatest reductions observed in M 955. While the loss in seed dry weight might indicate a minor role in P homeostasis, it might also be due to some other effect such as Ins (**1**) deficiency, as hypothesized for several maize genotypes (Raboy et al., 2000). Since "low phytate" mutations greatly perturb the chemistry of two important cellular compounds, phosphate and Ins (**1**), one would expect many downstream effects on seed chemistry, physiology, and gene expression/regulation for genes in pathways involving phosphate or Ins (**1**). For example, a mutation in one of the soybean genome's MIPS genes reduces seed Ins P_6 (**17**) and increases seed Pi (Hitz et al., 2002). The reduced supply of Ins (**1**) in this mutant's seed also reduces the raffinose/stachyose series of compounds. In light of the numerous pathways dependent on Ins (**1**) or phosphate supply and/or homeostasis, it is surprising that mutations like M 955 are in fact viable.

The main selective advantage for Ins P_6 (**17**) accumulation in seeds might be in efficient P and mineral storage (Raboy et al., 2000). Efficient storage and retrieval of P and minerals might be essential for survival and successful germination in the natural environment in which the progenitors to cultivated species evolved. It is not known how the large shifts in seed P composition observed in *lpa* genotypes, from Ins P_6 (**17**) to Pi, might impact P or mineral leaching, seed and grain production and storage, or disease, predation or stress susceptibility. The lack of a severe impact on seed phenotype (other than P and Ins phosphate phenotype) and seed function of these *lpa* mutants might simply reflect the fact that they primarily impact a relatively inert seed fraction. The bulk of Ins phosphate that accumulates in seed tissues is sequestered and deposited in specialized compartments known as protein storage vacuoles, and therefore removed from cellular metabolism. Perhaps only low levels of cellular Ins P_6 (**17**) (relative to the total mass that accumulates as a sequestered storage product), levels like that observed in M 955 seed, is sufficient to provide substrate for the potential multitude of non-storage cellular processes.

4. Experimental

4.1. Plant materials

An M_2 population containing random, sodium azide-induced mutations was generated in the barley cultivar "Harrington" using the seed treatment method as described by Nilan et al. (1973). M_3 seed harvested from individual M_2 plants were screened for the high inorganic P (HIP) phenotype associated with the *lpa* trait (Raboy et al., 2000). Five M_3 seeds were sampled from each M2 plant, individually crushed with a hammer blow or lab press, and incubated overnight at 4 °C in 0.4 M HCl (10 μl per mg seed). The extracts were then briefly vortexed, and allowed to settle for a minimum of 0.5 h. Aliquots were assayed for Pi using the microtitre plate colorimetric assay as described (Larson et al., 2000; Raboy et al., 2000). Non-mutant barley seeds typically contain $\leqslant 0.7$ mg Pi g^{-1}. Seed testing for >1.0 mg Pi g^{-1} were deemed "HIP", and their extracts were then tested for a corresponding reduction in Ins P_6 (**17**), or unusual accumulations of other Ins phosphates, using a high voltage paper electrophoresis (HVPE) assay for acid-extractable P-containing compounds (Raboy et al., 2000).

Seed representing selected M_2 progenies were field-planted. Progenies either homozygous or segregating for selected mutations were identified using the HIP and HVPE assays. Plants containing barley *lpa* mutations were crossed to the parental cultivar Harrington. The F_1 seed obtained were used to produce F_2 progenies, or used to produce BackCross$_1$ (BC_1) progeny, which in turn were used to produce BC_2 and subsequent backcross generations. Self-pollination of a given BC generation produced BC_x F_2 progenies segregating for a given mutation. These were used for inheritance studies, and to isolate sibling BC_x F_3 and subsequent progenies homozygous for a given mutant or wild-type allele.

Homozygous wild-type or non-mutant (in terms of seed phytic acid) maize seed of the inbred line A619, an A619 BC_5F_4 line homozygous for maize *lpa*1-1, and an A619 BC_2F_4 line homozygous for *lpa* 2-2, the second recessive allele of the *lpa*2 locus, were planted in a field nursery and self-pollinated, providing seed for this study. Homozygosity for the *lpa*2-2 allele used here produces seed with a phenotype similar to seed homozygous for the *lpa*2-1 allele originally reported (Raboy et al., 2000).

4.2. Quantitative analyses of seed P and Ins P fractions

Samples of mature seeds were dried for 48 h at 60 °C. These were then milled to pass through a 20-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., 2000). Aliquots of tissue (typically 0.5–1.0 g) 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. Ins phosphates are expressed as their P (atomic weight 31) content to facilitate comparisons between seed P fractions. Seed Pi 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) TCA/25 mM $MgCl_2$.

4.3. HPLC

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). Four Ins penta*kis*phosphates were kindly provided by Dr. Brian Phillippy, USDA Southern Regional Research Center, for use as chromatographic and NMR standards. These were Ins(1,2,3,4,6)P_5 (**11**), Ins(1,2,3,4,5)P_5 (**12**) and/or its enantiomer Ins(1,2,3,5,6)P_5 (**12**), Ins(1,2,4,5,6)P_5 (**14**) and/or its enantiomer Ins(2,3,4,5,6)P_5 (**15**), and Ins(1,3,4,5,6)P_5 (**16**). Samples of seeds were dried and milled as described above. Two methods of sample preparation were used. For "Phenotyping-HPLC", flour (40 g) was extracted in 0.4 M HCl (100 ml) overnight. Following centrifugation (15,000 *g*, 30 min), 200 μl of supernatant was diluted with dd H_2O (to 1.0 ml) and passed through a 0.2 μm filter. Aliquots (200 μl) were then fractionated as indicated below. For "10X-HPLC", aliquots (10 ml) of the same supernatants were first diluted with dd H_2O (to 20 ml) in a 30-ml Corex centrifuge tube, and 15 mM $FeCl_3$/0.2 M HCl (5 ml) was added. These were heated at 90 °C for 30 min, and centrifuged (8000 *g*, 30 min). The ferric Ins phosphate precipitate was then treated with 1.5 M NaOH (1.0 ml, 0.5 ml in the case of M 955), converting the ferric Ins phosphate to soluble Na Ins phosphate and an FeOH precipitate. The solutions (1.0 ml) were transferred to 1.5-ml microfuge tubes, and centrifuged (12,000 rpm, 10 min). An aliquot (200 μl) of supernatant was then diluted to 1.0 ml, passed through a 0.2 μm filter, and an aliquot (200 μl) was then fractionated indicated below.

Samples were fractionated on a Dionex IonPac AS7 anion-exchange column, equipped with a Dionex IonPac AG7 guard column, 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 1.0 ml min^{-1}: initial condition, 100% Buffer A; 1–45 min a linear gradient from 0 to 80% 0.5 M NaCl pH 4.0, in 10 mM methyl piperazine pH 4.0 (Buffer B); 45–60 min 20–100% Buffer A. The column eluent was mixed with metal dye detection

colorimetric reagent (1.5% $FeCl_3$:0.15% sulfosalicylic acid) at a flow rate of 0.5 ml min^{-1}, using an Upchurch PEEK high pressure mixing tee (VWR) and a Eldex Model B-100-S metering pump (Eldex Laboratories, Inc., Menlo Park, CA, USA), and the mixture passed through a 100 cm reaction coil prior to peak detection via absorbance at 550 nm. Ins phosphate in a sample peak was calculated using a standard curve obtained via the analysis of seven Ins P_6 standards, prepared using commercially-obtained Na Ins P_6 (Sigma). These standards contained 25, 50, 75, 100, 125, 150 and 200 nM Na Ins P_6, which yielded area units ($\times 10^6$) of 5.9, 14.5, 22.0, 31.1, 39.1, 45.3, and 59.5, respectively.

4.4. Purification and structural identification of Ins phosphates in barley and maize non-mutant seed and in barley lpa2-1 seeds

The objective was to purify and isolate as free acids soluble Ins phosphates found in non-mutant barley seed (cv. Harrington), non-mutant maize seed (inbred A 619) and barley *lpa*2-1 seed, and then to determine their structures using NMR. In most samples mixtures of Ins phosphates were obtained. In some cases (Fig. 6A) an Ins phosphate was purified to relative homogeneity. Seed (100–500 g) was ground with a coffee grinder and extracted in 0.4 M HCl (1.0–2.0 l) 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. In initial experiments supernatants containing Na Ins phosphates were neutralized with HCl and loaded onto preparative Dowex 1X2-400 anion exchange columns (packed volume 5 ml). These were eluted with a 0.0–0.4 M HCl linear gradient (400 ml) or a 0.4 M HCl isocratic gradient (400 ml), and 5 ml fractions were collected. Fractions containing Ins phosphates were identified following acid digestion of fraction aliquots, and colorimetric assay for P in the digests. In subsequent experiments, Na Ins phosphate-containing supernatants were fractionated using an FPLC equipped with a Pharmacia mono-Q 5/5 column. The column was equilibrated with 10 mM methyl piperazine pH 4.0 (Buffer A), and the sample then loaded at a flow rate of 0.5 ml min^{-1}. The column was then eluted (flow rate of 0.5 ml min^{-1}) with the following gradient: 1–120 min a linear gradient from 0 to 30% 0.5 M NaCl pH 4.0, in 10 mM methyl piperazine pH 4.0 (Buffer B); 120–145 min 100% Buffer B. One ml fractions were collected. Aliquots of each fraction were tested for the presence of Ins phosphates with a microtitre plate assay that used the same metal dye detection colorimetric reagent (1.5% $FeCl_3$:0.15% sulfosalicylic acid) employed in HPLC described above.

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 Savant SpeedVac Concentrator. The structures of these Ins phosphates were determined by a combination of one and two-dimensional (1D and 2D) 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 Varian Unity Inova-400 spectrometer. 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 ^1H NMR spectra were obtained at 399.943 MHz. ^1H 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 6738 Hz, pulse width 90° tipping angle. Typically, 16–32 scans with recycle delays of 4–6 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).

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