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Characterization of the storage of phosphorus, inositol phosphate and cations in grain tissues of four barley (*Hordeum vulgare* L.) *low phytic acid* genotypes

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Abstract

Phosphorus (P), phytic acid (*myo*-inositol hexa*kis*phosphate or Ins P_6) and mineral storage were studied in grains homozygous for barley (*Hordeum vulgare* L.) *low phytic acid* (*lpa*) mutations named *lpa*1-1, M 635, M 955, and *lpa*2-1. In wild-type (WT) grain both the embryo and rest-of-grain (aleurone layer) contained about 10 and 90% of whole-grain total P and Ins P. Most of the inositol phosphate (Ins P) was phytic acid. Proportional reductions in both embryo and aleurone layer Ins P contributed to whole-grain Ins P_6 reductions in M 635 and M 955, with little effect on embryo or aleurone layer total P. In terms of total P these mutations show no grain-tissue-specificity. In contrast, whole-grain Ins P_6 reduction in *lpa*1-1 and *lpa*2-1 is solely or largely aleurone layer specific. In *lpa*1-1 the distribution of total P shifted in part, from rest-of-grain to embryo, with a net reduction in total P. Electron microscopy showed a general reduction, as compared with WT, in electron-dense globoids in both aleurone and scutellum cells in all mutants except *lpa*2-1, whose globoid morphology appeared indistinguishable from WT. Energy-dispersive X-ray analyses of P, K, Mg, Ca, Fe, and Zn indicated that, generally, relative levels were similar in mutant and WT aleurone layer and scutellum tissues. Likely inorganic P found in *lpa* grains is stored as salts of K and Mg, as is Ins P_6 in WT grain.

Keywords: Barley (*Hordeum vulgare* L.); Embryo and aleurone globoids; Grains; Inositol phosphates; *Low phytic acid*; Phosphorus

1. Introduction

Phytic acid (*myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate or Ins P_6) is the main storage form of phosphorus (P) in seeds/grains, where it frequently accounts for 1% or more of the dry weight and 50–80% of the total P [1–4]. Phytic acid is an effective chelator of cations. It is deposited in seeds/grains as phytate salts of mineral cations such as K, Mg, Ca, Fe, Zn and Mn. Such salts are called phytate. In mature grains phytate is organized into spherical inclusions called globoids that are in turn found within protein bodies [5]. When viewed with the transmission electron microscope, these globoids are naturally-electron-dense. Phytate deposits are also observed to occur transiently in various tissues and subcellular compartments during grain development [6,7]. Thus phytate deposition is thought to play an important role in storage and homeostasis of both P and some other mineral nutrients during grain development and maturation.

There is considerable interest in *low phytic acid* (*lpa*) mutants of major seed crops since such seeds may: (1) be more nutritious because of improved bioavailability of P, Ca, Fe and Zn when seeds/grains are eaten by man and other monogastric animals, and (2) reduce P pollution of groundwater and surface waters by reducing P in manure [8]. *Low phytic acid* mutants, such as those isolated in maize [8–10], rice [11], soybean [12,13], barley [14–16] and wheat [17], also provide a valuable study system to aid understanding

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of: (1) the synthetic pathways of Ins phosphates in developing grains, (2) how electron-dense globoids form, and (3) the maintenance of homeostasis of P and several minerals in cells. For example, the maize *lpa*2 gene encodes a variant of the Ins(1,3,4) P_3 5-/6-kinase gene family [18]. This enzyme was first characterized in studies of mammalian cells as acting *in vivo* on a substrate derived from Ins(1,4,5) P_3, in turn the product of the canonical phosphatidylinositol phosphate pathway [19]. Study of developing *lpa*2 maize grain provided evidence that synthesis of Ins P_6 proceeds, at least in part, via phosphatidylinositol phosphate early-intermediates, rather than solely via a step-wise phosphorylation of soluble inositol phosphates [20,21]. A reduced function mutation in a soybean sequence encoding a D-*myo*-inositol-3-P_1 synthase (MIPS), the enzyme that converts glucose 6-P to Ins(3) P_1, results in both reduced grain phytate and reduced grain raffinosaccharides [12]. This provides genetic evidence that *de novo*, seed-specific synthesis of Ins(3) P_1 is critical to net seed phytate accumulation.

Dry, non-mutant or wild-type (WT) barley grains contain about 0.38% total P and 1.02% Ins P_6 on a whole-grain dry weight basis [4]. In the homozygous condition the four barley *lpa* mutations studied here, *lpa*1-1 (formerly M 422), M 635, M 955 and *lpa*2-1 (formerly M 1070), result in whole-grain Ins P_6 reductions of 50–60, 70–75, 90–95 and 50–60%, respectively [8,14]. In M 635 and M 955 the reductions in whole-grain Ins P_6 are matched almost entirely by increases in grain inorganic phosphate (Pi). Barley *lpa*2-1 is phenotypically similar to the two alleles of the maize *lpa*2 locus in that reductions in whole-grain Ins P_6 are accompanied by increases in both Pi and specific inositol phosphates (Ins P) with five or fewer phosphate esters, primarily Ins(1,2,3,4,6) P_5 and Ins(1,2,4,6) P_4 (and/or its enantiomer Ins(2,3,4,6) P_4 since the methods used here do not distinguish between enantiomers) [14]. These lower inositol phosphates are not called phytic acid but functionally act like phytic acid in that they serve as a store of inositol and P, in addition to providing binding sites for various cations. Initial analyses of barley *lpa*1-1 indicated that Ins P_6 reductions appeared to be accompanied solely by increases in Pi [16]. However, more recent HPLC assays [14] indicated that while decreases in Ins P_6 in barley *lpa*1-1 grain were largely accompanied by increased Pi, there was also a small but reproducibly detectable increase in the same Ins P_5 that accumulates in barley *lpa*2-1, Ins(1,2,3,4,6) P_5. Thus these four barley *lpa* mutations provide a model consisting of a set of mutations whose effects on grain P and Ins P range from relatively moderate (*lpa*1-1) to extreme (M 955), and whose grain phenotypes indicate that these mutations alter two or more steps in the Ins and Ins P pathways.

In research reported here four barley *lpa* mutations [8,14,16] were used to study the effect of perturbing grain Ins P_6 accumulation on P, Ins P and mineral distribution and deposition in globoids in the mature cereal whole-grain and grain parts. Mature grains (caryopses) produced by grass species including barley are composed of endosperm and germ (embryo axis and scutellum) regions surrounded by outer protective pericarp/testa layers [22]. Barley grain anatomy is illustrated in [22]. While the bulk of the endosperm is the starchy endosperm, the outermost part of the endosperm forms the aleurone layer, which in barley is typically two to three cells thick [22,23]. The scutellum, which is the biggest portion of the embryo, is a storage structure for the germ (embryo) and absorbs nutrients released from the endosperm. The embryo and aleurone layer cells are rich in lipid bodies and protein bodies [24–26]. Protein bodies in barley aleurone layer and embryo cells contain phytate-rich globoids [25,27,28]. Greater than 80% of the Ins P_6 in small grains like barley is localized in the aleurone layer with the remainder in the germ, whereas the reverse is observed with maize [29]. The research reported here provides a major addition to our understanding of *lpa* mutant barley grains in that it investigated the composition of mineral nutrient stores and the structure of phosphorus-rich deposits in embryo and aleurone layer cells to determine if they were affected in the same way by the different *lpa* mutations. The evidence we present is consistent with globoids in the *lpa* mutant grains being a mixture of Ins P_6, Pi, perhaps other inositol phosphates with less than six phosphate groups, as well as other plant mineral nutrients such as K, Mg, Ca, Fe and Zn.

2. Materials and methods

2.1. Plant material

Mature barley (*Hordeum vulgare* cv. Harrington) grains were provided by the USDA-ARS, Small Grains and Potato Research Unit, Aberdeen, Idaho. Grains used in this study were produced in the year 2000 in the same nursery located at the University of Idaho's Tetonia Research Center. Homozygous *lpa* mutations (*lpa*1-1, M 635, M 955 and *lpa*2-1) were isolated in the cv. Harrington. Homozygous Harrington grain was used as the wild-type control.

2.2. Preparation of grain fractions for chemical analyses

Normal appearing and undamaged grains were separated into two fractions, the "embryo" and the "rest-of-grain" portions. To slightly soften the grain so that embryo removal was precise the grains were soaked for 2–5 h in 90% ethanol. This procedure was selected since phytate is not soluble in 90% ethanol but some tissue swelling occurs [30]. Analysis of the ethanol soaking solution revealed that very little P, in any chemical form, was released.

The percent moisture for whole grains and grain fractions was determined by heating samples in an oven for 2 h at 130 °C [31]. The results of quantitative measurements are presented on a dry weight basis.

2.3. Total P and inositol P analysis

Total P in the embryo and rest-of-grain fractions was determined using the procedures of Dorsch et al. [14]. Samples 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. Total P was determined following wet-ashing of aliquots (typically 150 mg) using a colorimetric assay of digest [32].

Anion-exchange HPLC analyses of grain inositol phosphates were performed using a modification of the method as described in [33,34]. 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, D/L-Ins(1,2,3,5,6) P_5, D/L-Ins(1,2,4,5,6) P_5, and Ins(1,3,4,5,6) P_5. Tissue samples were extracted in 0.4 M HCl (1.0 ml). Aliquots (0.2 ml) were diluted with dd H_2O (to 1.0 ml) and passed through a 0.2 μm filter. Aliquots (0.2 ml) were then fractionated on a Waters HPLC setup (No. 626 Pump, No. 600 S Controller and a No. 490 E Multiwavelength Detector) equipped with a Dionex IonPac AS7 anion-exchange column (with a Dionex IonPac AG7 guard column). The anion-exchange column 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% to 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 an 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 500 nm. Ins phosphate in a sample peak was calculated via comparison with a standard curve obtained via analysis of solutions of commercial Na Ins P_6 (Sigma–Aldrich).

2.4. Specimen preparation for environmental scanning electron microscopy

Environmental scanning electron microscopy (ESEM), in conjunction with X-ray microanalysis was used to determine the element content of selected cell regions in tissue that received no chemical treatment or metal coating. Grains from each type were cut with a razor blade so that mid-grain transverse sections were exposed for study. The aleurone layer was our main focus since about 85% of total grain P is in the endosperm but we also studied scutellum and starchy endosperm cells [35]. Samples were mounted on aluminium stubs and viewed without metal coating in an ElectroScan 2020 at 20 kV.

2.5. Scanning transmission electron microscopy

Scanning transmission electron microscopy (STEM) was used to determine the ultrastructure of globoids in scutellum and aleurone cells and to determine the element content of globoids in those two tissues using X-ray microanalysis. For each of the five grain types, eight typical grains were selected for preparation and study. Small pieces of scutellum at mid-embryo were dissected from dry grains, as were mid-grain pieces of aleurone layer (with attached pericarp and some starchy endosperm). All samples then were prepared using a low-water-content procedure [30]. Initial soaking in 85% ethanol for 24 h permitted some swelling of the tissue so that epoxy resin infiltration was improved in later steps while soluble phytate was retained [30]. No electron-dense stains were used. After the initial 85% EtOH soaking, samples were dehydrated in 100% EtOH and then propylene oxide. A slow (seven day) Spurr's resin infiltration series was used before the samples were placed in moulds and hardened at 70 °C for 8 h. To retain any water-soluble elements during microtomy [36], all sections, 1–1.5 μm thick, were cut dry using a glass knife and a Reichert OMU2 ultramicrotome. Sections were transferred to formvar-carbon coated copper grids using a cactus spine and flattened with a drop of absolute ethanol. Sections were studied for ultrastructural features using a JEOL 1200 EX-II TEMSCAN (JEOL, Tokyo) operating at 80 kV.

2.6. Energy-dispersive X-ray analysis

Elements present in selected cell structures were measured with PGT IMIX energy-dispersive X-ray analysis systems (Princeton Gamma Tech, Princeton, NJ) attached to an ElectroScan 2020 ESEM operated at 20 kV or a JEOL 1200 EX-II TEMSCAN operated at 80 kV with a beam current of ∼54 μA. For a given microscope system (ESEM or STEM) operating conditions such as detector distance, tilt, aperture, 60 s count time, accelerating voltage, etc., were kept constant. For ESEM studies, samples that had as flat a surface as possible were selected for mounting parallel to the stub surface. Both microscope systems were used to study mid-grain region aleurone cells and ground meristem (parenchyma) cells of mid-embryo scutella. Starchy endosperm cells were only evaluated with ESEM–EDX analysis. With ESEM–EDX analyses three grains of each of the five grain types were studied for element content in samples that received no preparation except for being cut with a razor blade. These analyses provided a means of determining if the specimen preparation procedures used for STEM–EDX analyses extracted or moved major amounts of the elements of interest. For each of the 15 grains studied, two analyses, each using a 1/4 raster area at 1000× magnification, were carried out over aleurone layer, scutellum and starchy endosperm cytoplasm regions.

With STEM–EDX analyses, where spatial resolution was better, naturally-electron-dense deposits (globoids) were

Table 1
Dry weights (mean ± S.D.) of whole-grain of barley wild-type (WT) and *low phytic acid* (*lpa*) genotypes and their dissected grain fractions

Genotype	Whole-grain before separation (mg per grain)	Embryo (mg per part)	Rest-of-grain (mg per part)
WT	56.0 ± 3.2	1.89 ± 0.21	54.6 ± 2.9
*lpa*1-1	51.6 ± 3.0	2.06 ± 0.22	50.0 ± 2.9
M 635	55.3 ± 2.8	1.98 ± 0.17	53.7 ± 2.8
M 955	48.0 ± 2.1	1.96 ± 0.17	46.3 ± 2.1
*lpa*2-1	53.0 ± 3.3	2.48 ± 0.21	51.0 ± 2.9

Total sample size for each genotype was 750 grains or grain parts, weighed in 16 batches.

spot analyzed. Spectra were saved if the count rate was over 1000 cps. Globoids of various diameters were included in the analyses. For each of the five grain types, 10–15 spectra were collected from different globoids in different cells of each of six grains for scutellum and from eight grains for aleurone. Thus there were 78–86 spectra for each tissue of each of the five grain types. Using the PGT IMIX system, counts were obtained for the Kα X-ray peaks of P, K, Mg, Ca, Cu, Fe, Mn and Zn by integrating X-ray counts in the window for each element as defined in [37]. Background subtraction was done by a computer program that joins points along the energy axis that are between the peaks characteristically found in globoids, and peak-to-background (P/B) ratios were calculated [38]. Corrections for peak overlaps (potassium Kβ peak overlaps the Ca Kα peak; Mn Kβ peak overlaps the Fe Kα peak; Cu Kβ peak overlaps the Zn Kα peak) were done [38]. Statistical significance between mean P/B ratios was determined by MINITAB's analysis of variance test. When a significant difference was established, Tukey's test was used to determine which of the means differed at $P = 0.05$ [39].

3. Results

3.1. Weights and total P of grain fractions

Samples of grain of each genotype were dissected into two fractions: "embryo" and "rest-of-grain". The embryo and rest-of-grain fraction weights of the different genotypes were similar with only two relatively small exceptions (Table 1). The mean embryo weight of *lpa*2-1 was about 20–30% greater than any of the other grain types and the mean rest-of-grain weight for M 955 was about 15% less than in WT. In all genotypes the concentration of total P was several times higher in the embryo than in the rest-of-grain fractions (Table 2). However, since the embryo fractions represented less than 5% of the whole-grain weight, in most genotypes the embryo still only contained about 10% of whole-grain total P. In grains homozygous for the M 635, M 955 or *lpa*2-1 mutations, both embryo and rest-of-grain total P concentrations, and thus whole-grain total P, were similar to WT. However, homozygosity for the *lpa*1-1 mutation resulted in a 28% increase in embryo total P concentration and a 21% decrease in rest-of-grain total P concentration, as compared with WT. The reduced rest-of-grain total P in *lpa*1-1 grain was sufficient to result in a whole-grain total P reduction, as compared with WT, of about 15%, which is similar to that previously reported [14].

The amount of P leaked into the 90% ethanol used to soak barley grains prior to embryo removal was very small. For both grain types the maximum leakage was 0.1% of the total P in the grain. The *lpa* mutants leaked more than the WT but the difference was not significant for the interpretation of results. The grain type, followed by the percentage of total P leaked into the ethanol, were as follows: WT, 0.01; *lpa*1-1, 0.10; M 635, 0.07; M 955, 0.08; and *lpa*2-1, 0.06.

Table 2
Total phosphorus (P) and total inositol phosphorus (total Ins P) in whole-grains and dissected grain fractions obtained from WT and *low phytic acid* barley genotypes

Genotype	Total P concentration ($mg\,g^{-1}$)			Total inositol P concentration ($mg\,g^{-1}$)		
	Embryo	Rest-of-grain	Whole-grain	Embryo	Rest-of-grain	Whole-grain
WT	12.58 ± 0.25	3.30 ± 0.12	3.64 ± 0.12	5.87 ± 0.13	2.19 ± 0.09	2.34 ± 0.08
*lpa*1-1	16.12 ± 0.90	2.60 ± 0.01	3.16 ± 0.02	8.06 ± 1.12	0.85 ± 0.02	1.15 ± 0.04
M 635	12.49 ± 0.12	3.54 ± 0.10	3.88 ± 0.09	2.58 ± 0.24	0.68 ± 0.02	0.75 ± 0.01
M 955	11.29 ± 0.13	3.97 ± 0.10	4.29 ± 0.09	0.48 ± 0.06	0.10 ± 0.0002	0.12 ± 0.004
*lpa*2-1	13.19 ± 0.43	3.56 ± 0.11	4.04 ± 0.09	4.44 ± 0.63	1.40 ± 0.09	1.55 ± 0.13
	Total P content (μg per part)			Total inositol P content (μg per part)		
WT	23.8 ± 0.5	179.9 ± 6.6	203.7 ± 6.1	11.1 ± 0.3	119.7 ± 4.7	130.8 ± 4.3
*lpa*1-1	33.2 ± 1.8	130.0 ± 0.7	163.2 ± 1.1	16.6 ± 3.3	42.7 ± 1.1	59.3 ± 2.2
M 635	24.7 ± 0.2	190.1 ± 5.3	214.8 ± 5.1	5.1 ± 0.7	36.6 ± 1.1	41.7 ± 0.4
M 955	22.1 ± 0.2	183.8 ± 4.6	205.9 ± 4.3	0.9 ± 0.2	4.6 ± 0.01	5.5 ± 0.2
*lpa*2-1	32.7 ± 1.1	181.6 ± 5.8	214.3 ± 4.7	11.0 ± 2.2	71.2 ± 4.6	82.2 ± 6.9

For both total P and total inositol P, $N = 2$. Values presented as mean ± S.D. Whole-grain values were obtained via summing of dissected fraction values. To facilitate comparisons, all values are expressed in terms of their phosphorus (atomic weight 31) content, and in terms of both concentration ($mg\,g^{-1}$) or amount (μg per part).

3.2. Inositol phosphates

Total Ins P (mean ± S.D., $N = 2$) values were derived from HPLC analysis of dissected fractions; *myo*-inositol tris-, tetra*kis*-, penta*kis*-, and hexa*kis*phosphate peaks were summed to give total Ins P. Whole-grain values were obtained via summing of dissected fraction values. In WT grain total Ins P represented 47% of embryo total P and 66% of rest-of-grain total P (Table 2). The levels of whole-grain total Ins P for each genotype analyzed here (Table 2), obtained by summing the values for each genotype's embryo and rest-of-grain fractions, are similar to those previously reported for these genotypes [8,14]. The reductions in whole-grain Ins P, as compared with WT, were 51, 68, 95 and 34% for grain homozygous for *lpa*1-1, M 635, M 955, and *lpa*2-1, respectively. In grain homozygous for the M 635 and M 955 mutations, the reduction (as compared with WT) in whole-grain total Ins P was found to be due to relatively proportional reductions in both embryo and rest-of-grain total Ins P (Table 2). In contrast, in grain homozygous for *lpa*1-1 embryo total Ins P was 37% greater than WT embryos. In grains homozygous for *lpa*2-1 embryo total Ins P concentration is reduced 24% as compared with WT (Table 2). However, since *lpa*2-1 embryo dry weight was increased 31% as compared with WT, when total Ins P is expressed on a content basis (as units per part) *lpa*2-1 embryo total Ins P (11.0 μg per embryo) is similar to WT (11.1 μg per embryo). Thus these results indicate that in both barley *lpa*1-1 and *lpa*2-1 homozygotes, embryos appear phenotypically similar to WT in terms of their total grain Ins P levels. For these genotypes the reduction in total Ins P was observed here to be due solely (*lpa*1-1) or largely (*lpa*2-1) to reductions in rest-of-grain total Ins P (Table 2).

Anion-exchange HPLC (Fig. 1) illustrated that the Ins P pattern or "phenotype" of the WT embryo and rest-of-grain fractions are very similar (Fig. 1A and F): Ins P_6 is the predominant Ins P in both tissues, representing >90% of the soluble Ins phosphates; the remaining Ins phosphates consist primarily of a series of four Ins penta*kis*phosphate peaks. These four Ins P_5 peaks were shown in previous work [14] to be (from shortest to longest retention time): the "5-OH" Ins(1,2,3,4,6) P_5; the "6-OH" Ins(1,2,3,4,5) P_5 and/or its enantiomer the "4-OH" Ins(1,2,3,5,6) P_5 (these methods do not distinguish between enantiomers); the "3-OH" Ins(1,2,4,5,6) P_5 and/or its enantiomer the "1-OH" Ins(2,3,4,5,6) P_5; and the "2-OH" Ins(1,3,4,5,6) P_5.

In barley *lpa*1-1, the whole-grain mutant phenotype was a reduced Ins P_6 content accompanied mostly by increased Pi but also by small increases in Ins(1,2,3,4,6) P_5 [14]. That phenotype was observed here only in the rest-of-grain fraction (Fig. 1G). The Ins P phenotype of the barley *lpa*1-1 embryo (Fig. 1B) appeared very similar to that of non-mutant WT tissues (Fig. 1A and F). In M 635 (Fig. 1C and H) and M 955 (Fig. 1D and I), HPLC analysis confirms that both the embryo and rest-of-grain fractions display mutant Ins P phenotypes, similar to each other and to that previously reported for whole-grain homozygous for these mutations [14]; proportional reductions in each of the Ins phosphates observed in WT tissues, contributed to a 68% reduction in total Ins P in M 635 and a 95% reduction in M 955. Finally, HPLC indicated that while both the embryo (Fig. 1E) and rest-of-grain (Fig. 1J) fractions of barley *lpa*2-1 clearly display a mutant Ins P phenotype, the mutant phenotype was more pronounced in the rest-of-grain fraction than in the embryo fraction. The rest-of-grain phenotype was very similar to that originally reported for whole-grain [14]. In *lpa*2-1 aleurone layers the reduced Ins P_6 is accompanied by increases in non-Ins P_6 inositol phosphates, particularly the "5-OH" versions of Ins P_4 and Ins P_5.

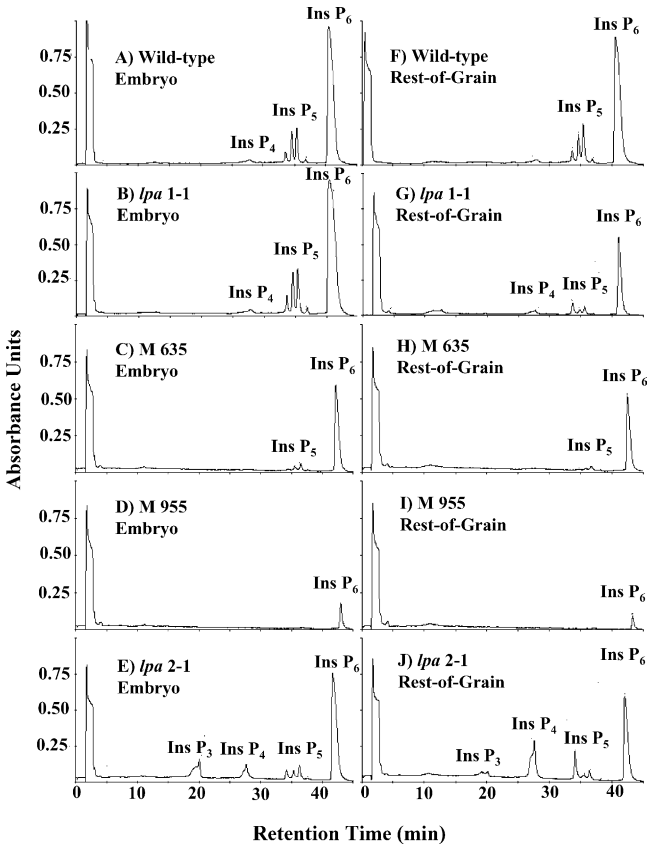

Fig. 1. Anion-exchange high-performance liquid chromatography (anion-exchange HPLC) of acid-soluble *myo*-inositol (Ins) phosphates in dissected grain fractions of "wild-type" (non-mutant) and *low phytic acid* (*lpa*) barley genotypes. (A)–(E) analyses of embryo fractions; (F)–(J) analyses of "rest-of-grain" fractions (all grain tissues remaining following removal of the embryo). Analyses of fractions obtained from wild-type, non-mutant grain used as a control are shown in (A) and (F). Analyses of fractions obtained from grain homozygous for *low phytic acid* 1-1, M 635, M 955 and *low phytic acid* 2-1 mutations are shown in (B) and (G), (C) and (H), (D) and (I), and (E) and (J), respectively. In addition to Ins P_6, Ins P_3 and Ins P_4 (stereoisomers of these latter two Ins phosphates are unknown), four Ins penta*kis*phosphates were observed. These were, in order of increasing elution time: Ins(1,2,3,4,6) P_5, D/L-Ins(1,2,3,5,6) P_5, D/L-Ins(1,2,4,5,6) P_5, and Ins(1,3,4,5,6) P_5.

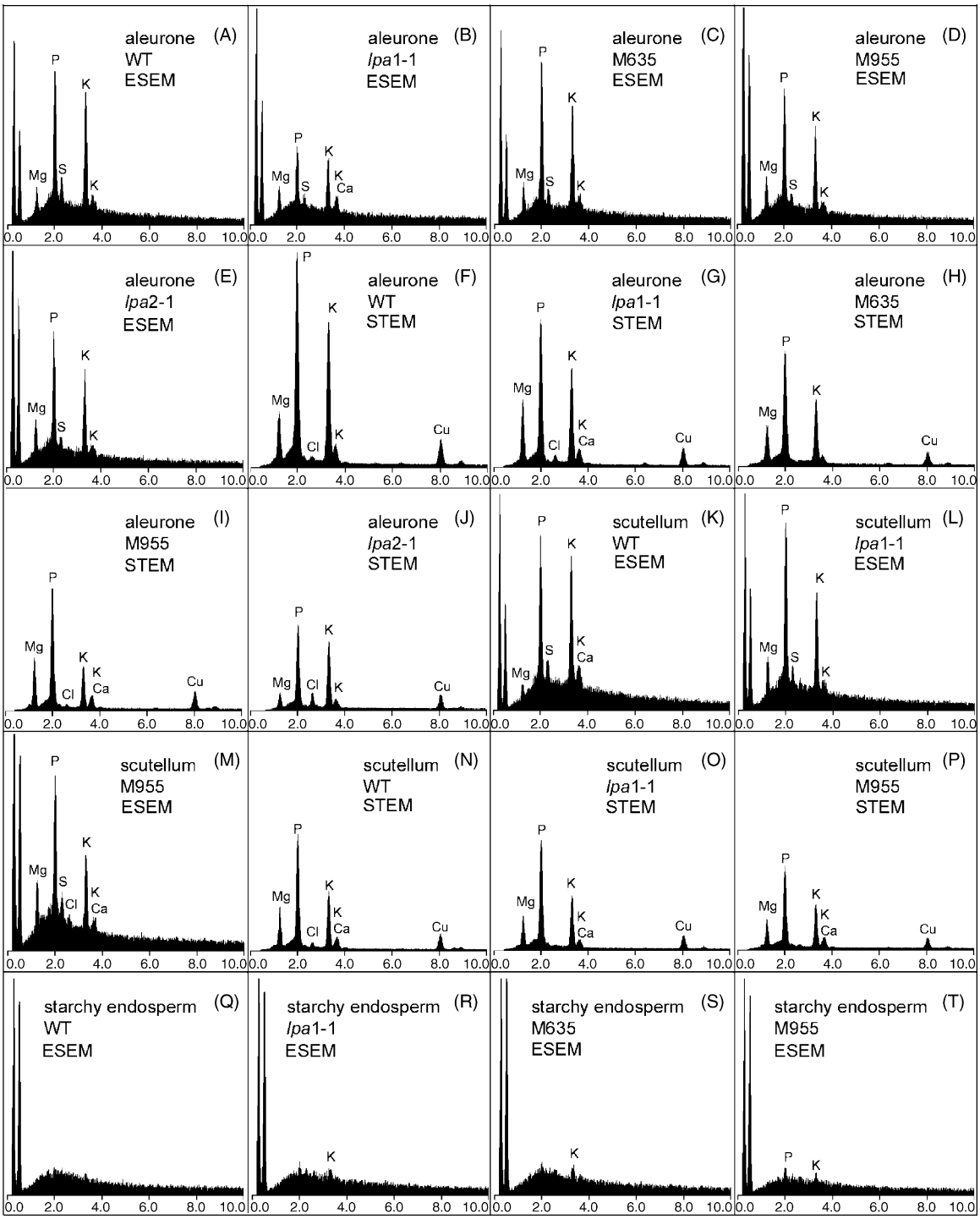

Fig. 2. EDX analysis spectra of rastered cytoplasm areas of cut dry grains using ESEM or electron-dense globoids in sections viewed in STEM mode. (A)–(J) are from aleurone cell cytoplasm, (K)–(P) are from scutellum cell cytoplasm, and (Q)–(T) are from starchy endosperm cell cytoplasm. All spectra presented are typical for a given grain type and tissue. Detector used for ESEM allows measurement of C and O (peaks seen on the left side of spectra). Beryllium window on the STEM detector eliminates X-rays from C and O. Horizontal axes show X-ray energy levels (0.0, 2.0, 4.0, 6.0, 8.0, and 10.0 keV). Vertical scales were made constant for a given spectra set ((A)–(E) full scale = 1000, (F)–(J) = 7100, (K)–(M) = 1000, (N)–(P) = 7100, (Q)–(T) = 1000). Copper grids used for sections gave artifact peaks for copper at 8.04 keV, Kα and 8.91 keV, Kβ. Chlorine in Spurr's resin may have contributed to Cl peaks in sectioned tissue. Principal X-ray energies in keV and lines of elements present are as follows: C Kα, 0.28; O Kα, 0.52; Mg Kα, 1.25; P Kα, 2.02; S Kα, 2.31; Cl Kα, 2.62; K Kα, 3.31, and K Kβ 3.59; Ca Kα, 3.69, and Ca Kβ 4.01; Fe Kα, 6.40; and Zn Kα, 8.63.

3.3. Energy-dispersive X-ray analyses

Square raster areas of cytoplasm from aleurone (Fig. 2A–E), scutellum (Fig. 2K–M) and starchy endosperm (Fig. 2Q–T) cells were studied with ESEM–EDX analysis to determine what the element composition was without any fixation or embedding. Since equipment operating conditions were kept constant and the vertical scale was set the same for a given tissue, it is valid to compare peak heights. Carbon and oxygen peaks were found in all areas in all grain types. In addition to C and O, the aleurone layer and scutellum cells of all grain types had peaks for P, K, Mg, and S. Of these four elements, P had the highest peaks, followed by K, then Mg and lastly S. In both aleurone layer and scutellum a few spectra revealed trace amounts of Zn or Mn. Traces of Si were more common in the aleurone cell cytoplasm than in scutellum cell cytoplasm. The scutellum and aleurone cell cytoplasm EDX analyses were quite similar for the WT and the four mutants, but the amounts of the elements present in the *lpa*1-1 were smaller in the aleurone layer. At a set vertical scale, the aleurone *lpa*1-1 peak heights were lower than for the aleurone of other grain types; also, the aleurone cell peaks were lower than those of the scutellum cytoplasm in the same *lpa*1-1 grains. With EDX analysis the starchy endosperm in all samples contained big peaks of C and O and very small peaks of P and K. In a few cases Mg, S, Si or Cl were barely detectable in the starchy endosperm cytoplasm.

EDX analyses in the STEM mode showed that globoids from aleurone layer cells (Fig. 2F–J) and scutellum cells (Fig. 2N–P) for the WT and all *lpa* mutants gave major peaks for P, K and Mg in approximately the same peak height proportions as seen with ESEM–EDX analyses. Phosphorus was the tallest peak, K was second tallest and Mg was third tallest. In some globoids traces of Ca and/or Fe and/or Zn were detectable. Globoid analyses usually lacked S, which was commonly present in rastered areas of cytoplasm in ESEM–EDX analyses.

EDX analysis results for electron-dense deposits or "globoids" from aleurone layer and scutellum cell sections for all grain types, presented as peak-to-background ratios (Table 3), showed that P, K, and Mg were the main mineral elements present and that Ca, Fe and Zn generally were present in low to trace amounts while Mn (data not shown) was not detectable. Since analytical conditions were kept constant for all analyses, P/B ratios provide a useful way of comparing different samples. The only clear and large difference in globoid mineral content among tissues was that in all genotypes except *lpa*1-1, scutellum globoids contained more than twice the level of Ca than did aleurone globoids. Concerning differences (or lack thereof) between genotypes, the four *lpa* mutants were all similar to WT in both aleurone layer and scutellum globoid element content. However, three statistically significant differences in mineral content of *lpa*1-1 globoids, as compared with WT and the other three *lpa* mutations, are worth noting. Aleurone layer globoid P in *lpa*1-1 was reduced as compared to WT, M 635, M 955 and *lpa*2-1, by about 25%. While the Fe level in *lpa*1-1, M 635 and M 955 were all significantly higher than WT, the largest increase, 2.6-fold, was with *lpa*1-1. Finally, the Ca, Fe and Zn levels of *lpa*1-1 scutellum globoids were reduced from 40 to 50% as compared with WT and the other *lpa* genotypes.

3.4. Globoid ultrastructure

Wild-type aleurone layer cells had globoids of various sizes but often they were 1–2 μm in diameter (Fig. 3A). The WT scutellum cells had more numerous and smaller (usually <1 μm) globoids clustered inside the protein bodies (Fig. 3F). The aleurone of *lpa* mutants (*lpa*1-1, M 635 and M 955 in Fig. 3B–D) had numerous smaller globoids but there was size variation, especially in M 635. The *lpa*2-1 mutant (Fig. 3E) had naturally-electron-dense deposits very similar in structure to the WT. In the scutellum, both the WT and all four *lpa* mutants, had numerous small globoids often less than 0.5 μm in diameter (*lpa*1-1 in Fig. 3G and M 635 in Fig. 3H and M 955 in Fig. 3I and *lpa*2-1 in Fig. 3J). The M 955 scutellum contained smaller and more widely spaced globoids than any other *lpa* mutant.

Table 3
Peak-to-background ratios (mean ± S.D.) derived from STEM energy-dispersive X-ray analysis of globoids in sections of aleurone layers and scutella of the wild-type (WT) and *low phytic acid* (*lpa*) genotypes of barley

Tissue	Genotype	Peak-to-Background Ratios					
		P	K	Mg	Ca	Fe	Zn
Aleurone layer	WT	7.66 ± 1.22	10.17 ± 2.26	3.94 ± 1.19	0.57 ± 0.34	0.27 ± 0.13	0.19 ± 0.10
	*lpa*1-1	5.65 ± 1.45	8.37 ± 2.99	4.85 ± 1.55	0.79 ± 0.77	0.70 ± 0.24	0.29 ± 0.13
	M 635	8.03 ± 1.18	11.02 ± 2.52	4.45 ± 1.69	0.36 ± 0.47	0.48 ± 0.22	0.25 ± 0.10
	M 955	8.02 ± 1.59	9.24 ± 2.65	5.12 ± 1.66	0.67 ± 0.78	0.43 ± 0.27	0.31 ± 0.19
	*lpa*2-1	7.22 ± 0.91	11.88 ± 2.73	2.99 ± 1.18	0.56 ± 0.37	0.17 ± 0.09	0.35 ± 0.36
Scutellum	WT	8.20 ± 1.73	11.83 ± 2.97	4.94 ± 1.50	1.73 ± 0.94	0.21 ± 0.18	0.45 ± 0.27
	*lpa*1-1	7.81 ± 1.45	9.87 ± 2.51	3.96 ± 1.05	0.89 ± 0.67	0.12 ± 0.11	0.25 ± 0.24
	M 635	7.33 ± 1.33	10.12 ± 2.64	4.71 ± 1.23	1.90 ± 1.08	0.30 ± 0.21	0.52 ± 0.35
	M 955	7.07 ± 1.36	10.10 ± 2.95	4.79 ± 1.50	1.96 ± 1.05	0.21 ± 0.14	0.47 ± 0.49
	*lpa*2-1	7.13 ± 1.59	12.08 ± 3.02	3.95 ± 1.14	2.39 ± 1.15	0.25 ± 0.23	0.40 ± 0.31

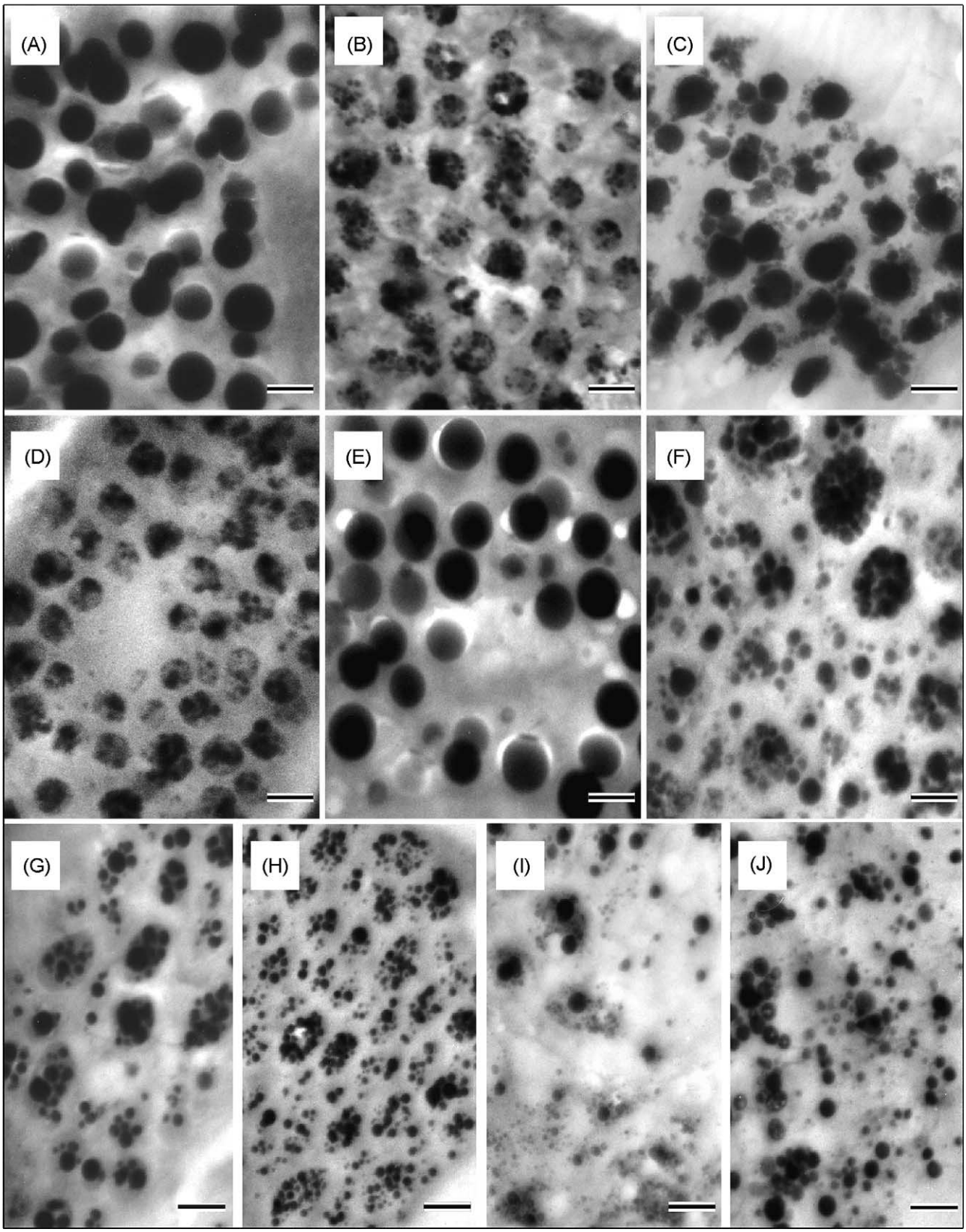

Fig. 3. STEM images of about 1–2 μm thick sections of aleurone layer cells (A)–(E) and scutellum cells (F)–(J). Scale bars = 2 μm. Since no electron-dense stains were used during low-water-content preparation, all black spheres are due to natural electron density of the globoids. (A) WT, (B) *lpa*1-1, (C) M 635, (D) M 955, (E) *lpa*2-1, (F) WT, (G) *lpa*1-1, (H) M 635, (I) M 955, and (J) *lpa*2-1.

4. Discussion

4.1. Effects of lpa mutations on P and Ins P in different tissues

The results presented here extend studies on whole-grains by reporting the most comprehensive study to date of possible tissue-specific effects of *lpa* mutations. Barley *lpa*1-1 is the first Ins P_6 pathways mutation identified to date whose reduced Ins P_6 phenotype clearly is specific to one tissue, in this case the aleurone layer, but not to other tissues such as the germ, that typically accumulate Ins P_6. Since there is no direct vascular or plasmodesmata connections to either endosperm or embryo, during grain development mineral nutrients have to move apoplastically from the integument to the aleurone layer and from the starchy endosperm to the embryo. Symplastic movement may occur between starchy endosperm cells. Clearly, in *lpa*1-1 there was no restriction of P supply to the developing embryo, which indicates that the aleurone layer of the endosperm also had to have access to adequate P during development. The *lpa*1-1 block in the aleurone layer's ability to serve as a sink for P may have resulted in an elevated supply of P to the embryo, and thus, resulted in an elevated embryo Ins P_6 concentration.

Barley *lpa*1-1 also differs from all other studied *lpa* mutations in that homozygosity for this aleurone-specific function also results in both a shift in P distribution and a net reduction in grain P. Since no such effects were found with the other mutations, it is the perturbation of a specific function in the Ins P pathways in barley *lpa*1-1, and not the ability of grains to synthesize Ins P_6 *per se*, that impacts distribution and net amount of grain P in this case. This grain-tissue-specific mutation is similar to several maize starch pathway mutations that alter the chemistry of starch, and at the same time reduce net uptake of carbon into the developing grain [40]. Since genetics is useful in engineering the chemistry of grain P, it is of practical importance in the development of grain crops for use in feeds and foods. Genetic manipulation of the total amount of grain total P is also of practical importance. Cultivars or hybrids that are agronomically acceptable and produce grains with reduced total P may provide a tool for enhanced P management in livestock production. Identifying functions that are specific to individual cell types or tissues may eventually permit the engineering of reduced grain phytate while limiting the negative impacts on agronomic performance to a minimum, a long-term objective in the development of low-phytate crops with acceptable agronomic performance [8]. The impact on grain P distribution and net grain P accumulation of the aleurone-specific barley *lpa*1-1 mutation does indicate that while Ins P_6 accumulation *per se* is not critical to P uptake and distribution, specific functions within the Ins P pathways may be. Identification of the function altered in this mutation would therefore represent a step beyond the definition of the structural pathway to Ins P_6, a step towards the elucidation of the evolution of divergent gene expression of these pathways specific to higher plant tissues.

Barley *lpa*2 also appears to be aleurone-specific, but it is not as clear-cut a case as *lpa*1. Previous studies have shown that MIPS expression in the developing rice grain is tissue-specific, in that it is localized to the germ and the aleurone layer, the sites of Ins P_6 assembly into globoids [41]. Expression of the maize *lpa*2 gene, encoding an Ins(1,3,4) P_3 5-/6-kinase, appears greatest during embryo development but also is detectable at low levels in other tissues [18], and no tissue-specific phenotype of a lesion in this gene has yet been reported. We have hypothesized, based on syntenous map positions and similarity in Ins P phenotype, that the maize and barley *lpa*2 loci encode orthologous functions [16]. Perhaps the fact that the greatest level of expression is in the germ of maize, *lpa2*, simply reflects the fact that in maize most Ins P_6 accumulates in the germ.

4.2. Tissue and subcellular localization of elements

These results demonstrate that the normal accumulation of P, K and Mg in the germ and aleurone tissues of the mature cereal grain, with a near-absence of these minerals in the mature starchy endosperm, is not dependent on the localized, tissue-specific synthesis of Ins P_6, as was indicated in an earlier study of developing rice grains [6]. Ogawa et al. [6] demonstrated that there was transient accumulation of P, K and Mg in the cereal starchy endosperm during development with subsequent redistribution by maturity to the final storage sites of the germ and aleurone. One possible effect of the *lpa* mutation could have been a shift of elements into the starchy endosperm. However, comparison of ESEM–EDX analysis of cytoplasm areas of dry grains of all types revealed no major shift of elements to the starchy endosperm. Even in grains homozygous for M 955, where less than 10% of WT levels of Ins P_6 was observed, the distribution of P, K and Mg, and their concentration in specific cell types like the aleurone layer, was indistinguishable from WT. No shift of elements to the starchy endosperm occurred in *lpa*1-1 rice grains [42].

Otegui et al. [7] had shown that Ins P_6 synthesis and deposition seems important to transient storage of Mn and Zn in chalazal endosperm early in seed development in *Arabidopsis thaliana*, findings that are in many ways similar to that of the studies of transient storage of P, K and Mg during endosperm development in rice [6]. In our results, in three of four *lpa* mutations, perturbation of Ins P_6 synthesis had little effect on the mature grain localization of Ca, Fe and Zn. This finding raises a question concerning the importance of the proposed role for Ins P_6 synthesis and phytate salt deposition in transient heavy metal storage during grain development. In relation to WT the mutant phenotype of barley *lpa*1-1 had elevated aleurone globoid Fe accompanied by decreased scutellum globoid Ca and Fe. This indicates that, at a minimum, specific functions in the Ins P pathways, and not the ability of grains to accumulate wild-type levels

of phytic acid per se, may be important to final storage in mature grains.

EDX analysis peak-to-background ratios are not quantitative measures, for example, in picomoles per cubic micrometer, but do permit useful comparisons, especially between values for the same element. P/B ratios help to minimize the influences of such factors as differences in specimen thickness, specimen surface topography, and specimen density. When struck with an electron beam different elements generate X-rays to different degrees [43]. Based on values for 80 kV electrons, in relation to P, the yield of Mg is a 37% underestimate and K is a 24% overestimate [44]. The elements Ca, Fe, Mn and Zn all are more readily detected than P so the small peaks for these elements do represent trace amounts and not reduced sensitivity of detection.

A comparison of EDX analysis of areas of cytoplasm studied with ESEM and globoids studied in STEM mode allowed us to conclude that the great majority of the elements P, K, Mg, Ca, Fe, and Zn present in barley scutellum and aleurone cell cytoplasm were located inside these electron-dense deposits. On the other hand, S mainly comes from non-globoid regions, likely protein. While statistically significant differences were found between P/B ratios for globoids in the different mutants compared to the WT, there were no radical differences in the distribution of the stored elements. In all cases P, K, and Mg were the main elements detected with lesser/trace amounts of Ca, Fe, and Zn. Calcium generally was much higher in scutellum globoids than aleurone layer globoids, a feature previously reported for Himalaya barley [45]. The lack of large-scale differences from WT occurred even in M 955 with its 95% reduction in Ins P_6. The increase in Pi in the mutants may have resulted in P and the cations accumulating in areas where globoids would have formed despite the lack of binding sites in the form of Ins P_6. In *lpa*2-1 and perhaps in the other mutants increases in lower Ins phosphates could also have contributed to the formation of clusters of electron-dense globoids.

4.3. Success with specimen preparation protocols

It was important to determine whether or not our specimen preparation procedures had any major effect on phosphorus, phytic acid or mineral deposits. In order to precisely dissect the embryos from the starchy endosperm and pericarp/testa/aleurone layer we developed a procedure of soaking the grains in 90% ethanol. The much improved precision in embryo isolation obtained with this procedure was large compared to the very small extraction of P into the soaking solution. At most, a thousandth of the total P present in the grain was leaked. For accurate localization of elements in sections we also had to determine that the low-water-content specimen preparation procedures designed to retain phytate in grain tissue while facilitating infiltration of epoxy resin and sectioning, was an acceptable procedure for grains of *lpa* mutants containing increased Pi. We accomplished this by comparing results of ESEM–EDX analysis with STEM–EDX analysis. X-ray microanalysis in ESEM had the great advantage that the only specimen preparation made was to cut the dry grain, but it suffered from poorer spatial resolution. Since the spread of electrons in a thick sample results in X-ray generation from a pear-shaped volume [43]. The similarity between the element composition of different cell regions studied with ESEM–EDX analysis and the element composition of electron-dense structures as determined by STEM–EDX analysis allowed us to conclude that the low-water-content preparation procedure and dry sectioning retained elements in place in *lpa* mutant grain tissues as well as it does in high phytate grain tissues.

4.4. Ultrastructure of mineral rich deposits

Our hypothesis was that major reduction in Ins P_6 would result in either smaller diameter globoids, fewer globoids, or both. In *lpa*1-1, the aleurone layer had more globoids of smaller diameter; however, in the scutellum, the globoids were relatively uniform in size but less frequent. Globoid diameter is very important since the volume in a globoid increases exponentially with increasing diameter. Thus a 0.75 μm diameter globoid has a volume of 0.2 μm^3, whereas 2.75 μm diameter globoid has a volume of 10.9 μm^3 [46]. One large 2.75 μm diameter globoid contains the volume of 55 globoids of 0.75 μm diameter. The relative mass of globoids often paralleled the level of Ins P_6 in a given mutant, as was observed in a study of rice *low phytic acid* 1-1 where globoids were also smaller and more numerous than in WT [42]. However, the reduction of electron-dense deposits we observed in some of the *lpa* mutants did not match the reduction in Ins P_6. It is likely that naturally-electron-dense inclusions must also come from lower Ins P's and even from areas rich in Pi. For example, *lpa*2-1 aleurone layer cells look very much like those in WT even though the Ins P was reduced by 34%. Aleurone layer cells of M 955 with a 95% reduction in Ins P_6 appeared structurally rather similar to *lpa*1-1 which had a 51% reduction. M 955 did have a greater frequency of globoids that were of less regular shape and also were less stable in the electron beam. This suggests that what appeared to be globoids may have been concentrations of Pi occupying the space normally occupied by Ins P_6 in most seeds. Residual Ins P, perhaps with the aid of pyrophosphate units may have maintained the naturally-electron-dense particles. In most grains and seeds the mineral nutrient reserve in globoids consists of mainly Ins P_6 molecules that are cross-linked by di- and tri-valent cations to form globoid aggregates. In most of the *lpa* mutants the aggregates we observed must be a more complex mixture of Ins P's, Pi, perhaps phosphorus in other forms such as polyphosphates, and multivalent cations.

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