## Developing Seeds of Arabidopsis Store Different Minerals in Two Types of Vacuoles and in the Endoplasmic Reticulum

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Mineral-accumulating compartments in developing seeds of Arabidopsis were studied using high-pressure-frozen/ freeze-substituted samples. Developing seeds store minerals in three locations: in the protein storage vacuoles of the embryo, and transiently in the endoplasmic reticulum (ER) and vacuolar compartments of the chalazal endosperm. Energy dispersive x-ray spectroscopy and enzyme treatments suggest that the minerals are stored as phytic acid (myoinositol-1,2,3,4,5,6-hexakisphosphate) salts in all three compartments, although they differ in cation composition. Whereas embryo globoids contain Mg, K, and Ca as cations, the chalazal ER deposits show high levels of Mn, and the chalazal vacuolar deposits show high levels of Zn. The appearance of the first Zn-phytate crystals coincides with the formation of network-like extensions of the chalazal vacuoles. The core of these networks consists of a branched network of tubular ER membranes, which are separated from the delineating tonoplast membranes by a layer of cytosolic material. Degradation of the networks starts with the loss of the cytosol and is followed by the retraction of the ER, generating a network of collapsed tonoplast membranes that are resorbed. Studies of fertilized fis2 seeds, which hyperaccumulate Zn-phytate crystals in the chalazal vacuolar compartments, suggest that only the intact network is active in mineral sequestration. Mineral determination analysis and structural observations showed that Zn and Mn are mobilized from the endosperm to the embryo at different developmental stages. Thus, Zn appears to be removed from the endosperm at the late globular stage, and Mn stores appear to be removed at the late bent-cotyledon stage of embryo development. The disappearance of the Mn-phytate from the endosperm coincides with the accumulation of two major Mn binding proteins in the embryo, the 33-kD protein from the oxygen-evolving complex of photosystem II and the Mn superoxide dismutase. The possible functions of transient heavy metal storage in the chalazal endosperm are discussed. A model showing how phytic acid, a potentially cytotoxic molecule, is transported from its site of synthesis, the ER, to the different mineral storage sites is presented.

## INTRODUCTION

Seed formation in flowering plants involves the coordinated development of biosynthetic activities in the embryo and in the surrounding endosperm. Even though the specific interactions and signal exchanges between these developing tissues remain to be elucidated, the interdependence of the embryo and the endosperm has been firmly established (Ray, 1997).

The endosperm supports embryogenesis by both mobilizing storage substances and providing developmental signals (Lopes and Larkins, 1993; DeMason, 1997). In plants in which the endosperm persists at maturity, such as in cereals, it stores reserves for the germinating embryo. In contrast, in species such as Arabidopsis, in which the endosperm is consumed by the embryo during development, the storage compounds accumulate in the mature embryo cells (Berger, 1999).

All seeds store minerals in the form of mineral deposits. These deposits are composed of phytin, a salt of phytic acid (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate) and its cations (mostly Mg, K, and Ca) (Lott et al., 1995). In mature seeds, phytin is located inside protein storage vacuoles (PSVs) in inclusions called globoids, which store up to 90% of the total P. During germination, phytin is decationized and then hydrolyzed sequentially by phytases to phosphate and a series of lower phosphoric esters of *myo*-inositol (Loewus and Murthy, 2000), which serve as important sources of P and cations for the germinating embryo (Raboy, 1997).

How phytic acid and cations are transported to the PSVs in the embryo, and the extent to which the endosperm controls these processes, have yet to be determined. Because the endosperm in Arabidopsis is a transient tissue, and seed formation is completed in a few days, it is conceivable that the function of the endosperm in mineral transport to the embryo could be limited to that of a simple conduit. Alternatively, the need to optimize the flow of mineral nutrients to the

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010486.

exponentially growing and mineral-storing embryo could require a more active role of the endosperm in these processes.

Polarization of the endosperm along two well-defined poles, chalazal and micropylar, is a common feature of many flowering plant seeds (Friedman, 2001). In Arabidopsis and many other members of Brassicaceae, the transient endosperm consists of three domains: micropylar, central, and chalazal (Brown et al., 1999; Sørensen et al., 2001). The chalazal domain is located next to the vascular bundle of the maternal tissue and, in some species, differentiates into a cyst with haustorium-like extensions that can penetrate the adjacent tissues (Pacini et al., 1975; Vijayaraghavan and Prabhakar, 1984; Nguyen et al., 2000). As a result of these structural features, the chalazal endosperm domain has been assumed to play an important role in the loading of storage compounds from surrounding maternal tissues into the developing seed (Nguyen et al., 2000, 2001).

Because phytin is highly soluble in water, conventional chemical fixation with aqueous fixative solutions does not allow its proper preservation for electron microscopy; thus, both its site of synthesis and its transport pathway within the cell have remained elusive. Using high-pressure freezing/freeze-substitution techniques, we were able to preserve water-soluble salts that accumulate during seed development in Arabidopsis. We found that, aside from the well-characterized phytin globoid crystals in PSVs of embryo cells, the developing seed also transiently stores minerals at two sites within the chalazal endosperm: phytic acid salts with a high proportion of Mn in the endoplasmic reticulum (ER), and phytic acid salts with a high concentration of Zn in specialized subdomains of the central vacuolar compartment. Analyses of mineral levels at different seed developmental stages, and the differential timing of mobilization of both Mn-phytate and Zn-phytate crystals, suggest that the chalazal domain is involved in the temporary storage of minerals that are translocated to the embryo at specific stages of development. We also show that the increase in expression levels of Mn-containing enzymes in the embryo coincides with the disappearance of Mn-phytate crystals from the endosperm.

## RESULTS

## The Chalazal Endosperm Domain Undergoes Many Changes in Parallel with the Development of the Embryo

To define the stages in embryo and endosperm development during which the different types of mineral deposits are produced and mobilized, we reexamined the process of Arabidopsis seed development in specimens preserved by high-pressure freezing/freeze-substitution techniques (Figure 1). In particular, precise staging of the embryos was important to correlate specific structural events in the endosperm with the changes in mineral distribution. Soon after fertilization, the endosperm undergoes several cycles of nuclear division and becomes increasingly curved (cf. stages I and II in Figures 1A and 1B). Furthermore, once the embryo reaches the quadrant stage (stage II; Figure 1B), changes in the distribution of the endosperm nuclei and concomitant alterations in the cytoplasm lead to the creation of three distinct subdomains within the large endosperm cells. The three domains are known as (1) the micropylar domain, where the embryo is located, (2) the chalazal domain adjacent to the vascular bundle of the maternal tissue, and (3) the central domain, which is occupied mostly by the central vacuole and a thin peripheral layer of cytoplasm (Otegui and Staehelin, 2000).

Cellularization of the endosperm starts in the micropylar domain during stage III, the late globular stage of embryo development (Figure 1C), and continues progressively toward the central domain (stages IV and V; Figures 1D and 1E). The chalazal domain remains in a syncytial state until very late in seed development (stage VI; Figure 1F). Indeed, cellularization only starts after the embryo has occupied most of the micropylar zone (between stages III and IV; Figures 1C and 1D). At maturity, the Arabidopsis seed consists of a fully developed embryo, a peripheral single layer of endosperm cells, and the seed coat (stage VIII; Figure 1H).

As soon as the chalazal domain is established (stage II; Figure 1B), it undergoes a process of extensive differentiation that continues through stage III of embryo development. Typically, this domain exhibits polyploid nuclei (Boisnard-Lorig et al., 2001), a dense cytoplasm, and extensive arrays of closely packed parallel ER cisternae (Figure 2). In addition, the chalazal domain exhibits a highly complex vacuolar system, Golgi stacks, plastids, mitochondria, and numerous vesicles. During stage III (globular stage), the syncytial cell wall that surrounds the chalazal endosperm domain becomes thicker, irregularly lobed, and highly enriched in callose, as indicated by the heavy anti– $\beta$ -1,3-glucan immunolabeling depicted in Figure 3.

## Three Different Cellular Compartments Accumulate Mineral Deposits of Different Cation Composition during Seed Development

We identified three different cellular compartments that accumulate minerals in the developing Arabidopsis seed: in the embryo, in the PSVs within which the well-known seed globoids are formed (Figure 4); and in the chalazal domain of the endosperm, in stacked ER cisternae (Figures 2 and 5) and specialized subcompartments of the central vacuole (Figures 2, 6, and 7).

Spherical phytin globoid crystals are seen first inside the developing PSVs of embryo cells during stage VII, the late bent-cotyledon stage (Figure 4). The two types of electrondense crystals in the chalazal endosperm appear much earlier, during stages I and II of seed development. The crystals located in the ER lumen consist of sheet-like elements (Fig-



Figure 1. Seed Developmental Stages in Arabidopsis.

Light microscopic images of longitudinally sectioned developing seeds illustrating the principal stages of embryo development (stages I to VIII) and concomitant changes in endosperm organization. The chalazal endosperm domains are outlined in red.

(A) Two-celled embryo stage. The embryo (E) is located at the micropylar zone (MZ) of the endosperm. The chalazal zone (CZ) is still undeveloped.

- (B) Quadrant stage. The endosperm is now organized into three domains: micropylar (ME), central (CE), and chalazal (CZE).
- (C) Globular stage.
- (D) Heart stage. Cellularization has started at the ME and continues toward the CE domain.
- (E) Torpedo stage.
- (F) Early bent-cotyledon stage. The CZE is still in a syncytial state.
- (G) Late bent-cotyledon stage. The CZE has now cellularized.
- (H) Mature embryo stage. The mature seed consists of a seed coat (SC), a single layer of endosperm (EN), and the embryo.

ures 2, 5, and 8), whereas those in the specialized vacuolar compartments are rounded and irregularly shaped (Figures 6 and 7).

To determine the elemental composition of the different crystals, we used high-pressure-frozen samples substituted in the absence of heavy metals, sectioned with a dry knife (Figure 5A), and analyzed by means of energy-dispersive x-ray spectroscopy (EDXS). The EDXS spectrum of the embryo globoids was characterized as seed phytin salts, with a main peak of P and minor peaks of Mg, K, and Ca but no Mn or Zn (Figure 4C). In contrast, the two types of crystalline deposits in the endosperm were highly enriched in Mn and Zn. Thus, whereas the ER crystals in the endosperm were composed mostly of Mn and P (Figure 5B), the crystals in the chalazal vacuolar system were rich in Zn and P, with minor amounts of Ca, K, and Mg (Figure 6B). A semiquantitative analysis of the cation:P ratios in these three types of crystals is shown in Table 1.

The presence of P and cations in both types of chalazal endosperm crystals suggests compounds with phosphate groups. To determine what kind of compounds are forming these crystals, we incubated sections of developing endosperm with phosphatase and phytase for 15, 5, and 1 min. As controls, we incubated the sections in buffer without enzyme (Figure 8). The sections were observed before and after treatment to study the level of crystal solubilization. We also performed the same procedure with sections of embryo tissues with phytin globoid-containing PSVs (Lott et al., 1995). In all cases, the enzyme treatments removed virtually all of the crystalline material within 1 min, whereas the buffer treatment alone caused a less complete breakdown of the crystals within the same time interval (Figure 8). These results suggest that the endosperm crystals are made of phosphate-containing salts highly soluble in aqueous solutions, most likely salts of phytic acid, such as those that form the globoids in the mature embryo.

## Mn-Phytate Crystals Appear First in the ER of the Chalazal Domain and Later in ER Cisternae throughout the Whole Endosperm

The first Mn-phytate crystals were detected within ER cisternae of the chalazal endosperm during stage II, the quadrant stage of embryo development. During stage III, the globular stage, the number of such crystals increased in the chalazal pole region (Figure 2). Subsequently, during the heart and torpedo stages, additional ER crystals began to appear throughout the whole endosperm, including the cellularized micropylar domain (data not shown). Finally, at the bent-cotyledon stage (stage VI), the ER Mn-phytate crystals started to disappear. The presence of crystals inside the ER may affect some of its functions, particularly when the crystals grow in star-like configurations and grossly distort the surrounding ER membranes, as illustrated in Figure 5C.

# Highly Specialized Vacuolar Subcompartments in the Chalazal Endosperm Store Zn-Phytate Crystals

Zn-phytate crystals (Figures 6A and 7) were seen first in the vacuolar compartments of the chalazal endosperm between



Figure 2. Chalazal Endosperm Domain at Stage III (Globular Stage).

Numerous sheet-like crystals (arrowheads) can be seen inside the expansive ER cisternae, which show extensive stacking. A branched vacuolar system (V) also pervades the entire chalazal domain, and in some places, extensive tubular membrane network structures can be seen within the vacuoles (stars). CV, central vacuole; ED, endothelium; N, nucleus. Bar = 200 nm.



Figure 3. Immunogold Localization of Callose in the Syncytial Cell Wall of the Chalazal Endosperm.

ED, endothelium; SCW, syncytial cell wall; V, chalazal vacuole. Bar = 50 nm.

stages I and II (two-celled and guadrant stages) and disappeared just before the endosperm started to cellularizethat is, during the late globular stage (between stages III and IV) of embryo development. The vacuolar subcompartments within which the crystals arose exhibited characteristic, highly convoluted membrane networks, which could be used to identify these subcompartments when the crystals were not seen (cf. Figures 6A, 7, and 9). As illustrated in the serial section micrographs of the chalazal endosperm region of a wild-type seed (Figure 9) and in the micrograph of the fis endosperm mutant (Figure 10A), the chalazal vacuolar compartments within which the Zn-phytate crystals arose were physically continuous with the large central vacuole of the endosperm cell. Thus, the crystals were produced in specialized extensions or subcompartments of the central vacuole and not in physically separate chalazal vacuoles.

Our micrographs also demonstrate that the Zn-phytate crystals were formed and grew in the aqueous phase of these specialized vacuolar domains and not within membranous vesicles within the vacuoles (Figures 6A and 7). Furthermore, in no instances did we observe vesicles in the vicinity of the chalazal vacuoles that contained electrondense deposits at the time of crystal formation. Occasionally, however, Zn-phytate crystals were observed close to or even partly surrounded by elements of the lattice-like membrane structures within the vacuolar subcompartments (Figures 7B and 10).

We are still uncertain about the exact mechanism of formation of these labyrinthic membrane networks, which arise from highly convoluted extensions of the chalazal vacuolar subcompartments and are delineated by a tonoplast membrane (Figure 7A). The core of the initially formed membrane network is composed of a branched network of ribosomefree, tubular ER membranes that are separated from the surrounding tonoplast membranes by a layer of cytosolic material (Figure 10B).

During stage III, the membrane networks undergo what appear to be a series of degradative changes. First, the more darkly staining cytosolic materials seem to be squeezed out of the membrane network, yielding a more lightly stained tonoplast/ER membrane network with narrower tubules (starred network in Figure 7B). Subsequently, the central ER tubules appear to be retracted, leaving behind a collapsed tonoplast membrane network that assumes a more latticelike appearance similar to the lipid-rich prolamellar bodies of etioplasts (arrowheads in Figures 6A and 7B). At that time, the lattice-like residual networks begin to disappear. Because the residual membrane networks in some instances still appear to be connected physically to the tonoplast membrane (Figure 9), they may be resorbed by lateral diffusion back into the tonoplast membrane, but further studies are needed to confirm this notion. Soon thereafter, the Znphytate crystals disappear from the chalazal region and cellularization starts in the micropylar domain of the endosperm.

To further characterize the membranes associated with the chalazal domain vacuoles, we immunolabeled sections of developing Arabidopsis seeds with an anti– $\alpha$ -TIP (tonoplast intrinsic protein) antibody to test for the presence of this protein.  $\alpha$ -TIP has been shown to be a marker for the tonoplast membrane of PSVs in embryo cells, which accumulate phytin globoid crystals (Figure 4) (Maurel et al., 1997). However, although the anti– $\alpha$ -TIP antibodies labeled the PSVs in our high-pressure-frozen samples (positive control; data not shown), the tonoplast membranes of the adjacent endosperm vacuole, including the vacuolar compartments in the chalazal region with the Zn-phytate crystals, were not labeled (data not shown).

## Zn and Mn Are Mobilized from the Endosperm to the Embryo at Different Developmental Stages

We studied the amount of Mn and Zn in whole seeds and isolated embryos at different developmental stages (stages III to VIII). Individual embryos were isolated and collected from stages VI, VII, and VIII (early bent-cotyledon, late bent-cotyledon, and mature embryo stages), and their mineral content was determined chemically. The amount of minerals in the endosperm seed coat was calculated by subtracting the contents in the embryo from the contents in the whole seed at the same developmental stage.

Figure 11 depicts the stage-dependent changes in Mn and Zn content per gram of dry weight as well as per indi-



Figure 4. Globoid Crystals in PSVs of the Embryo.

(A) Overview of stage VII embryo cells containing forming PSVs and plastids/chloroplasts (P). Bar = 1  $\mu m.$ 

**(B)** Detail of a developing PSV with numerous electron-dense globoids (arrows). Bar = 500 nm.

**(C)** EDXS spectrum of a globoid. Note the main peaks of P and minor peaks of K, Ca, and Mg. The K<sub>β</sub> peak at 3.58 keV overlaps the Ca<sub>α</sub> peak at 3.69 keV; thus, the peak is labeled K/Ca.

vidual whole seed, embryo, and endosperm plus seed coat. As shown in Figure 11A, the translocation of Mn from the endosperm to the embryo appeared to be initiated during stage VI and became substantial during stage VII (early bent-cotyledon and late bent-cotyledon stages), coincident



Figure 5. Mn-Phytate Crystals in the Chalazal Endosperm Domain.

 $({\rm A})$  Micrograph of an unstained section containing a sheet-like crystal in the ER lumen.

 $({\rm B})$  EDXS spectrum of an ER crystal demonstrating that Mn and P are the main minerals in these crystals.

**(C)** Star-shaped cluster of sheet-like crystals in the chalazal ER. Although most of the larger crystals were lost during sectioning and staining, the deformation of the ER membrane by these crystals is seen clearly (arrowhead).

Bars in (A) and (C) = 200 nm.

with the disappearance of the Mn crystals from the endosperm. On the other hand, more than half of the Zn was in the embryo by stage VI (early bent-cotyledon stage) (Figure 11B), the youngest stage from which we were able to isolate enough embryos ( $\sim$ 2000) for mineral analysis. These biochemical data are consistent with the findings of our structural studies, which showed that Zn-phytate crystals disappeared from the chalazal pole at the late globular embryo stage (between stages III and IV).

## The Increase in Mn Levels in the Embryo Coincides with Higher Levels of Expression of Mn-Containing Enzymes and Chlorophyll Content

We analyzed the expression of two major enzymes known to contain Mn in plants, the 33-kD protein from the oxygenevolving complex of photosystem II (PSII) and the Mn superoxide dismutase (MnSOD), in embryo stages V through VIII (torpedo to mature embryo stages). As illustrated in the protein gel blot shown in Figure 12A, the levels of both enzymes increased during stages V to VII and decreased in stage VIII, when the seed starts to desiccate in the final maturation phase. Similar changes were observed in the total chlorophyll content of the embryo (Figure 12B), with a steady increase in stages V through VII and a decrease in stage VIII. Because Zn is an essential component of >300 enzymes (Fox and Guerinot, 1998), we could not perform the equivalent study of expression levels of Zn-containing proteins.



Figure 6. Structure and Composition of a Zn-Phytate Crystal.

(A) Zn-containing crystal (asterisk) inside a chalazal vacuole with a convoluted membrane network at one end (arrowhead). M, mito-chondria. Bar = 500 nm.

(B) EDXS spectrum of a chalazal vacuolar crystal. Note the main peaks of P and Zn.



Figure 7. Chalazal Vacuoles with Tubular Membrane Network Appendages at Different Stages of Development.

(A) Chalazal vacuoles at stage II with Zn-phytate crystals (asterisks) and extensive tubular membrane network appendages (stars). Each tubular membrane network is continuous with the main vacuole and is delineated by a tonoplast membrane, which encompasses both cytosolic materials and tubular ER membranes (see Figure 10B for details). M, mitochondria; P, plastid/chloroplast.

(B) Higher magnification view of two distinct types of membrane networks (star and arrowhead) associated with a chalazal vacuole at stage III. Near the top edge, two ER-containing connections to the tonoplast surrounding the main vacuole are seen (small arrows). Note the smaller diameter of the cytosol-depleted tubules of the starred tonoplast-ER membrane network compared with the cytosol-rich network seen in (A). Adjacent to the starred tonoplast-ER network is a second type of membrane network (arrowheads) that consists of collapsed tonoplast membranes only. This type of network also exhibits a more geometrical architecture. Bars in (A) and (B) = 500 nm.

## In Fertilized *fis2* Seeds, Hyperaccumulation of Zn-Phytate Crystals Coincides with a Lack of Breakdown of the Membrane Network in the Chalazal Vacuolar Compartment

We also analyzed the accumulation pattern of crystals in seeds from *FIS2/fis2* plants pollinated with wild-type pollen. In these plants, 50% of the ovules inherit the mutant *fis* mu-

tation. When these ovules are fertilized by wild-type pollen, the seed develops but is arrested at early stages of development (Chaudhury et al., 1997). Pollinated *fis* seeds characteristically show an enlarged chalazal endosperm (Sørensen et al., 2001). We observed that many seeds aborted before the embryo reached the globular stage. Our micrographs demonstrate that the chalazal domain in these seeds accumulated more Zn-phytate crystals in its chalazal vacuolar



Figure 8. Effects of Phytase Treatment on Mn-Containing Crystals in Epoxy Sections.

(A) Control buffer treatment. Sections were incubated in sodium acetate buffer, pH 6, heated at  $55^{\circ}$ C for 1 min.

**(B)** Phytase treatment (0.5% [w/v] enzyme in the same buffer and conditions as in control treatment). Note that after the 1-min phytase treatment, the crystal is not dissolved completely and residual material still is seen in the section. Bars in **(A)** and **(B)** = 100 nm.

system than the wild-type endosperm. Many of these crystals appeared trapped between the highly convoluted membranes of the membrane network (Figure 9).

In addition, the development of the membrane network associated with the chalazal vacuolar compartment of the fertilized *fis2* seeds appeared to be blocked in an early developmental stage, because very few collapsed tonoplast membrane lattice structures—that is, degradative membrane configurations (Figure 7B)—could be seen (data not shown). The accumulation of Mn-phytate crystals in the ER did not appear to be affected in these seeds (i.e., the number of these crystals remained at control levels). EDXS analysis of both vacuolar and ER crystals of the fertilized *fis2* seeds demonstrated that their compositions were the same as those of the corresponding wild-type endosperm crystals (data not shown).

### DISCUSSION

Previous studies have reported the presence of globoids in the PSVs of embryos and have made cryptic references to crystal-like structures in the endosperm of members of the Brassicaceae, such as Arabidopsis (Mansfield and Briarty, 1990, 1992) and *Capsella bursa-pastoris* (Schulz and Jensen, 1974). However, because of the less than optimal structural preservation of the samples produced in those studies, they provided no information about what the crystals were made of, when they were formed, and when they were mobilized. The greatly improved structural preservation of cells and crystalline deposits in our high-pressurefrozen samples has enabled us to obtain answers to all of these questions.

The principal findings of this study are as follows: (1) developing Arabidopsis seeds store minerals at three locations, in the PSVs of the embryo, and transiently at two sites in the chalazal endosperm (the ER and vacuolar compartments); (2) the minerals appear to be stored as phytate salts in all three compartments; (3) the chalazal ER accumulates Mn-phytate crystals and the chalazal vacuolar compartments accumulate Zn-phytate crystals; (4) the accumulation of the Zn-phytate deposits coincides with the formation of characteristic tubular membrane network extensions of the chalazal vacuoles; and (5) the disappearance of the Mn-phytate crystals from the endosperm coincides with the accumulation of Mn and of two major Mn binding proteins in the embryo.

## The Chalazal Endosperm May Store Mn- and Zn-Phytates in Special Compartments to Avoid the Toxic Effects of these Heavy Metals

Mn and Zn are two heavy metals that can produce toxic effects in plants (Marschner, 1995). This toxicity appears to be one of the reasons why Mn and Zn are stored in the ER and in the vacuolar subcompartments of the chalazal endosperm in Arabidopsis seeds. The sequestration of Zn into vacuoles in the context of Zn detoxification has been shown to occur in several species (Verkleij et al., 1998; Lasat et al., 2000), and for this reason, the accumulation of Zn in chalazal vacuoles should not be considered a novel concept. In contrast, the accumulation and storage of Mn in the ER is a

Table 1.      Semiquantitative Estimation of Cation:P Ratios in Seed Crystals						
Tissue	Crystal Type	Mn:P	Zn:P	Ca:P	Mg:P	K:P
	Mn-phytate	1.68 ± 0.1	< 0.1	0.14 ± 0.03	< 0.1	0.1 ± 0.01
Endosperm	Zn-phyate	<0.1	$0.5\pm0.01$	$0.45\pm0.04$	0.11 ± 0.01	0.1 ± 0.01
Embryo	Globoid crystals	_a	<0.1	$0.3\pm0.03$	$0.1\pm0.04$	$0.22\pm0.02$

Values were obtained by dividing net counts of different cations by net counts of P from 10 EDXS spectra. <sup>a</sup>-, not applicable.



Figure 9. Three Serial Sections Showing the Connection between a Chalazal Vacuole and the Central Vacuole.

Chalazal vacuoles are recognized easily by their convoluted tonoplast (stars). The position of the connecting site between the vacuoles is indicated in the three sections by arrowheads. CV, central vacuole. Bar in (A) = 500 nm.

novel feature that adds to an already long list of functions performed by the ER (Staehelin, 1997). Whether the Mn-Paccumulating ER sites contain special sets of transporters and enzymes related to this function has yet to be determined. Mn could be sequestered into the ER by ECA1, a  $Ca^{2+}$  ATPase in Arabidopsis that appears to catalyze both  $Ca^{2+}$  and  $Mn^{2+}$  transport into the lumen of the ER and the Golgi (Liang et al., 1997). However, if this were the case, it is difficult to understand why the Mn-phytate crystals in the ER are essentially devoid of Ca.

Tolerance of heavy metals in plants can be achieved by different mechanisms, each with its own metal specificity (Van der Zaal et al., 1999). In the Zn hyperaccumulator plant Thlaspi caerulescens, tolerance to high levels of Zn is achieved by sequestering Zn into vacuoles (Lasat et al., 2000). This sequestration appears to be aided by the complexing of Zn with phytic acid, as shown by the formation of Zn-phytin crystals in the vacuoles of root cortical cells of the Zn-tolerant plant Deschampsia caespitosa grown in the presence of high concentrations of Zn (Van Steveninck et al., 1987). High rates of Zn transport across the tonoplast also have been shown to be essential for Zn detoxification in the Zn-tolerant plant Silene vulgaris (Verkleij et al., 1998). The transport of heavy metal ions into vacuoles is mediated by different tonoplast transporters (Shaul et al., 1999; Maeshima, 2001). Recently, a candidate for Zn transport across the tonoplast called ZAT was identified in Arabidopsis. Overexpression of this protein in Arabidopsis induces Zn tolerance (Van der Zaal et al., 1999).

## Structural Changes of the Tubular Membrane Networks Associated with the Chalazal Vacuoles Appear to Reflect Changes in Their Functional Activity

The Zn-phytate crystal–forming vacuoles in the chalazal endosperm also are characterized by the presence of large appendages of tubular networks of tonoplast/ER membranes (Figures 7A and 10). The increase in tonoplast surface area associated with these membrane networks could be a mechanism for accommodating the large numbers of membrane transporters needed for the rapid translocation of phytic acid into these specialized chalazal vacuolar domains (see below). A similar type of tonoplast membrane network has been observed in cells involved in the formation of calcium oxalate crystals in their vacuoles (Horner and Wagner, 1980).

As illustrated in Figures 7 and 10, the tonoplast and ER tubular networks undergo several structural changes during their development. Forming networks always seem to be composed of concentric ER and tonoplast membrane tubules separated by cytosolic material, with the ER tubes located in the center and the tonoplast membrane tubes located on the outside (Figure 10B). Degradation of the networks appears to commence with the removal of the cytosolic material from the space between the two membranes. This is followed by the withdrawal of the central tubular ER membranes, leaving behind an empty network of collapsed tonoplast-derived membranes that gradually disappear (Figures 6, 7B, and 9).

We postulate that only the networks composed of ER tubules, cytosolic materials, and tonoplast membranes are



Figure 10. Chalazal Endosperm Domain of a Fertilized fis2 Seed.

(A) Overview. The chalazal vacuolar system appears highly branched and contains numerous Zn-phytate crystals associated with a membrane network (open arrow). It also is clearly connected to the central vacuole (CV). The cytoplasm contains many stacked ER cisternae and approximately the same number of Mn-phytate ER crystals (arrowheads) as seen in wild-type endosperm domains. ED, endothelium. Bar =  $2 \mu m$ .

(B) Detailed view of a membrane network in the chalazal vacuolar system of a *fis2* endosperm. At this magnification, the ER cisternae located inside the tonoplast membrane tubules and the cytosolic materials separating the two membrane systems can be seen clearly. Individual Mnphytate crystals (asterisks) appear trapped between the tubules of the membrane network. Bar = 200 nm.

involved actively in the transport of phytic acid into the vacuoles. This hypothesis is supported by the finding that in the *fis2* mutant, which hyperaccumulates Zn-phytate crystals in the chalazal vacuolar compartments (Figure 10), most development stops before the onset of the breakdown of the membrane networks. Thus, many large Zn-P crystals are formed under conditions in which very few collapsed tonoplast membrane network regions are observed.

Another interesting aspect of the chalazal vacuolar system is its continuity with the central vacuole. This physical continuity could facilitate a rapid and efficient mobilization and translocation of Zn, P, and other mineral nutrients from the chalazal to the micropylar domain of the endosperm, where the embryo is located. In addition, we have shown that  $\alpha$ -TIP is not present in the tonoplast of the chalazal vacuolar system. This aquaporin is characteristic of PSVs (Maurel et al., 1997), and it has been suggested that its distribution correlates with phytin deposits (Raboy, 1997). However, its absence in chalazal vacuoles, which accumulate important amounts of Zn-phytates, does not support this correlation.

## Sequestration of Mn and Zn in Different Compartments of the Chalazal Endosperm May Facilitate Differential Mobilization to the Embryo

The storage of Mn and Zn in different compartments of the chalazal endosperm likely is related to the differential timing of mobilization of these two heavy metals from the endosperm to the embryo. Thus, Zn translocation from the endosperm appears to be initiated when the embryo reaches the late globular stage of development (stage III/IV; Figure 1), as indicated by the disappearance of Zn-containing crystals from the vacuoles of the chalazal endosperm. In contrast, the mobilization of Mn appears to occur somewhat later, at the early bent-cotyledon stage (stage VI; Figure 1).

Although both Mn and Zn are incorporated into the embryo (Figure 11), neither of these heavy metals accumulates in the globoid crystals of the PSVs (Figure 4). This finding suggests that the embryo does not store either Mn or Zn in PSVs for future use during seed germination. Instead, these heavy metals appear to be used immediately for the assembly of metalloproteins and other molecules as the need arises during embryo development.

Plant cells produce many enzymes that are activated by Mn<sup>2+</sup>, but only two major Mn-containing enzymes have been identified to date, MnSOD and the 33-kD protein of the oxygen-evolving complex of PSII (Marschner, 1995). This fact has enabled us to test the hypothesis that the transiently stored Mn in the ER cisternae is used for the synthesis of these metalloproteins in the embryo.

SODs are present in all aerobic organisms and catalyze the conversion of superoxide ( $O_2^{-}$ ) to hydrogen peroxide ( $H_2O_2$ ), which is dismutated subsequently into  $H_2O$  and  $O_2$  by peroxidases and catalases (Marschner, 1995; Kliebenstein et al., 1998). MnSOD is confined to mitochondria, and in tobacco, its activity parallels the activity of the respiratory electron transport chain (Bowler et al., 1988). As illustrated in Figure 12A, MnSOD expression increased during embryo development, reaching its highest level at the bent-cotyledon stage (stage VII). This finding suggests that the metabolic activity, and therefore the rate of mitochondrial respiration in the embryo cells, is highest during this stage of embryo development. Interestingly, this stage coincides with the filling phases of PSVs and lipid bodies (Mansfield and Briarty, 1992).

The PSII complex absorbs light energy, produces excited electrons that are used to reduce plastoquinone, and catalyzes the oxidation of water to produce electrons for the photosynthetic electron transport chain. The water-splitting reaction takes place in the oxygen-evolving complex, in which a cluster of four Mn atoms stabilized by the 33-kD protein serves as the catalytic core (Nield et al., 2000). As expected, the highest expression level of the 33-kD protein at the bent-cotyledon stage coincides with the highest concentration of total chlorophyll in the developing embryo (Figure 12).



Figure 11. Changes in the Amounts and the Distribution of Mn and Zn in Developing Seeds of Arabidopsis.

The analysis was performed in samples corresponding to stages III to VIII. The dissected seeds and embryos were analyzed by inductive coupled plasma mass spectroscopy. Curves show the amount of mineral per gram of whole seed dry weight. Bars show the amount of mineral per whole seed (stages III to V), embryo, and endosperm plus seed coat (stages VI to VIII). The amount of heavy metals in the endosperm seed coat for stages VI to VIII was calculated by subtracting the metal content of the embryos from the metal content of the whole seeds. Arrows indicate the developmental stages during which the Mn- and Zn-phytate crystals disappear from the endosperm.

How the developing embryo uses the endosperm Zn stores is less clear. Zn is an essential component of >300 enzymes, including proteins required for DNA and RNA metabolism, cell division, and protein synthesis (Marschner, 1995). Several structural motifs found in transcriptional regulatory proteins are stabilized by Zn, including the Zn finger, Zn cluster, and RING finger domains (Fox and Guerinot, 1998). Because such a large number of proteins contain Zn, we were not able to study their expression levels during embryo development. However, it is interesting that Zn stores disappear from the endosperm at the late globular stage, when tissue specification is first shown by differential gene expression (West and Harada, 1993). Thus, one could hypothesize that at least part of the endosperm Zn stores are incorporated rapidly into the embryo to form part of both cell division and transcriptional regulatory complexes



Figure 12. Protein Gel Blot of Mn-Containing Enzymes, and Total Chlorophyll Content of Embryos during Stages V to VIII of Seed Development.

(A) The two Mn-containing enzymes were MnSOD and the 33-kD protein of the oxygen-evolving complex of PSII. Actin was used as a loading control.

**(B)** Total chlorophyll content in the embryos was determined for the same developmental stages shown in **(A)**.

involved in the tissue specification and organ differentiation processes.

Interestingly, in fertilized fis2 seeds, in which the embryo aborts before reaching the globular stage, the number of Zn-phytate crystals in the chalazal endosperm is increased. FIS genes encode proteins that have homology with Polycomb proteins, which regulate gene expression in Drosophila (Sørensen et al., 2001). As mentioned above, this hyperaccumulation of Zn-phytate crystals in fertilized fis2 seeds may be attributable, in part, to the inability of the endosperm to degrade in a timely manner the membrane networks that appear to be involved in the pumping of Zn and phytic acid into the chalazal vacuoles. A second cause of this hyperaccumulation could be the stalled development of the embryos, which prevents them from using the Zn-phytate stores in the endosperm. However, because so little is known about endosperm development in fis mutants (Grossniklaus et al., 2001), there may be other causes for the Zn crystal hyperaccumulation in the fis2 mutant.

## Why Does the Chalazal Endosperm Domain Transiently Store Mn and Zn?

The chalazal domain has been described as a cyst of dense cytoplasm with haustorium-like extensions (Pacini et al., 1975; Mansfield and Briarty, 1990; Boisnard-Lorig et al., 2001) that could be involved in the transfer of nutrients from maternal tissues to the embryo (Nguyen et al., 2000). We have shown that as soon as the chalazal domain is established, the ER and vacuoles undergo extensive differentiation and transiently accumulate Mn and Zn, respectively. These events could explain some of the features that characterize the chalazal domain, such as the differential gene expression pattern, the cellular ultrastructure, and the presence of polyploid nuclei, which frequently occur in metabolically active plant cells (Lopes and Larkins, 1993).

This leaves unanswered the more fundamental question of why the chalazal endosperm stores such high levels of Mn and Zn. Mn and Zn have been reported to have low to intermediate mobility in the phloem, whereas other essential mineral nutrients, such as K, Mg, P, S, and N, exhibit high mobility (Marschner, 1995). Thus, because the Arabidopsis seed matures in a very short time (~240 to 290 h after fertilization; Mansfield and Briarty, 1992), the endosperm might begin accumulating low/intermediate-mobility minerals soon after fertilization to ensure an adequate supply of these micronutrients for the rapidly developing embryo.

Because there are no symplastic connections between maternal tissues and the endosperm (Patrick and Offler, 2001), the presence of lobed cell walls or even transfer celllike walls in the chalazal endosperm of many flowering plants has been interpreted as a mechanism to facilitate solute transport between the adjacent maternal tissues and the developing seed (Vijayaraghavan and Prabhakar, 1984). In this context, the accumulation of callose in the chalazal cell wall (Figure 3) is puzzling, because this Glc polymer has been shown to restrain apoplast transport in seeds (Yim and Bradford, 1998). One possible explanation is that callose may function as a selective barrier to the transport of molecules into the endosperm from the surrounding maternal tissues.

#### How and Where Is Phytic Acid Synthesized?

The discovery of phytin in two compartments of the chalazal endosperm as well as in the PSVs of embryos highlights both the unique physical properties of this phosphate-rich molecule and our limited knowledge of how and where it is made. The biosynthetic pathway of phytic acid can be divided into two steps: the formation of inositol-3-phosphate [Ins(3)P<sub>1</sub>], and the addition of the remaining phosphate groups by a series of kinases. The sole source of Ins(3)P<sub>1</sub> is Glc-6-P, which is converted into Ins(3)P<sub>1</sub> by the enzyme Ins(3)P<sub>1</sub> synthase (Raboy et al., 2000). In plants, both cytosolic and chloroplastic forms of Ins(3)P<sub>1</sub> synthases have been characterized (Loewus and Murthy, 2000). Recently, pRINO1 from rice, a cDNA clone highly homologous with  $lns(3)P_1$  synthases from other plants and yeast, was isolated, and its transcript levels were shown to increase dramatically during the accumulation of globoids in scutellum and aleurone cells (Yoshida et al., 1999). These results strongly suggest that  $lns(3)P_1$  synthesis is the first committed step in phytic acid biosynthesis. Two studies, one in the monocot *Spirodela polyrhiza* and the other in the slime mold *Dictyostelium discoideum*, led to the biochemical characterization of the sequential phosphorylation steps that convert  $lns(3)P_1$  to phytic acid (Stephens and Irvine, 1990; Brearley and Hanke, 1996; van Haastert and van Dijken, 1997).

What is still missing from these studies of phytic acid biosynthesis pathways is precise information about where the different enzymes are located. Greenwood and Bewley (1984) reported the presence of globoid-type granules in ER cisternae of developing castor bean endosperm cells that produce PSVs. This finding led to the proposal that phytic acid is synthesized in association with the ER and is deposited in the ER lumen, from which it is transported in vesicles to the PSVs. Whether the kinases associated with these reactions are located on the cytosolic or the lumenal side of the ER membranes has yet to be determined. Similarly, there is no information on the transport system that delivers the InsP to the ER lumen. Nevertheless, the demonstration

#### ER Mn-InsP<sub>e</sub> 1) InsP<sub>6</sub> Mn + Mn-rich crystal Mn InsF InsP Ins(3)P CYTOPLASM InsP<sub>6</sub> ER Zn Ca Mg K VACUOLE VACUOLE 2) Zn/Ca/Mg/K InsPa InsP<sub>e</sub> Zn-P rich crystal tonoplast EMBRYO в Ca Mg K InsP<sub>n</sub> Ca/Mg/K InsP<sub>6</sub> InsP InsP<sub>4</sub> globoid ER PROTEIN STORAGE VACUOLE GOLGI

## A CHALAZAL ENDOSPERM

Figure 13. Model of Phytic Acid Synthesis and Its Translocation to Mineral-Storing Sites in Developing Arabidopsis Seeds.

(A-1) Phytic acid  $(InsP_6)$  is synthesized by enzymes associated with the ER and is deposited immediately into the ER lumen. Sequestration of the phytic acid in the ER lumen prevents it from complexing with cytosolic cations and thereby perturbing the ion balance of the cells. In the chalazal endosperm, phytic acid forms crystals in two locations: Mn-rich crystals in the ER and Zn-rich crystals in the vacuoles.

(A-2) Phytic acid for the vacuoles diffuses into the ER tubules that form the core elements of the membrane networks. There, transporters transfer the phytic acid first across the ER and then across the tonoplast membrane into the vacuole, where it interacts with the cations that are to be stored. The geometry of the networks reduces the potential of phytic acid cation crystals from forming in the cytosol.

(B) In the embryo, the transport of phytic acid from the ER to the PSV follows the secretory pathway, as suggested by others. Yet to be determined is whether Golgi-to-PSV transport of phytic acid occurs via multivesicular bodies or by a more direct route.

of Mn-phytic acid crystals in the lumen of ER cisternae of the chalazal endosperm lends further support to the idea that the ER lumen is the site where phytic acid is stored initially after its synthesis.

## Phytic Acid Is a Potentially Toxic Compound and May Require Special Cellular Structures for Transport to Mineral Storage Sites

From a cell biological perspective, the sequestration of phytic acid into the ER lumen may be required to offset the propensity of phytic acid to form complexes with many different types of cations and the need for cells to carefully regulate the ionic concentration of their cytoplasm. These two considerations seem to dictate where phytic acid-containing deposits are formed in cells and how phytic acid is transported to mineral storage sites. Figure 13 presents three models that illustrate how phytic acid might be transported to the three mineral storage sites described in this study. Common to all of these models is a hypothetical transport system that delivers phytic acid or precursor molecules to the ER lumen. To date, no phytic acid transporters have been described in eukaryotic cells (S. Shears, personal communication). However, because phytic acid has been reported to be present in the ER lumen (Figure 5) (Greenwood and Bewley, 1984), a mechanism must exist for transporting phytic acid or precursor molecules from the cytosol into the FR lumen

In the case of the Mn-storing crystals (Figure 13A-1), the situation is the simplest, because the phytic acid–containing Mn crystals are formed directly in the ER lumen. Most likely, crystal formation occurs close to the transporters that deliver the  $Mn^{2+}$  ions into the ER lumen, where they can complex with phytic acid that has already been sequestered. What prevents  $Ca^{2+}$ , which is found normally at high concentrations in the ER, from also becoming incorporated into the crystals remains unknown. One possibility is that the ER in the chalazal endosperm does not accumulate  $Ca^{2+}$  to high concentrations.

More speculative is the model for the translocation of phytic acid from the ER lumen into the chalazal endosperm vacuoles, where the Zn-phytate crystals are formed (Figure 13A-2). Here, we postulate that the phytic acid in the lumen of the ER can diffuse readily into the ER tubules that form the core of the membrane networks associated with the chalazal vacuoles. Inside these networks, the phytic acid would be translocated across the ER membrane into the confined space between the ER and the tonoplast membrane, from which it would be transported across the tonoplast into the vacuole. Inside the vacuole, the binding of phytic acid with Zn and other cations would lead to the formation of the crystalline deposits.

Why do the cells need to form highly complex ER/tonoplast membrane networks to transfer phytic acid from the ER to the chalazal vacuoles? One reason could be to provide more space for the phytic acid transporters, which would accelerate the transport of phytic acid into the vacuoles. Equally important might be the need to create a semisequestered membranous compartment in which phytic acid could be transported efficiently from the ER to the vacuoles without perturbing the cationic composition of the cytosol.

The third model depicting the transport of phytic acid from the ER to the PSVs (Figure 13B) corresponds to the pathway for the transport of storage proteins from the ER to the PSVs via Golgi stacks. During this transport, all of the products remain within the lumen of vesicles and cisternae and are not released into the cytosol, but how these products get from the Golgi to the PSVs remains controversial. Jiang et al. (2001) recently reported evidence suggesting that the globoid crystals inside PSVs might be surrounded by a membrane containing vacuolar H<sup>+</sup>-pyrophosphatase and y-TIP. The origin and functional significance of such membranes remain to be determined. We tried to detect this postulated globoid membrane in our high-pressure-frozen specimens, but the heavy staining of the globoid contents precluded us from doing so (Figure 4). In contrast, we are fairly certain that there is no membrane surrounding the Zn-phytate crystals in the chalazal vacuoles (Figures 6A and 7). This latter finding is consistent with the idea that globoidtype crystals can form in vacuoles in the absence of a limiting membrane. Further studies are needed to define more precisely how phytic acid is transported from its site of synthesis, the ER, to the different types of mineral-storing compartments.

### METHODS

Seeds of *Arabidopsis thaliana* (Landsberg *erecta* wild type) were planted in Metro-mix 200 growing medium with Arabidopsis controlled-release fertilizer 17-6-12 plus micronutrients (Lehle Seeds, Round Rock, TX) and watered with distilled water. Plants were grown in growth chambers at 24  $\pm$  1°C with a 14-h-light/10-h-dark cycle. Developing seeds were excised at various times after anthesis.

Male-sterile *pi/pi* FIS2/*fis2* mutant seeds, which were kindly provided by Abdul Chaudhury (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia), were grown in the same conditions. Flowers were pollinated with wild-type pollen. From these plants, ~50% of the ovules inherit the *fis* mutation and abort during development. These seeds are recognized easily because of their larger size and delayed development. Some of these seeds were excised and processed as described for wild-type seeds.

#### **High-Pressure Freezing/Freeze Substitution**

Whole developing wild-type seeds and fertilized *fis2* seeds were removed from the siliques, loaded in sample holders filled with a solution of 0.1 M Suc, frozen in a Baltec HPM 010 high-pressure freezer (Technotrade, Manchester, NH), and then transferred to liquid nitrogen for storage. Substitution was performed in 2% (w/v)  $OsO_4$  in anhydrous acetone at  $-80^{\circ}$ C for 120 h and was followed by slow warming to room temperature during a period of 2 days. After several acetone rinses, samples were teased from the holders and infiltrated in Epon resin according to the following schedule: 5% (v/v) resin in acetone (4 h), 10% resin (12 h), 25% resin (12 h), and 50, 75, and 100% resin (24 h at each concentration). Polymerization was performed at 60°C.

For energy-dispersive x-ray spectroscopy (EDXS) analysis, high-pressure-frozen samples were substituted in 2% glutaraldehyde in acetone and embedded in Epon. For immunolabeling with anti– $\alpha$ -TIP antibody, some high-pressure-frozen samples were substituted in 0.1% (w/v) uranyl acetate plus 0.2% (v/v) glutaraldehyde in acetone at  $-80^{\circ}$ C for 72 h and warmed to  $-20^{\circ}$ C for 24 h. After several rinses with acetone, samples were infiltrated with Lowicryl HM20 (Electron Microscopy Sciences, Fort Washington, PA) during 48 h and polymerized at  $-50^{\circ}$ C under UV light for 72 h.

#### Immunolabeling

For immunodetection of callose, we used a monoclonal antibody against  $\beta$ -1,3-glucan from Biosupplies (Parkville, Victoria, Australia) on samples substituted in 2% OsO<sub>4</sub> in acetone and embedded in Epon. For immunolabeling of  $\alpha$ -TIP, we used a polyclonal anti– $\alpha$ -TIP antibody (Johnson et al., 1989) and samples embedded in Lowicryl HM20. Both types of specimens were sectioned, placed on formvarcoated nickel grids, and blocked for 20 min with a 5% (w/v) solution of nonfat milk in TBST (Tris-buffered saline plus 0.1% [v/v] Tween 20). Sections were incubated with primary antibodies diluted 1:20 (anti– $\alpha$ -TIP) and 1:10 (anti-callose) in a solution of 2.5% (w/v) nonfat milk in TBST (0.1% Tween 20) at room temperature for 1 h. The sections were rinsed in a stream of TBST (0.5% Tween 20) and then transferred to the secondary antibody (goat anti-rabbit IgG [1:50] in TBST) conjugated to 15-nm gold particles for 1 h. Control experiments were performed by omitting the primary antibody.

#### EDXS

Sections were cut with a dry knife to avoid washing out soluble mineral deposits, mounted on copper grids, and coated with carbon. EDXS studies were performed with a JEOL JEM 2000 EXII electron microscope (Akishima, Tokyo, Japan) fitted with a Kevex Delta Electron Detector Spectrometer (Noran Instruments, Middleton, WI) with a Quantum Thin Window detector. Spectra were acquired for 300 s at  $\times$ 10,000 magnification, an accelerating voltage of 100 kV, and with the sections tilted between 20 and 30° toward the detector. Ten spectra of each of the three types of crystals found in this study were obtained. Net counts were obtained by subtracting the background from the peaks.

#### **Total Metal Analysis**

Approximately 1000 seeds and 1500 to 2000 embryos of each developmental stage were collected for mineral content determination. The analysis was performed at ACZ Laboratories (Steamboat, CO). Samples were digested with repeated additions of nitric acid and hydrogen peroxide and refluxed in a hot-water bath for a total of 4 h. After filtration and centrifugation, samples were analyzed by inductively coupled plasma mass spectroscopy using a Fisons VG PlasmaQuad II STE ICP-MS (inductively coupled plasma mass spectrometer; Middlesex, UK), and the contents of  $Mn^{55}$  and  $Zn^{66}$  were quantified. Quality control included the use of internal standards and the analysis of one duplicate, one blank (reagent only), and one laboratory control sample of known composition for each sample.

#### **Phytase and Phosphatase Treatments**

Sections embedded in Epon resin and mounted on copper grids were incubated in drops of alkaline phosphatase (Sigma, St. Louis, MO) and crude phytase from wheat (Sigma) for 1, 5, and 15 min. For phosphatase treatment, grids were incubated in 0.2% (w/v) enzyme in 1 mM MgCl<sub>2</sub> in 100 mM Gly buffer, pH 9.4, and heated to 37°C (Greenwood and Bewley, 1984). For phytase incubation, 0.5% (w/v) enzyme in sodium acetate buffer, pH 6, heated to 55°C was used.

#### Protein Extraction and Protein Gel Blot Analysis

Embryos at stages V to VIII (torpedo, bent-cotyledon, late bent-cotyledon, and mature stages) were excised from developing seeds and homogenized in extraction buffer containing 60 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 1% (v/v) bromphenol blue. Protein concentration was determined with a kit from Bio-Rad (Hercules, CA) based on the Lowry method (Lowry et al., 1951). Proteins were resolved by SDS-PAGE on 12% (w/v) gels and electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA) using a semidry transfer technique. The polyclonal anti-MSD1 antibody, which recognizes Mn superoxide dismutase from Arabidopsis (Kliebenstein et al., 1998), was used at a concentration of 1:2000, and the anti-33-kD protein antibody (Camm et al., 1987) was used at a concentration of 1:3000. We used the monoclonal antibody anti-actin (Andersland et al., 1994) to ensure equal loading of cytoplasm protein. Immunoblots were developed using alkaline phosphatase-conjugated antibodies and were visualized by reaction with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

#### **Chlorophyll Determination**

Embryos at stages V to VIII were grounded in *N*,*N*-dimethylformamide and sonicated for 15 min. After centrifugation, the total chlorophyll content in the supernatant was determined by spectrometry according to the calculations of Inskeep and Bloom (1985). For every stage, three replicate samples consisting of 20 embryos were analyzed.

## ACKNOWLEDGMENTS

We thank Abdul Chaudhury for providing the *fis2* mutants, Maarten Chrispeels (University of California, La Jolla) for the anti- $\alpha$ -TIP antibody, Richard Cyr (Pennsylvania State University, University Park) for the anti-actin monoclonal antibody, and Patricia L. Conklin (Cornell University, Ithaca, NY) and Robert Last (Cereon Genomics, Cambridge, MA) for the anti-MSD1 antibody. We also thank Kim Davidson (Colorado State University, Fort Collins) for assistance with the EDXS analysis, William Young (Measurement Standards, Colorado Department of Agriculture, Denver) for weighing seed and

embryo samples, and Jotham Austin for comments on the manuscript. This work was supported by National Institutes of Health Grant GM 59787 to L.A.S. and by a grant from the Antorchas Foundation to M.S.O. M.S.O. is a researcher at Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Received November 6, 2001; accepted March 1, 2002.

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