

Induction of Lipid Metabolic Enzymes during the Endoplasmic Reticulum Stress Response in Plants¹

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The endoplasmic reticulum (ER) stress response is a signal transduction pathway activated by the perturbation of normal ER metabolism. We used the maize (*Zea mays*) *floury-2* (*fl2*) mutant and soybean (*Glycine max*) suspension cultures treated with tunicamycin (Tm) to investigate the ER stress response as it relates to phospholipid metabolism in plants. Four key phospholipid biosynthetic enzymes, including DG kinase and phosphatidylinositol (PI) 4-phosphate 5-kinase were up-regulated in the *fl2* mutant, specifically in protein body fractions where the mutation has its greatest effect. The third up-regulated enzyme, choline-phosphate cytidylyltransferase, was regulated by *fl2* gene dosage and developmental signals. Elevated accumulation of the fourth enzyme, PI 4-kinase, was observed in the *fl2* endosperm and soybean cells treated with Tm. The activation of these phospholipid biosynthetic enzymes was accompanied by alterations in membrane lipid synthesis and accumulation. The *fl2* mutant exhibited increased PI content in protein body membranes at 18 d after pollination and more than 3-fold higher triacylglycerol accumulation in the endosperm by 36 d after pollination. Incorporation of radiolabeled acetate into phospholipids in soybean culture cells increased by about 30% with Tm treatment. The coordinated regulation of ER stress related proteins and multiple components of phospholipid biosynthesis is consistent with signaling through a common pathway. We postulate that the plant ER stress response has an important role in general plant metabolism, and more specifically in integrating the synthesis of protein and lipid reserves to allow proper seed formation.

Seed development requires coordination between formation of a pre-emergent embryo and synthesis of the major starch, lipid, and protein storage reserves. Regulation of storage reserve synthesis is complex because of a need to keep the individual components in their proper ratios for the development of mature, viable seeds that will desiccate and subsequently germinate properly. Little is known about how these processes are regulated or how seeds adapt to perturbations in the metabolic pathways essential for their growth and maturation. Because the endoplasmic reticulum (ER) is the site for processing of secretory proteins and formation of membrane and storage lipids, it is likely to play a key role in coordinating the metabolism of proteins and lipids during seed development.

One of the simplest systems for exploring intracellular communication between protein and phospholipid biosynthesis is the formation of protein bodies in maize (*Zea mays*). These membrane-bound organelles arise directly from the ER as ordered aggregates of storage proteins within the ER lumen. Much attention has been given to the packaging of storage proteins within protein bodies (Okita and Rogers, 1996), yet little is known about vesiculation of the membrane to form the limiting boundaries of the organelles. Because maize protein bodies represent terminal vesicles, they do not require additional complex sorting signals or docking proteins. Nevertheless, the transition from flattened ER cisternae to distinct spherical protein bodies must involve some changes in membrane organization in response to storage protein accumulation during seed fill.

A more dramatic alteration in ER membranes is seen in the endosperm of the maize mutant, *floury-2* (*fl2*). Protein bodies derived from the ER are deeply convoluted in this mutant and remain clustered near nuclei rather than dispersed throughout the endosperm (Zhang and Boston, 1992). In addition, ER-resident molecular chaperones that are essential for proper protein folding and secretion increase markedly (Fontes et al., 1991; Marocco et al., 1991; Li and Larkins, 1996; Wrobel et al., 1997). The primary defect in the *fl2* mutant is a single point mutation in a 22-kD α -zein storage protein that prevents the signal peptide of the zein from being cleaved (Coleman et al., 1995). Instead, the signal peptide remains attached and anchors the zein to the membrane (Gil-

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likin et al., 1997). Because the mutant zein gene is expressed specifically in the seed, the rest of the maize plant is phenotypically normal.

The chaperone induction and changes in membrane phenotype previously observed in the *fl2* endosperm are similar to characteristics of mammalian and yeast cells during ER stress. Accumulation of unfolded proteins in the ER triggers a well-defined signal transduction pathway in yeast called the unfolded protein response (UPR; for review, see Chapman et al., 1998; Kaufman, 1999). The most upstream component of the UPR identified to date is a yeast transmembrane protein kinase named Ire1p (Cox et al., 1993; Mori et al., 1993). This Ser/Thr kinase is activated by dimerization and phosphorylation in response to unfolded protein accumulation from the ER lumen (Shamu and Walter, 1996). Subsequent signal transduction through a series of intermediates leads to transcriptional induction of a number of ER resident proteins, including the molecular chaperones, binding protein (BiP), and protein disulfide isomerase (PDI; for molecular chaperone review, see Gething and Sambrook, 1992; Boston et al., 1996). Recent evidence from mammalian research is suggestive that BiP is constitutively bound to Ire1p and that the release of BiP following introduction of ER stress is the signal for Ire1p activation (Bertolotti et al., 2000). BiP dissociation likely occurs because of its higher affinity for unfolded proteins than Ire1p. UPR components appear to be conserved from yeast to mammalian systems (Foti et al., 1999), and the existence of an *IRE1* homolog in *Arabidopsis* (N. Kozumi and M.J. Chrispeels, personal communication) suggests that a similar signal transduction pathway is likely to be present in plants.

Several lines of evidence suggest a connection between phospholipid biosynthesis and UPR signaling. Overexpression of integral membrane proteins is correlated with an increase in phospholipid biosynthesis, proliferation of ER, and induction of the UPR (Chapman et al., 1998 and refs. therein). Such membrane proliferation is presumably a cellular accommodation to depletion of free chaperones and/or proteins involved in import into the ER and represents a perceived need to increase membrane surface area. The stimulus for phospholipid biosynthesis has been suggested by Cox et al. (1997) to be transduced through the same Ire1p-mediated signaling cascade that controls molecular chaperone production.

The *fl2*-mediated induction of ER stress occurs in a tissue devoted primarily to synthesis of storage reserves and gives us the means to determine whether or not protein and lipid metabolism are coordinated during protein body formation in seeds. Cell cultures provide an additional system to investigate the ER stress response in plants without the pleiotropic effects of the *fl2* mutation. A number of pharmacological agents, including tunicamycin (Tm), an inhibitor N-linked glycosylation of proteins, can be used to

induce an ER stress response in cell cultures. Tm is a potent inducer of the ER stress response (Watowich and Morimoto, 1988; Lee, 1992) and has been shown to increase mRNA and protein accumulation of ER-resident molecular chaperones in plants (D'Amico et al., 1992; Denecke et al., 1995; Wrobel, 1996).

In this study we report that four key phospholipid biosynthetic enzymes are up-regulated in *fl2* protein bodies during early kernel development. Levels of phosphatidylinositol (PI) in *fl2* protein body membranes and total triacylglycerols in the *fl2* endosperm are increased when compared with their normal counterparts. In addition, expression levels of PI 4-kinase and overall phospholipid synthesis are elevated in soybean (*Glycine max*) cells in which an ER stress response is chemically induced. The data presented here provide new insights into coordination of the ER stress response and phospholipid metabolism in plants.

RESULTS

Enzymes Assayed

We assayed protein bodies of normal and *fl2* maize for activities of enzymes that are believed to be involved at regulatory or rate-limiting steps in phospholipid biosynthesis (Fig. 1). Choline-phosphate cytidylyltransferase (CCT, enzyme 1), is considered to be the rate-limiting step in the biosynthesis of phosphatidylcholine (PC), the major phospholipid component of most eukaryotic membranes (for a review of plant phospholipid metabolism, see Ohlrogge and Browse, 1995). The lipid kinases diacylglycerol (DG) kinase (enzyme 2), PI 4-kinase (enzyme 3), and PI 4-phosphate 5-kinase (PIP 5-kinase, enzyme 4) are enzymes that catalyze the biosynthesis of phosphatidic acid (PA), PI 4-phosphate (PIP), and PI 4,5-bisphosphate (PIP₂), respectively. Although these phospholipids are minor components of most membranes, they have been implicated in the regulation of vesicle trafficking and other essential signaling pathways (De Camilli et al., 1996; Roth, 1999).

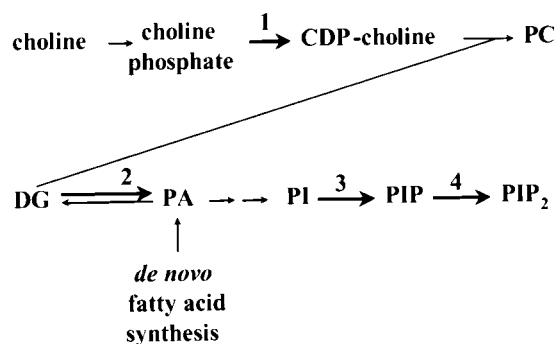


Figure 1. Abbreviated pathway showing reactions of phospholipid metabolism assayed in this study. Bold arrows highlight reactions catalyzed by CCT (1), DG kinase (2), PI 4-kinase (3), and PIP 5-kinase (4).

CCT Activity Increases with *fl2* Gene Dosage

To determine if CCT activity was affected by the *fl2* mutation we analyzed enzyme activity associated with protein bodies of normal and *fl2* kernels. Because of the triploid nature of maize endosperm, a gene dosage series ranging from zero to three copies of a given gene can be generated by reciprocal crosses between homozygous parental lines. Figure 2 shows the effects of *fl2* gene dosage on CCT activity at 18 d after pollination (DAP). Although a single copy of the *fl2* gene had no significant effect on the enzyme activity associated with protein bodies, two and three copies resulted in increases of about 1.2- and 1.5-fold, respectively. This dosage effect corre-

lates well with our previous findings that visible morphological effects of *fl2* on protein bodies increase minimally with one copy of the mutant gene, and become progressively more severe with the introduction of two and three copies (Zhang and Boston, 1992). Levels of BiP RNA (Boston et al., 1991) and protein (Zhang and Boston, 1992) also increase incrementally with each dose of the *fl2* gene (Boston et al., 1991; Wrobel et al., 1997). CCT activities in corresponding ER fractions were not significantly different regardless of *fl2* gene dosage (Fig. 2B).

CCT Activity Changes during Seed Development

To obtain a profile of the differences in CCT activity associated with normal and *fl2* protein bodies during seed development we assayed endosperm at developmental stages from 10 to 36 DAP. During early seed fill, CCT activity was high in normal and *fl2* maize (Fig. 3). As the seed matured, CCT activity remained high in *fl2* protein bodies, but dropped in normal ones, with the largest differences appearing at 14 to 18 DAP. At later stages of endosperm maturation, CCT activity dropped overall and differences between normal and *fl2* protein bodies decreased.

Lipid Kinase Activities Increase in *fl2* Protein Bodies

The activities of enzymes previously implicated in vesicle formation, DG kinase, PI 4-kinase, and PIP 5-kinase, were assayed in protein bodies from kernels harvested 18 and 28 DAP. An increase in each of these activities in protein body fractions was observed in *fl2* relative to normal maize. Table I shows the averages of duplicate values obtained from a single experiment. Because considerable variation was observed in the total amount of radioactivity incorporated among independently isolated protein body fractions, the results of five separate experiments were analyzed as the relative increase in enzyme activity in *fl2* versus normal for each independent experiment. These results are shown in Table I as mean ratios \pm SE. To determine if differences in lipid kinase activities at 18 DAP were specific to protein bodies, ER-enriched fractions from the Suc gradients were assayed. No significant differences were observed (data not shown). By 28 DAP, the mean lipid kinase activities tended to be higher in *fl2* protein bodies than in normal protein bodies at the same developmental stage, but the increases were not significant.

PI 4-Kinase Expression Increases in *fl2* Protein Bodies

To determine if up-regulation of lipid kinases was due, at least in part, to an increase in enzyme amounts, we investigated the accumulation of PI 4-kinase by immunoblot analysis. Protein body fractions from normal and *fl2* endosperm and a microso-

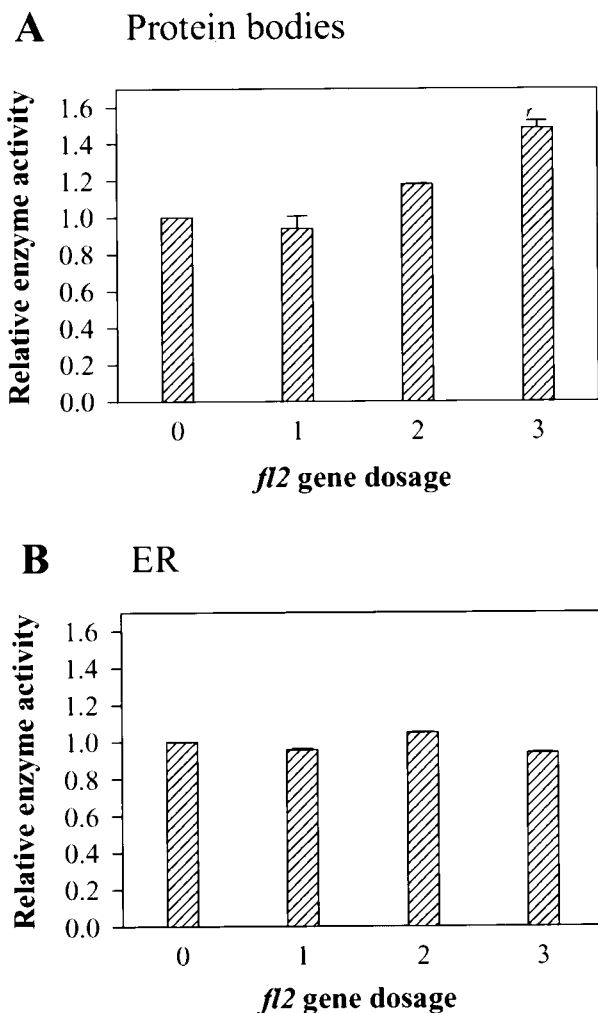


Figure 2. Relative CCT activity of protein body fractions in relation to *fl2* gene dosage. CCT activity is expressed as fold induction over activity in normal endosperm. A, Protein body fractions (mean normal value was 0.237 nmol min⁻¹ mg⁻¹ protein). B, ER fractions. Proteins were extracted from kernels harvested 18 DAP with the triploid endosperm genotypes +/+ (0 doses), +/+/*fl2* (one dose), +/*fl2*/*fl2* (two doses), and *fl2*/*fl2*/*fl2* (three doses) and were fractionated by centrifugation through Suc gradients. Bars represent means of three separate experiments \pm SE.

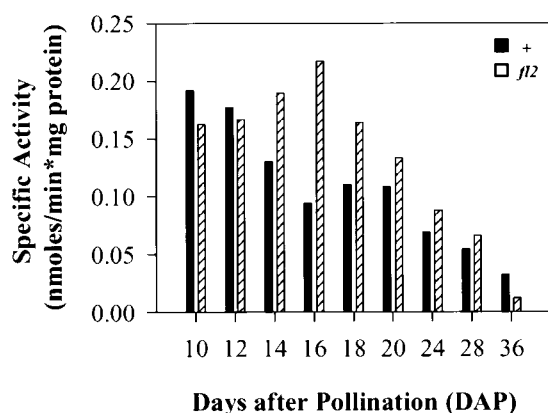


Figure 3. CCT activities of protein body fractions during endosperm development. Black bars, Normal protein bodies; hatched bars, *fl2* protein bodies. Data from a representative experiment are shown. Similar trends were seen in four independent experiments.

mal membrane fraction from carrot cells grown in suspension culture were subjected to SDS-PAGE as described in "Materials and Methods." A band of approximately 65 kD was detected in maize and carrot (Fig. 4). This band most likely corresponds to the low- M_r PI 4-kinase that has been previously described by Westergren et al. (1999) to have PI 4-kinase activity in spinach. A second cross-reacting band with an apparent molecular mass of 77 kD was prominent in *fl2* samples, but not in samples from carrot microsomes unless the blots were exposed for much longer times (data not shown). Both cross-reacting proteins were much more abundant in *fl2* protein bodies than in normal ones and were not seen in identical blots probed with an equal concentration of preimmune serum (data not shown). The isoforms of PI 4-kinase have not been investigated in detail in maize, but the existence of multiple isoforms has been reported in spinach, carrot, and Arabidopsis (Stevenson et al., 1998; Westergren et al., 1999; Xue et al., 1999). A duplicate immunoblot probed with anti-BiP antibody is shown for comparison.

Table 1. DG, PI, and PIP kinase activities of normal (+) and *fl2* protein bodies

DAP	Product	Activity ^a		Ratio (<i>fl2</i> /+)	Mean Ratio \pm se ^b
		+	<i>fl2</i>		
18	PA	0.59	1.30	2.2	3.2 \pm 0.45
	PIP	0.24	0.49	2.0	2.9 \pm 0.70
	PIP2	0.01	0.03	3.0	2.5 \pm 0.33
28	PA	0.40	0.55	1.4	1.6 \pm 0.27
	PIP	0.14	0.19	1.4	1.6 \pm 0.43
	PIP2	0.01	0.02	2.0	1.8 \pm 0.57

^a Specific activities (nmol min⁻¹ mg⁻¹ protein) represent an average of duplicate reactions from one experiment. ^b Final column (far right) shows mean of ratios (*fl2*/+) from five separate experiments \pm se.

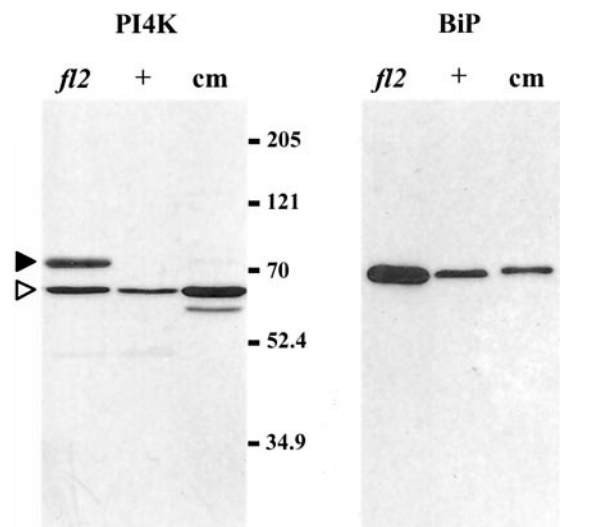


Figure 4. Immunoblot analysis of proteins from *fl2* and normal (+) protein bodies. Samples contained equal amounts of protein (150 μ g) from protein bodies; equivalency was confirmed by staining duplicate gels with Coomassie Brilliant Blue (data not shown). Protein (100 μ g) from carrot microsomes (cm) was used as a positive control. Left, Immunoblot probed with anti-PI4K antibody. Arrowheads mark major cross-reacting species (white arrowhead, 65-kD band; black arrowhead, 77-kD band). Right, Immunoblot of a duplicate membrane probed with anti-BiP antibody. Sizes and relative mobilities of prestained protein markers are indicated to the right of the PI4K immunoblot.

PI 4-Kinase Expression Increases with Chemical Induction of the ER Stress Response

The common feature between the *fl2* mutation and chemical treatment of cell cultures with Tm is induction of an ER stress response. We investigated the expression of PI4-kinase in response to chemical induction of an ER stress response as a way of verifying that the effect on lipid metabolism is directly due to the ER stress response and not due to pleiotropic effects of the mutation. Soybean suspension cultures incubated with Tm showed a strong induction of the molecular chaperones BiP and PDI compared with control cultures, as judged by immunoblot analysis of whole-cell extracts (Fig. 5, A and B). PI4K immunoblots of the same samples also showed increases in response to chemical induction (Fig. 5C). Modest increases in molecular chaperone and PI4-kinase accumulation were observed over the time course of these experiments in control cells treated only with the *N,N*-dimethylformamide solvent. Similar results were observed with untreated soybean cultures, however, suggesting that the abundance of these proteins also changes with culture age (data not shown).

One limitation to immunoblot analysis is that the observed signal represents the combined accumulation of protein produced before and after the application of the stress-inducing compound. To overcome this limitation we performed short-term radiolabeling and immunoprecipitation of treated

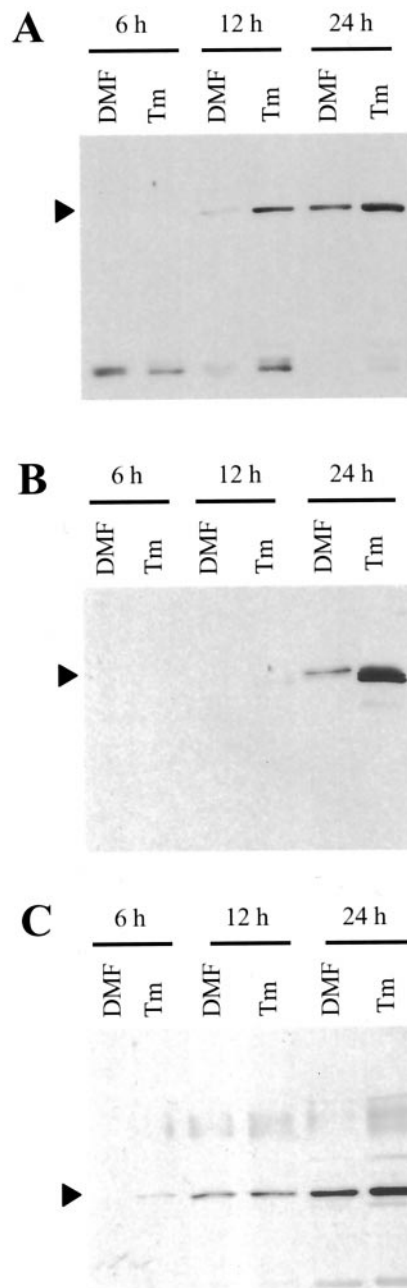


Figure 5. Immunoblot analysis of proteins from soybean cells treated with Tm. Proteins were extracted from whole cells as described in "Materials and Methods" and equal protein loading was confirmed by staining of duplicate gels with Coomassie Brilliant Blue (data not shown). A, Immunoblot probed with anti-BiP antibody. The arrowhead indicates BiP cross-reacting band of about 75 kD. B, Immunoblot probed with anti-PDI antibody. The arrowhead indicates cross-reacting PDI doublet. The slight shift in mobility of PDI in Tm-treated cells represents the inhibition of glycosylation. C, Immunoblot probed with anti-PI4K antibody. The arrowhead indicates a 65-kD cross-reacting band.

soybean cells to determine the effects of the Tm treatment on protein synthesis. Immunoprecipitation from whole-cell extracts revealed a strong induction of the ER stress response by Tm as shown by in-

creased synthesis of the chaperones BiP, PDI, and calreticulin (Fig. 6A). Immunoprecipitation of newly synthesized proteins from ER-enriched fractions showed induction of BiP and PI4K expression in as little as 6 h after Tm treatment (Fig. 6, B and C). The

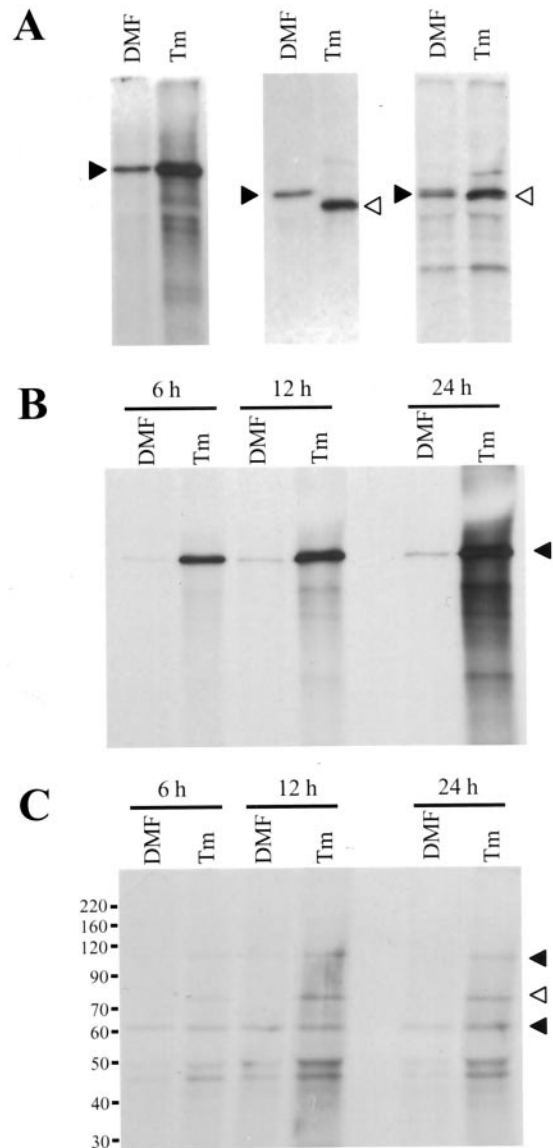


Figure 6. Immunoprecipitation analysis of soybean cell cultures treated with Tm. A, Protein from whole-cell extracts labeled in vivo was immunoprecipitated with anti-BiP antibody (left), anti-calreticulin antibody (center), or anti-PDI antibody (right). Cells were treated with Tm for 12 h prior to a 30-min incubation with [35 S] protein labeling mix. Arrowheads designate major cross-reacting bands. Calreticulin and PDI cross-reacting species are both glycosylated and show a shift to the non-glycosylated form with Tm treatment (white arrowheads). B, Protein from ER-enriched fractions immunoprecipitated with anti-BiP antibody. C, Protein from ER-enriched fractions immunoprecipitated with anti-PI4K antibody. Sizes and relative mobilities of prestained markers are indicated to the left of the figure. Black arrowheads designate bands similar in size to previously characterized PI4-kinases and the white arrowhead indicates band of similar size to the 77-kD band observed in maize (Fig. 4).

multiple cross-reacting proteins (known active forms are designated by the arrowheads to the right of the figure) all increase in response to chemical treatment.

Normal and *fl2* Protein Body Membranes Differ in Composition

The differences in activities of lipid metabolic enzymes suggested that the phospholipid composition of *fl2* protein bodies may differ from that of normal protein bodies. To investigate whether or not membranes were affected by the *fl2* mutation we quantified the amounts of the major phospholipids from protein body and ER fractions. Protein body membranes from the *fl2* mutant contained much lower levels of PA and higher levels of PI than normal protein body membranes (Fig. 7A). Amounts of PA, PI, PC, phosphatidylethanolamine, and phosphatidylglycerol in ER did not differ significantly between normal and *fl2* samples (Fig. 7B). PIP and PIP₂ were not detected in this assay.

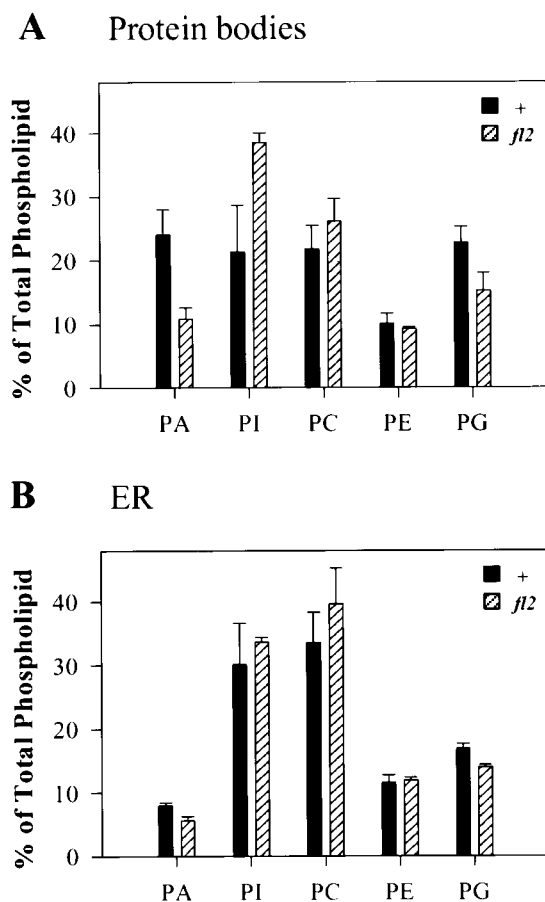


Figure 7. Phospholipid content of normal and *fl2* protein body and ER fractions separated by Suc density gradient centrifugation. PA, PI, PC, phosphatidylethanolamine (PE), and PG are expressed as percentages of total polar lipids in the sample as quantified by gas chromatography. A, Protein body profile. B, ER profile. Means are from three independent experiments \pm SE. Black bars, Normal; hatched bars, *fl2*.

Triacylglycerol Accumulation Increases in *fl2* Endosperm

Martiniello et al. (1978) compared seed quality traits between 142 normal inbred lines and their *fl2* counterparts and noted that the *fl2* lines displayed, on average, 32% higher oil content. In normal maize inbreds, 78% of total lipid in mature maize kernels, mostly in the form of triacylglycerol, is found in the embryo, whereas only 17% is contained in the endosperm (Weber, 1978). Because *fl2* is an endosperm-specific trait we questioned whether or not the reported increases in total seed lipid resulted from an increase in triacylglycerol accumulation specifically within the endosperm. To test this possibility, endosperm and embryo were dissected from *fl2* and normal kernels harvested at 18, 28, and 36 DAP and were dried for 2 d to obtain accurate dry weights prior to lipid extraction and analysis. As shown in Table II, endosperm triacylglycerol levels were greatly affected by the mutation. At 18 DAP, triacylglycerol levels were 1.3-fold higher in *fl2* and by 36 DAP, they were more than 3-fold higher in *fl2* than in normal endosperm. In contrast, triacylglycerol content in the *fl2* embryos was not significantly different from the content of normal embryos. In endosperm and embryo, total PL content in *fl2* was similar to that of normal seeds.

Acetate Incorporation into Lipid Species Increases in Treated Soybean Cells

If induction of ER stress does indeed lead to increased phospholipid biosynthesis, we might expect to see this reflected as an increase in acetate incorporation into these species in induced cell cultures. Investigation of lipid synthesis in soybean cells revealed that overall incorporation of acetate into various lipid species was up-regulated with induction of the ER stress response by Tm (Table III). An increase in acetate incorporation of about 30% was seen in response to treatment with Tm for 12 and 24 h. TLC separation of the radiolabeled compounds revealed a general overall increase in the synthesis of each of the major phospholipids (data not shown) as opposed to specific increases in any one phospholipid species.

DISCUSSION

The ER mediates synthesis and storage of proteins, phospholipids, and triacylglycerols in the developing seed. Our findings suggest that phospholipid metabolism and protein production in protein bodies are coordinately regulated through a common ER pathway that shares many characteristics of the ER stress response. Examination of phospholipid metabolism in the *fl2* mutant revealed alterations in enzyme activities and the phospholipid composition of protein body membranes. Given that PC is a major structural phospholipid of ER and protein body membranes, it is not surprising that signals eliciting the need for

Table II. Triacylglycerol (TG) and total polar lipid (PL) levels of normal (+) and *fl2* endosperm and embryo

Endosperm and embryo lipid levels are expressed as milligrams of lipid per gram of dry wt and are means of three experiments \pm SE.

	DAP	Endosperm		Embryo	
		+	<i>fl2</i>	+	<i>fl2</i>
TG	18	4.5 \pm 1.10	6.0 \pm 1.30	160 \pm 7.5	170 \pm 8.6
	28	1.7 \pm 0.53	3.2 \pm 0.63	79 \pm 8.0	89 \pm 10
	36	1.4 \pm 0.27	4.6 \pm 0.41	81 \pm 8.4	73 \pm 4.3
PL	18	1.30 \pm 0.11	1.30 \pm 0.17	5.2 \pm 1.30	4.5 \pm 1.1
	28	0.27 \pm 0.07	0.38 \pm 0.02	2.1 \pm 0.22	2.6 \pm 0.4
	36	0.11 \pm 0.01	0.17 \pm 0.02	2.0 \pm 0.03	2.8 \pm 0.9

increased membrane biogenesis would result in enhanced activity of CCT, the rate-limiting enzyme in PC synthesis. The greatest differences in CCT activity were seen from 14 to 18 DAP. It has been previously shown that at 14 and 18 DAP, mRNA and protein of the major zeins decrease in the *fl2* mutant when compared with normal maize (Jones, 1978; Lopes et al., 1994). Together with our observations, these data suggest that the reduction in zein accumulation in the *fl2* mutant coincides with the increases in CCT activity.

Because the products of the DG, PI, and PIP kinase reactions represent relatively minor membrane components, we suggest that the increases in their activities are less likely to be needed for membrane biogenesis and instead are related to the roles these phospholipids play in secretory vesicle formation. PA and polyphosphoinositides are involved in secretory vesicle formation in mammalian and yeast cells and are essential for vesicle trafficking (Roth, 1999). These lipids are necessary for recruiting proteins to the vesicle surface for vesicle budding, docking, and fusion signals (De Camilli et al., 1996). For example, in yeast the *SEC14* gene encodes a PI transfer protein that is necessary for the formation of secretory vesicles. The mutation in *sec14*, however, can be suppressed by overexpressing PI 4-kinase (Hama et al., 1999). In our studies, increases in PI 4-kinase activity were accompanied by increases in the amount of PI 4-kinase protein (Figs. 4–6). These increases are suggestive of regulation at the level of transcription and/or translation. Because more than one cross-reacting band was observed in samples displaying an ER stress response, further studies are needed to determine which of the cross-reacting proteins is responsible for the enzyme activity measured in vitro. However, all bands were increased as a result of the ER stress response induced by the *fl2* mutation or chemical treatment.

It is likely that protein body formation in maize is controlled in a manner similar to vesicle budding and that the *fl2* mutant zein may interfere with this process. Investigation of *fl2* endosperm morphology reveals deeply invaginated protein bodies that are clus-

tered near nuclei rather than being dispersed throughout the cell (Zhang and Boston, 1992). If the *fl2* mutation perturbs the process of protein body formation, then the seed might be expected to increase PA and/or PI production to facilitate normal protein body formation under the stress imposed by accumulation of the mutant zein in the ER membrane. Despite the fact that higher DG kinase activity was observed in *fl2* protein bodies, PA levels were significantly lower than those of normal protein body membranes (Fig. 5A). It is interesting that the decrease in PA was accompanied by a concomitant increase in PI (and enhanced PI 4-kinase and 5-kinase activities). Taken together, these observations lead us to suggest that the *fl2* mutation causes an enhanced flux of phospholipid metabolites through the phosphoinositide pathway, at the expense of net PA accumulation.

Previous studies have identified a number of specialized ER subdomains with differing morphological and biochemical properties such as non-random localization of mRNAs, unequal distribution of molecular chaperones, and localized assembly and packaging of proteins into protein bodies (Okita and Rogers, 1996). Thus, the concept of protein bodies as protein-filled extensions of the ER as a whole may be overly simplified. We hypothesize that the *fl2* mutant zein specifically perturbs the regions of the ER involved in protein body biogenesis.

In addition to mediating the synthesis of phospholipids and seed storage proteins, the ER controls production of triacylglycerols that are stored as oil bodies. With the exception of DG acyltransferase, the final enzyme in triacylglycerol synthesis, all other steps of the triacylglycerol pathway are shared with phospholipid biosynthetic pathways (Ohlrogge and Browse, 1995). Thus, signals leading to increased phospholipid synthesis during seed development may concomitantly give rise to increased triacylglycerol accumulation in the seed (assuming DG acyltransferase activity is not limiting). Our results demonstrate that the *fl2*-specific increase in triacylglycerol levels was largely confined to the endosperm where *fl2* is expressed, as opposed to the embryo, the primary site of oil storage in the maize kernel. Moreover, increases in triacylglycerol content in *fl2* endosperm are in agreement with the previ-

Table III. Increases in acetate incorporation into lipid species after incubation of soybean cells with Tm for 12 and 24 h

Increases in acetate incorporation (counts per minute) are expressed as ratios of Tm treated/DMF treated.

Replicates	12 h	24 h
1	1.35	1.64
2	1.20	1.30
3	1.43	1.00
4	1.47	1.26
Average \pm SE	1.36 \pm 0.06	1.30 \pm 0.13

ously documented increase in total oil content in *fl2* kernels averaged over 142 inbred lines (Martiniello et al., 1978). These results suggest that the triacylglycerol content of crop species may be influenced by the ER stress response pathway during seed development.

Taken together, the results presented here demonstrate that the *fl2* mutation affects lipid biosynthesis, as well as the storage protein composition of maize seeds. Chemical induction of the ER stress response in soybean cell cultures showed similar results. In both cases the ER stress response in plants lead to increases in molecular chaperone levels and lipid biosynthesis. In maize kernels, ER stress specifically leads to alterations in protein body membrane composition and morphology and increases in phospholipid biosynthetic enzyme activities. Future studies will be directed toward understanding the underlying mechanisms that coordinate the integrated regulation of protein and lipid metabolism in plants.

MATERIALS AND METHODS

Plant Materials

Maize (*Zea mays*) inbred W64A (normal) and its near isogenic mutant, W64Afloury-2 (*fl2*), were grown at the North Carolina Central Crops Research Station (Clayton) in the summers of 1998 and 1999. Maize kernels were harvested at specific DAP, frozen in liquid nitrogen, and stored at -80°C .

Soybean (*Glycine max*) cell cultures were grown according to Abustait et al. (1985) and were passaged into fresh media weekly. Cultures were treated 4 d after passage with Tm (Calbiochem, La Jolla, CA) as previously described (Wrobel et al., 1997). Control cultures for the Tm samples were treated with an equal amount of its solvent *N,N*-dimethylformamide.

Fractionation of ER and Protein Bodies

All centrifugation and extraction steps were performed at 0°C to 4°C . Buffer A [100 mM {Tricine *N*-[2-hydroxy-1,1-Bis(hydroxymethyl)ethyl]glycine}-NaOH, pH 7.5, 1 mM EDTA, and 10 mM KCl] was used for enzyme activity assays. Buffer B (10 mM Tris-HCl [pH 8.5 at 25°C], 10 mM KCl, and 5 mM MgCl_2) was used for immunoblot and lipid analyses. Homogenization was carried out in buffer A made 20% (w/v) Suc and 10 mM dithiothreitol or buffer B made 7.2% (w/v) Suc and 10 mM dithiothreitol as noted below for specific procedures. For maize protein extractions, endosperm was removed from kernels and was ground in buffer (1:2, w/v) with a mortar and pestle. Homogenates were subjected to centrifugation at 300g for 10 min to remove cell debris. Supernatants were applied to discontinuous Suc gradients (Larkins and Hurkman, 1978) prepared as 2-mL steps of 2.0, 1.5, and 1.0 M Suc in buffer A or B and mixed at the 2.0 M/1.5 M interface by gently moving a curved Pasteur pipette through the interface six to 10 times. Gradients were subjected to centrifugation at

156,000g for 30 min at 4°C in a swinging bucket rotor. ER and protein bodies were collected from the 1.0 M/1.5 M and 1.5 M/2.0 M interfaces, respectively. Cellular fractions were subjected to centrifugation at 5,000g for 10 min in a tabletop centrifuge (protein bodies) or 100,000g for 15 min in a fixed angle rotor (ER).

An alternative method of fractionation was used to obtain protein for the CCT activity assay. After centrifugation at 300g to remove cell debris, supernatant was subjected to a 5,000g centrifugation for 10 min to separate protein bodies (5,000g pellet) from ER and other membranes (5,000g supernatant). Supernatant was subjected to centrifugation at 100,000g for 15 min in a fixed angle rotor. Resulting protein body and ER pellets from either method were resuspended in homogenization buffer and were quantified for protein using the Bio-Rad Protein Assay Kit I, with a bovine γ -globulin protein standard (Bio-Rad, Hercules, CA).

CCT Activity Assay

Protein bodies prepared in buffer A (50 μg in 25 μL of buffer) were added to an equal volume of CCT reaction buffer (Kinney et al., 1987) containing 4 mM [methyl- ^{14}C]choline-phosphate (50 mCi mmol^{-1} , NEN Life Science Products, Boston). Reactions were incubated in a water bath at 30°C for 15 min and were stopped by heating at 95°C for 1.5 min. Product (cytidine diphosphate-choline) and substrate (choline-phosphate) were separated by thin-layer chromatography (TLC) on 60Å silica plates (Whatman, Ann Arbor, MI) with a developing solvent of equal parts 95% (v/v) ethanol and 2% (w/v) NH_4OH . Bands were visualized with a Bioscan System 500 imaging scanner, scraped, and quantified by liquid scintillation spectroscopy in a liquid scintillation analyzer (Packard TRI-CARB 2100TR, Packard Instrument Company, Meriden, CT).

Lipid Kinase Activity Assay

Protein body and ER fractions from Suc gradients made with buffer A were resuspended in 30 mM Tris-HCl (pH 7.2 at 25°C) and were assayed for endogenous lipid kinase activities. Reactions of 20 μg of protein from protein body fractions or 2 μg of protein from ER fractions in 30 mM Tris-HCl (pH 7.2), 7.5 mM MgCl_2 , 1 mM NaMolybdate, 0.01% (v/v) Triton X-100, and 0.9 mM [γ - ^{32}P]ATP (0.2 Ci mmol^{-1} ; 50- μL total reaction volume) were incubated for 10 min at room temperature and stopped with 1.5 mL of chloroform:methanol (1:2, v/v). Lipid products were extracted and analyzed by TLC as previously described (Cho et al., 1995).

Immunoblotting

Protein bodies from Suc gradients made with buffer B were washed twice by dilution in buffer B followed by a 5,000g centrifugation at 4°C for 10 min. The final pellet was resuspended in buffer B containing 0.15 M NaCl, made 1% (v/v) Triton X-100, and were allowed to mix on a Nutator

(Innovative Medical Systems, Ivyland, PA) for 1 h at 4°C. Prior to fractionation through 8% (w/v; PI 4-kinase) or 10% (w/v; BiP) SDS-polyacrylamide gels, one volume SDS-PAGE sample buffer (Laemmli, 1970) was added and samples were boiled for 5 min. For soybean cells, protein was extracted by grinding cells in 2× SDS-PAGE sample buffer (4 mL g⁻¹) using a mortar and pestle. Samples were boiled for 5 min and were subjected to centrifugation at 16,000g for 2 min prior to fractionation through 8% (w/v; PI 4-kinase) or 10% (w/v; BiP and PDI) SDS-polyacrylamide gels.

The PI 4-kinase immunoblots were probed with antibodies raised against a recombinant protein encoding the C-terminal one-third of AtPI4K α as described by Stevenson et al. (1998) except that proteins were transferred in a submerged system in 10 mM CAPS [3-(cyclohexylamino)propanesulfonic acid], pH 11, and 20% (v/v) methanol for 1.5 h at 40V to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). Proteins for BiP immunoblots were transferred in a similar manner and the membranes were probed with monoclonal antiserum raised against spinach BiP (ID9, StressGen Biotechnologies, Victoria, BC, Canada; 1:10,000 in Tris-buffered saline [TBS]) and goat anti-mouse IgG conjugated to horseradish peroxidase (Bio-Rad; 1:30,000 dilution in TBS). Three low- M_r proteins are often seen in immunoblot analysis of soybean cells, but are not recognized by the monoclonal BiP antibody in maize or in immunoprecipitations from soybean cells. The expression of these proteins does not seem to follow the BiP expression pattern with various treatments (data not shown). Proteins for PDI blots were transferred as described above and the membranes were probed with polyclonal antiserum raised against recombinant castor bean PDI (Coughlan et al., 1996; 1:5,000 dilution in TBS) and goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad; 1:30,000 dilution in TBS). Membranes were blocked with 5% (w/v) non-fat dry milk in TBS prior to incubation with primary antibody and cross-reacting material was visualized by chemiluminescent detection according to Stevenson et al. (1998).

Protein Labeling

At the designated times after treatment, 1-mL aliquots of soybean suspension culture cells were transferred to a 24-well tissue culture plate (Becton-Dickinson, Lincoln Park, NJ). Cells were incubated with Easytag Expre³⁵S³⁵S-protein labeling mix (NEN Life Science Products) for 30 min and were washed according to the protocol described by Malik et al. (1999) prior to being frozen in liquid nitrogen and kept at -80°C until analysis.

ER-enriched fractions for immunoprecipitation were prepared by grinding the frozen cells in buffer A made 20% (w/v) Suc using a mortar and pestle with a small amount of glass beads. Cellular debris was removed by a 2,000g centrifugation for 2 min and the supernatant was applied to a discontinuous Suc gradient prepared as a 0.6-mL step of 1.5 M Suc and a 1.0-mL step of 1.0 M Suc, both in buffer A. The remaining fractionation steps were identical to those described above for fractionation of ER from maize.

Immunoprecipitation

All immunoprecipitation steps were conducted at 0°C to 4°C. Radiolabeled cells were resuspended at a ratio of 2 mL g⁻¹ in lysis buffer consisting of 20 mM sodium phosphate buffer (pH 7.5), 500 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) NP-40, 0.02% (w/v) sodium azide, and 0.5% (w/v) sodium deoxycholate and were disrupted using a microtube sample pestle (Research Products International, Mt. Prospect, IL) with a small amount of glass beads. After centrifugation at 16,000g for 2 min, the resulting supernatant was saved for analysis. In an alternate manner, ER-enriched fractions were obtained from soybean cells by Suc gradient fractionation as described above.

As a clearing step, 25 μ L of a 50% (v/v) slurry of Protein-A agarose (Life Technologies, Rockville, MD) in TBS was added to each sample (40 μ g of protein per incubation diluted to 200 μ L with lysis buffer). After clearing, proteins were incubated with antibodies against spinach BiP (1D9, StressGen Biotechnologies; 1 μ L), calreticulin (Coughlan et al., 1997; 1 μ L), or the C-terminal one-third of AtPI4K α (Stevenson et al., 1998; 8 μ L) for 4 h at 4°C on a Nutator, followed by incubation with Protein-A agarose on a Nutator for 1 h at 4°C. After washing the beads three times with lysis buffer, we resuspended the samples in 20 μ L of 2× SDS-PAGE sample buffer, boiled them for 5 min, and then separated proteins by SDS-PAGE. Gels were incubated with En³Hance autoradiography enhancer (NEN Life Science Products) for 1 h and in 5% (v/v) glycerol (4°C) for 30 min at room temperature. Gels were dried under vacuum onto filter paper (Whatman, Clifton, NJ) and were exposed to x-ray film at -80°C.

Lipid Analysis

Endosperm was homogenized in buffer B, left undisturbed for 15 min on ice to allow starch to settle, and then decanted prior to a low-speed centrifugation at 80g for 5 min and fractionation through Suc gradients as described above for separation of ER and protein bodies. Fractions from the gradients were diluted to the refractive index of the homogenization buffer and were subjected to centrifugation at 5,000g in a tabletop centrifuge (protein bodies) or 100,000g in a fixed angle rotor (ER). ER and protein body pellets were resuspended in buffer B prior to extraction of the lipids into the organic phase by overnight incubation in chloroform:methanol (2:1, v/v) at -20°C.

For total PL and triacylglycerol analysis, five to seven kernels of each phenotype were dissected. Endosperm and embryos were separated from each other and were placed in a drying oven (80°C) for 2 d prior to measuring of dry weights. Dried samples were pulverized with a mallet and lipids were extracted in chloroform:methanol (2:1, v/v) overnight at -20°C. Particulates were removed from the lipid extract by vacuum filtration through an solid-phase extraction cartridge with a 60- μ m exclusion size (Alltech Associates, Deerfield, IL).

Lipid extracts were evaporated under nitrogen at 50°C, resuspended in 100 μ L of chloroform:methanol (2:1), and applied to a 60-Å silica gel plate (Whatman). Lipid species

were resolved by TLC with a developing solvent of petroleum ether:diethyl ether:acetic acid (80:20:1, v/v) to separate total PL and triacylglycerol or chloroform:methanol:concentrated NH_4OH (60:30:1.5, v/v) to separate individual phospholipid species. All lipids separated by TLC were visualized with 2,7-dichlorofluorescein in 95% (w/v) ethanol and were identified by comigration with known standards. The regions of the TLC plate corresponding to individual lipid species were extracted with hexane and quantified by gas chromatography (Browse et al., 1986).

[^{14}C]Acetate Incorporation

An aliquot of cells was removed at the designated time after treatment with Tm and was incubated with shaking at room temperature for 2 h with [^{14}C]-acetic acid (NEN Life Science Products) at a concentration of $0.5 \mu\text{Ci mL}^{-1}$. After labeling, cells were harvested by centrifugation at 5,000g and were frozen at -80°C until analysis.

Labeled cells were thawed on ice and rinsed with ice-cold 5% (w/v) TCA. Lipids were extracted, dried under nitrogen, resuspended, and resolved by TLC as described above. Bands were visualized and quantified with an imaging scanner (500, Bioscan System, Washington, DC).

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