FINAL TECHNICAL REPORT
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Title: Coordination of Endoplasmic Reticulum (ER) Signaling During Maize Seed Development
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Research supported by DOE Grant Number DE-FG02-00ER15065 focused on characterization of the cellular processes that control accumulation of seed storage reserves, one of the most important sources of renewable fixed carbon and nitrogen found in nature. We hypothesized that that the central organelle for synthesis of oil and protein reserves, the endoplasmic reticulum (ER), also provides the critical cellular control for not only the total amount of reserves but also the balance between oil and protein that contributes to seed quality and value. Our studies focused primarily on two cellular mechanisms for maintaining ER homeostasis in the presence of functional perturbations from synthesis and trafficking of mutant proteins. The first is the ER stress response and the second is ER associated degradation (ERAD), a pathway for targeted protein degradation.

During the funding period, we analyzed maize mutants that do not properly synthesize, transport, and package storage proteins into protein bodies. These mutants exhibited an ER-stress response that we linked to enhanced accumulation of triacylglycerols and phospholipids as well as activation of key phospholipid biosynthetic enzymes and alterations in membrane lipid synthesis and accumulation. Our work resolved a longstanding controversy underlying the nature of these mutants by revealing the molecular defects responsible for the phenotype. We also made important advances in identifying the protein machinery for ERAD and ER stress. Using a soybean system, we discovered a novel synergistic response that integrates the pathways for ER and osmotic stress. These contributions have enhanced our understanding of intracellular communication among biosynthetic, trafficking and degradative pathways for proteins. Longer term, this insight into the regulation of seed metabolic flux should provide new opportunities for improving protein content and stability in grains. Publications acknowledging support from DOE award DE-FG02-00ER15065 are listed at the end of this report.

Major findings

Lipid metabolism and ER stress

We used both maize and soybean systems to investigate the ER stress response as it relates to phospholipid metabolism in plants (Shank et al., 2001). We made the key discovery that inducing a strong unfolded protein response (UPR) in the ER leads to an increase in triacylglycerol and phospholipid accumulation in endosperm, and a change in signaling kinases. Using maize mutants and soybean suspension cultures treated with pharmacological agents to induce ER stress, we found induction of important phospholipid biosynthetic enzymes, including diacylglycerol kinase, phosphatidylinositol 4-phosphate 5-kinase, choline-phosphate cytidylyltransferase, and phosphatidylinositol 4-kinase. The activation of these phospholipid biosynthetic enzymes was accompanied by alterations in membrane lipid synthesis, elevated accumulation of phosphatidylinositol and triacylglycerol content, and enhanced incorporation of radiolabeled acetate into phospholipids. These findings support our hypothesis that signaling through a common pathway is responsible for coordinated regulation of ER stress responses and

multiple components of phospholipid biosynthesis. Moreover, they are suggestive that the plant ER stress response has an important metabolic role in integrating the synthesis of protein and lipid reserves to allow proper seed formation.

Mutations responsible for the ER stress phenotype

Our findings that the opacity mutants, *Defective endosperm B30* (*De*-B30*) and *Mucronate* (*Mc*), exhibited a chronic ER stress response in developing endosperm challenged a longstanding assumption that these were regulatory mutants. We hypothesized that the mutant phenotypes were caused by defective storage proteins and investigated the molecular basis for the ER stress phenotype. For *Mc,* using transcriptome profiling, restriction fragment linked polymorphisms and 2D gel electrophoresis, we identified a 38-bp deletion (nucleotides 406–444 after the initiation codon) in a 16 kD gamma zein gene and showed that it creates a frame-shift mutation affecting the carboxy terminal third of the protein (Kim et al., 2006). For *De*-B30,* we identified a novel zein protein and a 19-kD alpha-zein cDNA in which proline replaced serine at the 15th position of the signal peptide (Kim et al., 2004). The mutant *Mc* and *De*-B30* alleles each recreated the opaque, ER-stress phenotype when expressed in transgenic maize. Thus, the *Mc* and *De*-B30* phenotypes result from mutations in zein genes, leading to the ER stress response in developing endosperm.

Protein Disulfide Isomerase –Phylogeny and Expression Analysis

Protein disulfide isomerases (PDI's) are critical components of both normal metabolism and the ER stress response as they facilitate proper formation of the highly disulfide-bonded storage proteins. RNA profiling data revealed induction of a number of proteins related to the redox state of disulfide bonds but an ill-defined superfamily structure. We used a bioinformatic approach to identify potential protein disulfide isomerase (PDI) sequences (Houston et al., 2005). Based on a genome-wide search of Arabidopsis, we produced a comprehensive list of 104 genes encoding proteins with thioredoxin domains. Phylogenetic analysis showed that evolutionary relationships of proteins with thioredoxin domains correlated with conserved enzymatic activities. Using a combination of bioinformatic, phylogenetic and biochemical tools we characterized this complex PDI-like clade in maize, Arabidopsis and rice and identified new, single-thioredoxin domain proteins as integral members. Although two of these (quiescin-sulfhydryl oxidase-like and adenosine 5'-phosphosulfate reductase-like) had putative non-isomerase enzymatic activities encoded by an additional domain, two others resembled small single-domain PDIs from *Giardia lamblia*, a basal eukaryote, and from yeast. Mining of maize expressed sequence tag and RNA-profiling databases along with RT qPCR analysis showed extensive variation in expression throughout the plant and in response to ER stress. The major PDI, in particular, accumulated to high levels during seed fill and was induced even more in the presence of mutant storage proteins.

Maize ERAD Machinery Differentially Affected by ER Stress

The removal of misfolded proteins from the lumen of the endoplasmic reticulum and subsequent delivery to cytosolic proteasomes is one of the quality control mechanisms present in the protein secretory pathway. We identified four maize *Der1-like* genes (*Zm Derlins*) and showed through complementation analysis that they are functional homologs of Der1p, a yeast protein implicated in ERAD (Kirst et al., 2005). *Zm Derlin* genes are expressed at low levels throughout the plant, but appear prevalent in tissues with high activity of secretory protein accumulation, including

developing endosperm cells. Expression of three of the four *Zm Derlin* genes increased during ER stress, with *Zm Derlin1-1* showing the strongest induction. Subcellular fractionation experiments localized Zm Derlin proteins to the membrane fraction of microsomes. In maize endosperm, Zm Derlin proteins were found primarily associated with ER-derived protein bodies regardless of the presence of an ER stress response.

Integration of ER- and Osmotic-Stress Pathways

Our earlier work had revealed integration of the ER stress pathway with metabolic pathways involving phospholipids (Shank et al., 2001). To investigate further the adaptive responses of the ER, we used soybean which had previously been shown to induce the molecular chaperone BiP in response to osmotic stress. Global expression profiling on soybean leaves exposed to polyethylene glycol treatment (osmotic stress) or to pharmacological agents revealed the expected major branches of the ER-stress response such as molecular chaperones and ERAD proteins, as well as specific osmotically regulated changes linked to dehydration (Irsigler et al., 2007). Surprisingly, a small proportion (5.5%) of total up-regulated genes represented a shared response that seemed to integrate the two signaling pathways. The genes showed similar induction kinetics and a synergistic response to the combination of osmotic- and ER-stress-inducing treatments. Thus, in addition to identifying ER-stress and osmotic-stress-specific responses in soybean, our global expression-profiling analyses provided a list of candidate regulatory components, which may integrate the osmotic-stress and ER-stress signaling pathways in plants.

Educational contributions; student research supported by DOE funds

Postdoc		
John Rao Thoguru		

Graduate Students		
Name	**Degree**	
Karin Shank	M.S.	
Mariana Kirst	Ph.D.	
Norma Houston	Ph.D.	
Jian Wu	Ph.D.	
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Undergraduate Students		
Leah Hewett	2003	
Jennifer Doss	2004-5	
Shanna Chriscoe	2004	
Mahmoud Chehab	2003	
Sherry Yang	2004-5	
Kristen Lendechy	2004	
Harry Lopez	2005, 6	
Heather McPherson	2007	
Kyle Gadzeck,	2008-9	
Danny Wrenn	2009	
Priti Anand	2009	
Anisha Anthony	2009	

Publications acknowledging DOE support

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