KIRST, MARIANA. Identification and characterization of maize Derlins in response to endoplasmic reticulum stress. (Under the direction of Rebecca S Boston).

Derlin or Derlp-like proteins are endoplasmic reticulum (ER) localized membrane proteins that are required for proper degradation of secretory misfolded substrates. In this work I identified two classes of Derlin proteins in maize. Those classes were named ZmDerlin1 and ZmDerlin2. Functional complementation studies using a yeast mutant for Derlp showed that both ZmDerlin classes of protein are functionally conserved with the yeast Derlp. Maize has four genes that encode proteins from the ZmDerlin1 and ZmDerlin2 classes. Expression analysis of those four genes in maize tissues exhibiting ER stress showed that three of the four ZmDerlin genes were up-regulated by ER stress.

I also observed that ZmDerlin1 and ZmDerlin2 proteins have different accumulation patterns in response to ER stress although both are preferentially associated with the protein body fraction in seeds at mid-stages of development. ZmDerlin1 shows increased accumulation in the endosperm of seeds that produce defective storage proteins while ZmDerlin2 is more abundant in the endosperm tissue of seeds that synthesize normal proteins.

The difference in the accumulation of those two classes of ZmDerlin proteins was also observed in several maize tissues and throughout seed development. Despite gene expression, detected through massively parallel signature sequencing, accumulation of the ZmDerlin proteins was not observed in all the tissues analyzed. In some cases, the ZmDerlin2 protein was not detected even though the messenger RNA was associated with polysomes.

Besides characterizing the two classes of ZmDerlin proteins in maize I also performed iterative sequence searches using ER-associated degradation (ERAD) protein sequences from yeast, plants and animals. Those searches led to the identification of several putative proteins that have homology to the described ERAD machinery. In summary, this work supports the presence of functional ERAD machinery in plants.
IDENTIFICATION AND CHARACTERIZATION OF MAIZE DERLINS
IN RESPONSE TO ENDOPLASMIC RETICULUM STRESS

By

Mariana E Kirst

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APPROVED BY:

Chair of Advisory Committee
Wendy F. Boss

Heike Winter-Sederof
Ralph Dewey
BIOGRAPHY

Mariana was born and raised in Rio de Janeiro, Brazil. She is the first of two daughters for Ana and Rubens Esmeraldo. Mariana moved to Brasilia where she studied biological sciences at the University of Brasilia and did research at the Embrapa genetics resource center.

It was during her senior year in college that she met her husband Matias. They moved to North Carolina in the beginning of 2000 when Matias started the PhD program in genetics and genomic sciences. Mariana enrolled in the PhD program at the Botany/Plant Biology Department at NCSU in the summer of 2001. Since then she has conducted research in ERAD in plants, a field that has proven to be both challenging and exciting. Upon completion she is moving to Gainesville both to join Matias and raise their first child, Lucas, to be born in October. Following her “maternity leave” she’ll look for a research position at the University of Florida.
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## TABLE OF CONTENTS

List of Tables..........................................................................................................................vii
List of Figures............................................................................................................................viii
List of abbreviations....................................................................................................................ix

Chapter 1: **Introduction** ........................................................................................................1
  General introduction .................................................................................................................1
    ER quality control overview ...............................................................................................1
    The unfolded protein response .........................................................................................2
    Uncovering the ER-associated degradation pathway ......................................................3
    Ubiquitination and the proteasome are involved in ER degradation ...............................4
    ERAD substrates are transported through the ER membrane .........................................5
  ER associated degradation pathway .....................................................................................6
    ERAD routes .......................................................................................................................6
    ERAD substrate targeting ....................................................................................................8
    ERAD retro-translocation pores .......................................................................................11
    ERAD substrate removal and degradation .......................................................................13
  Perspectives in the ERAD field .............................................................................................14
  Thesis Plan ..............................................................................................................................15
Reference ..................................................................................................................................16

Chapter 2: **Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress** ...............23
  Introduction ..........................................................................................................................24
  Results ..................................................................................................................................25
    Discovery of maize Derlin genes by RNA profiling .........................................................25
    Plant Derlin genes re grouped into two subfamilies .........................................................26
    Derlin proteins are conserved across kingdoms ...............................................................27
    Zm Derlin proteins are integral membrane proteins .......................................................27
    Zm Derlin1-1 and Zm Derlin2-1 genes complemen the yeast Der1 phenotype ..................28
    Zm Derlin mRNAs are expressed throughout the plant ...................................................29
    Expression of Zm Derlin genes is differentially induced during ER stress ....................29
    Zm Derlin1 and Zm Derlin2 proteins associate with protein bodies ..............................31
  Discussion ............................................................................................................................31
  Materials and Methods ........................................................................................................34
    Plant material ......................................................................................................................34
    GeneCaling Analysis .........................................................................................................34
    Sequence Alignment .........................................................................................................34
    Subcellular fractionation .....................................................................................................34
    Immunoblot Analysis .........................................................................................................34
    Quantitative Expression Analysis of Derlin genes by MPSS ..........................................34
    RT-PCR Analysis ...............................................................................................................34
    Quantitative RT-PCR Analysis .........................................................................................35

iv
Yeast Complementation of Ire1/Der1 mutants

References

Chapter 3: Uncovering proteins from the endoplasmic- reticulum-associated degradation pathway in plants through comparative genome analysis

Abstract

Introduction

ERAD

ER quality control in plants

Link between the unfolded protein response and ER degradation

ERAD in plants

Methodology

ERAD proteins in plants

Cdc48

Ufd1

Hrd1

Hrd3

Doa10

Ubiquitin-conjugating enzymes

EDEM/Htm1

Der1/Derlin

Conclusions and perspectives

References

Chapter 4: Characterization of Derlin1 and -2 in maize tissues and throughout seed development

Abstract

Introduction

Results

ZmDerlin proteins tissue distribution

ZmDerlin1 and 2 proteins are present in seeds but only ZmDerlin1 is present in ear shoots

ZmDerlin proteins are initially associated with the ER

ZmDerlin proteins are differently extracted at later stages of seed maturation

ZmDerlin1 protein is efficiently immunoprecipitated from 18 DAP fl2 endosperm

Discussion

Materials and Methods

Plant material

Protein extraction

Discontinuous Sucrose Gradient

Immunoprecipitation Assays

Immunoblot Analysis

Polysomal fractionation and RT-PCR reactions

References
Chapter 5: Conclusions and Perspectives ......................................................... 93
References ........................................................................................................ 95
LIST OF TABLES

Chapter 2: Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress
   Table 1: Putative Derlin homologs from maize, rice, and Arabidopsis................26
   Table 2: Relative abundance of Zm Derlin MPSS EST’s........................................29

Chapter 3: Uncovering proteins from the endoplasmic-reticulum-associated degradation pathway in plants through comparative genome analysis
   Table 1: ERAD proteins in yeast, human and plants..............................................60
LIST OF FIGURES

Chapter 2: Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress

Figure 1: Differences in expression levels of Zm cDNA fragments detected by GeneCalling .................................................................26
Figure 2: Protein sequence alignment of Derlin homologs..........................27
Figure 3: Immunoblots showing association of Zm Derlin1 with microsomal membranes..............................................................28
Figure 4: Complementation of a yeast Ire1/Der1 double mutant with Zm Derlin1-1 (ZmD1-1) and Zm Derlin2-1 (ZmD2-1)........................................28
Figure 5: Comparison of Zm Derlin gene expression by semiquantitative (A and B) and quantitative (C).......................................................30
Figure 6: Accumulation of Z.m Derlin proteins in normal and mutant endosperm tissue........................................................................31
Figure 7: Distribution of Zm Derlin proteins in subcellular fractions of normal and fl2 endosperm tissue..................................................31

Chapter 3: Uncovering proteins from the endoplasmic-reticulum-associated degradation pathway in plants through comparative genome analysis

Figure 1: Amino acid alignment of Ufd1 sequences..................................63
Figure 2: Amino acid alignment of putative Arabidopsis, human and yeast Hrd1 sequences.................................................................65
Figure 3: Comparison of amino acid sequences for animal ubc6e and predicted ubc6e plant proteins..............................................................68
Figure 4: Bayesian consensus tree of Derlin proteins resulting from analysis of protein sequences using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001)........70

Chapter 4: Characterization of Derlin1 and -2 in maize tissues and throughout seed development

Figure 1: Immunoblots showing accumulation of ZmDerlin1 protein in several maize tissues from normal W64A inbred line .................85
Figure 2: Association of ZmDerlin RNAs with polysomes..........................86
Figure 3: Immunoblots showing accumulation of ZmDerlin1 and ZmDerlin2 proteins in normal and fl2 samples from ear shoots to seeds at mid-stages of maturation........................................................................87
Figure 4: Distribution of ZmDerlin1 and ZmDerlin2 proteins in subcellular membrane fractions of leaf, ear shoot and kernel tissues from a normal W64A maize line..............................................................88
Figure 5: Distribution of ZmDerlin proteins across developmental stages in normal and fl2 seeds................................................................89
Figure 6: Immunoblots showing peptide competition assay for ZmDerlin1 protein......................................................................................92
LIST OF ABBREVIATIONS

aa – amino acid
A1PiZ - Z variant of alpha-1-protease inhibitor
AAA-ATPase – ATPases associated with diverse cellular activities
BACE457Δ - pancreatic isoform of human β-secretase
β-ME – β-mercaptoethanol
BiP – binding protein
BLAST – Basic local alignment search tool
bp – base pair
CFTR - cystic fibrosis transmembrane conductance regulator
CNX – calnexin
CPY – carboxypeptidase yscY
CRT – calreticulin
De*-B30 – defective endosperm B30 endosperm mutant
Δgpαf – N-glycosylation site mutant alpha-factor precursor protein
Der – degradation in the ER
Derlin – Der1-like
DTT – DL-Dithiothreitol
EDEM – ER degradation enhancing mannosidase-like protein
ER – endoplasmic reticulum
ERAD – endoplasmic reticulum associated degradation
ERSE - endoplasmic reticulum stress response element
fl2 – floury-2 endosperm mutant
GFP – green fluorescence protein
HMGR – enzyme involved in sterol synthesis
Hrd – HMG-CoA reductase degradation
Htm1 – homologous to mannosidase I
Man8GlcNAc2 and Man5GlcNAc2 - monoglucosylated N-glycans that combine glucose (Glc), Mannose (Man) and N-acetylglucosamines
Mc – mucronate endosperm mutant
MHC I - major histocompatibility complex class I
MLO – mildew resistance O
MW – molecular weight
NHK – α1-antitrypsin variant null Hong Kong
PDI – protein disulphide isomerase
PrA – proteinase yscA
RTA – plant protein ricin A-chain
TCR - T cell antigen receptor
Ubc – Ubiquitin conjugating enzyme
Ufd1 – ubiquitin fusion degradation 1
UPR – unfolded protein response
INTRODUCTION
General introduction

Proper synthesis and secretion of secretory proteins is necessary for appropriate cellular functions and organism development. Continuous synthesis of abnormal proteins, could lead to accumulation of toxic aggregates in the lumen of the endoplasmic reticulum (ER). To cope and prevent aggregate accumulation the ER has a quality control mechanism. One of the components of the ER quality control is the ER-associated degradation pathway (ERAD).

The ERAD pathway was identified almost two decades ago by experiments in animal cells that showed that the degradation associated with the ER was occurring in the cytosol instead of by ER resident proteases. However, despite the fast advances made in the ERAD research that led to increased knowledge of the pathway components and their functions, much is still left to be studied, especially in plants. With the advance in the research a variety of substrates degraded by the ER pathway were identified. This diversity of substrates degraded by different mechanisms led to questions of how they are recognized and which are the components involved in the degradation of each specific substrate.

In this chapter I will briefly present and discuss the initial work that led to the discovery of the ERAD pathway and its many segments that are shared with other pathways. I will primarily focus on work done in yeast and animal cells because most of what is known about ERAD today was learned in those organisms. The subsequent chapters consist of our work to understand ERAD in plants.

ER quality control overview

The ER is the organelle responsible for the synthesis and initial assembly and modification of secretory proteins. Molecular chaperones, protein disulfide isomerases and modifying enzymes present in the lumen of the ER are responsible for assuring that newly synthesized proteins acquire their native conformation (Ellgaard and Helenius 2003). Some of those ER resident proteins are part of the quality control system, a mechanism for ensuring that only correctly assembled proteins traffic through the secretory pathway. The quality control system can be divided in three parts, (1) ER-associated degradation (ERAD), (2)
trafficking of proteins to the lysosome or vacuole for future degradation and (3) unfolded protein response (UPR; Brodsky and McCracken 1999).

The unfolded protein response

The unfolded protein response (UPR) is elicited by the production and accumulation of misfolded proteins in the lumen of the ER. The main function of the UPR is to reduce the accumulation of abnormal proteins by triggering a signal transduction pathway that leads to translational attenuation and up-regulation of components involved in reducing the accumulation of misfolded proteins. The up-regulated genes encode molecular chaperones and genes that regulate metabolism and the redox environment, and degradation components present in the ER (Travers et al., 2000).

The ER senses the accumulation of misfolded proteins by three sensors. Each of the sensors encodes a protein that spans the membrane of the ER once and has a common mechanism to perceive the accumulation of misfolded proteins (Rutkowski and Kaufman, 2004). In cells not exhibiting ER stress the molecular chaperone BiP is found associated with the UPR sensors, a condition that changes upon stress. Treatment of cells with tunicamycin or DTT, agents that cause the accumulation of misfolded proteins in the lumen of the ER, causes conformational changes in the sensors due to release of the molecular chaperone BiP (Bertolotti et al., 2000, Okamura et al., 2000, Shen et al., 2005).

One of the unfolded protein response sensors, Ire1p, is present in all eukaryotes from yeast to mammals, including plants. When activated, Ire1p becomes phosphorylated and activates its site-specific endonuclease which leads to the mRNA splicing of the constitutively transcribed bZIP transcription factor HAC1, in yeast, or XBP1, in mammals (Sidrauski and Walter 1997, Yoshida et al., 2001). The spliced transcription factor binds to cis-acting elements present in UPR-induced genes leading to increased expression of molecular chaperones and components involved in reducing the accumulation of misfolded proteins (Mori et al., 1996, Travers et al., 2000).

In addition to Ire1 proteins, animal cells also contain an additional kinase that acts as a transducer for ER stress, PERK/PEK. PERK activation leads to phosphorylation of the common translation initiation factor eIF2alpha (Harding et al., 1999), which results in translational attenuation by inhibiting the initiation of protein synthesis. In wheat cells,
limited synthesis of new proteins due to phosphorylation of the serine residue from eIF2alpha by the pPKR kinase, an RNA-dependent protein kinase, has been described (Langland et al., 1995, Langland et al., 1996). This mechanism, however, has not yet been observed as a response to the accumulation of misfolded proteins in the lumen of the ER in plants.

Besides the two kinase transducers, animal cells have an additional sensor for ER stress, the transmembrane protein ATF6. Upon ER stress activation, ATF6, which is present in the ER membrane, is translocated to the Golgi where it is cleaved from the membrane to allow transport into the nucleus. There it enhances the expression of molecular chaperones and XBP1 mRNA by binding to a cis-acting endoplasmic reticulum stress response element (ERSE) region in those genes (Haze et al., 1999, Yoshida et al., 2000). Binding of ATF6 to the ERSE region in XBP1 leads to increased accumulation of the unspliced form of XBP1 mRNA, the Ire1p substrate, ultimately leading to up-regulation of molecular chaperones and ER degradation components.

Uncovering the ER-associated degradation pathway

Despite the initial belief that aberrant ER lumenal proteins were degraded by proteases present in the lumen of the ER, work from the early-to-mid 1990’s in mammalian and yeast cells determined that secretory proteins were transported out of the ER and degraded in a polyubiquitin, proteasome dependent manner (Ward et al., 1995, Pilon et al., 1997). Much of the initial progress made in determining that a compartment in close proximity to the ER was the site of degradation of unassembled or incomplete forms of multimeric complexes and mutated proteins was due to the study of human diseases. For example, observations that partial complexes or individual subunits from the T cell antigen receptor (TCR) were not expressed on the surface of T cells prompted investigation of the pathway responsible for degrading these TCR subunits in transfected fibroblast cells (Lippincott-Schwartz et al., 1988). Primary indications that degradation was occurring within the ER or in a closely related compartment came from endonuclease H resistance and sucrose gradient experiments in transfected fibroblast cells. Treatment of fibroblast cells with endonuclease H, which does not cleave N-linked oligosaccharides processed in the Golgi, showed that TCR alpha chains do not acquire resistance to the enzyme while sucrose density fractionation showed that the TCR alpha chains co-fractionated with ER enzymes. Similar conclusions were reached for
the Z variant of alpha-1-protease inhibitor (A1PiZ) in mouse hepatoma cells (Le et al., 1990). Treatment of cells containing the A1PiZ variant with drugs that inhibit ER to Golgi transport had no effect on A1PiZ degradation. The accumulation of a recombinant truncated form of A1PiZ containing an ER retention signal (KDEL) in the ER suggested that degradation of the recombinant A1PiZ form occurred following export from the ER but prior to reaching the Golgi apparatus.

In yeast, the ER degradation pathway was discovered by the use of yeast strains deleted for components involved in protein transport from ER to vacuole and vacuolar proteases (Finger et al., 1993, McCracken and Kruse 1993). That those yeast mutant cells were capable of efficient protein degradation independent of vacuolar proteases indicated the existence of an alternative degradation pathway. Also, like in animal cells, A1PiZ proteins in yeast showed sensitivity to endonuclease H digestion suggesting that they accumulate in the ER (Le et al., 1990). Efficient degradation of A1PiZ proteins in yeast cells deficient in vacuolar activity suggested that degradation of A1PiZ is independent of the vacuole.

**Ubiquitination and the proteasome are involved in ER degradation**

Although a proteolytic pathway to remove proteins from the ER had been identified, many questions remained. This was in part due to the lack of knowledge of which proteins were involved in degrading the substrates. Because the cytosol had been proposed to participate in the degradation of the ER substrates (Lippincott-Schwartz et al., 1988) and the proteasome had previously been found associated with microsomes and involved in the degradation of proteins under several stress conditions, Finger et al., (1993) tested if the proteasome was involved in ER degradation by using yeast cells mutated in subunits of the proteasome. However, they did not observe a change in the accumulation of the misfolded substrates, CPY* or PrA*, in stationary phase cells lacking subunits to the proteasome. The lack of activity of the proteasome led to the conclusion that it was not a major degradative component of the ER degradation pathway. Nevertheless, subsequent experiments using proteasome inhibitors to reduce the accumulation of the mutated form of the cystic fibrosis transmembrane conductance regulator (CFTR, ΔF508) in animal cells implicated the proteasome in the ER degradation (Jensen et al., 1995, Ward et al., 1995). In cells treated with proteasome inhibitors there was an increase in the accumulation of polyubiquitinated
forms of the mutated CFTR protein. Subsequently, use of proteasome inhibitors in conjunction with mutant yeast cells blocked CFTR degradation and confirmed that the proteasome was also required for the degradation of misfolded proteins in yeast cells (Werner et al., 1996). The presence of ubiquitinated misfolded ER proteins in cells treated with proteasome inhibitors corroborated the function of the proteasome in ER degradation. To confirm the requirement of modification by ubiquitin molecules for proper degradation a dominant negative form of ubiquitin, K48R, was co-transfected into animal cells with CFTR ΔF508. The incorporation of the K48R ubiquitin form into the mutant protein led to a substantial increase in the accumulation of both soluble and insoluble misfolded protein (Ward et al., 1995).

Due to the fact that proper ubiquitination requires the action of ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligase enzymes (E3) researchers initiated the investigation of specific enzymes that were localized in the ER and involved in the ubiquitination process. Werner et al., (1996) investigated the function of the ubiquitin-conjugating enzyme ubc6 in the ubiquitination process of A1PiZ by using yeast ubc6 deletion mutants. Although A1PiZ accumulation was not stabilized in null ubc6 cells, experiments in cells lacking the ubiquitin conjugating enzyme 7 (ubc7p) showed increased accumulation of a mutated vacuolar protein, carboxypeptidase Y (CPY*; Hiller et al., 1996). Those results suggest that different ubc proteins are required for degradation of distinct ER substrates. Similar conclusions were reached for the E3 ligase enzymes involved in ERAD. Distinct ERAD substrates have different accumulation patterns in yeast cells lacking specific E3 ligase enzymes (Bordallo et al., 1998, Swanson et al., 2001, Haynes et al., 2002).

**ERAD substrates are transported through the ER membrane**

Although the substrates used to uncover the ER degradation components were mostly soluble ER luminal proteins, the E3 ligase enzymes found to be involved in tagging ER substrates are integral ER membrane proteins and the E2 ligases are either membrane associated or soluble cytosolic proteins (Hiller et al., 1996, Bordallo et al., 1998, Swanson et al., 2001, Haynes et al., 2002). The fact that most of the ubiquitin tagging and degradation machinery is present in the cytosol suggested the existence of a pore to allow transport from the lumen of the ER to the cytosol. The primary candidate for such a transport role was the
Sec61 complex. Use of mutant yeast cells lacking the Sec61 or the Sec63 subunits of the translocon led to increased accumulation of misfolded proteins indicating that the translocon was involved in ER degradation (Plemper et al., 1997). However, the average diameter of the translocon does not allow for a misfolded protein to be in its globular form suggesting the association of molecular chaperones with misfolded proteins to maintain a translocation competent structure. To test the association of molecular chaperones with ER degradation substrates, temperature sensitive yeast mutant cells for genes that encode molecular chaperones were assayed for substrate accumulation under the restrictive temperature. In those cells, ER degradation substrates either accumulated as aggregates (Plemper et al., 1997, Nishikawa et al., 2001) or had reduced degradation rates (McCracken and Brodsky 1996). Association of molecular chaperones with ER degradation substrates was also observed in animal cells. The molecular chaperone calnexin was shown to associate with the immature form of CFTR ΔF508, a known ERAD substrate, until it was degraded (Pind et al., 1994).

ER associated degradation pathway

The ER degradation pathway turned out to be more complex than earlier expected when it was thought that proteases present in the ER or in a nearby compartment were responsible for degrading misfolded secretory proteins. This pathway is now known to share components with the folding machinery present in the lumen of the ER as well as with the proteolytic pathway in the cytosol. Current work is still uncovering the complexity of the ER degradation pathway in a diverse group of organisms, with increased emphasis on yeast genetics. The occurrence of different groups of misfolded proteins, i.e. membrane and soluble, suggests the existence of diverse routes which may or may not require the Sec61 translocon, or transport to other secretory compartments.

ERAD routes

Soluble and membrane-associated secretory proteins are targeted for degradation by a still unclear mechanism although molecular chaperones have been proposed to present the misfolded proteins for degradation. The best studied system to present misfolded proteins for degradation is one used for glycosylated proteins. Those proteins go through a cycle of mannose modifications and associate with the molecular chaperone calnexin prior to
degradation. Interestingly, some substrates are transported to the Golgi and back to the ER prior to degradation. Although it is not clear what signals this transport and which E3 ligase enzyme will tag the substrate with ubiquitin, distinct E3 ligase enzymes are required for subsets of substrates.

The E3 ligase used for most substrates studied to date is the RING finger E3 ligase enzyme Hrd1. Yeast mutants showed that Hrd1 is involved in the degradation of several ERAD substrates including CPY*, the sterol synthetic enzyme HMGR and ΔF508 CFTR (Bordallo et al., 1998, Bays et al., 2001a, Gnann et al., 2004). The function of Hrd1 is regulated by Hrd3 (Gardner et al., 2000). Co-immunoprecipitation assays showed that Hrd1 and Hrd3 proteins interact through a transmembrane domain and genetic analysis placed them in the same pathway as the Sec61 complex and the integral membrane protein Der1p (Bordallo et al., 1998, Gardner et al., 2000). The E3 ligase Hrd1 also acts in concert with specific ubiquitin conjugating enzymes (Biederer et al., 1996, Hiller et al., 1996, Friedlander et al., 2000).

Another E3 ligase present in the ER membrane of yeast cells is the protein Doa10. This protein was initially reported as required for the degradation of the cytosolic transcription factor Matα2 in yeast cells (Swanson et al., 2001). Doa10 contains a RING finger motif and a WW domain that can function in binding either substrates or cofactors. For the degradation of Matα2 and Deg1-containing substrates Doa10 functions in the same pathway as Ubc6 and Ubc7 and in complementary pathways to Ubc4/Ubc5 enzymes (Swanson et al., 2001). In the ER, Doa10 is involved in the degradation of the membrane protein Ubc6 but not the glycosylated substrate CPY*. Like the Hrd1 enzyme, Doa10 is found in protein complexes with cytosolic components fundamental for ER degradation (Swanson et al., 2001, Schuberth and Buchberger 2005).

A third E3 ligase involved in eliminating misfolded substrates from the ER lumen in yeast cells is Rsp5p. Substrates degraded by Rsp5p are first transported to the Golgi or a later secretory compartment prior to degradation. Rsp5p is a HECT domain E3 ligase enzyme that was identified in yeast cells lacking Hrd1 protein and overexpressing the soluble substrate CPY* (Haynes et al., 2002). Because cells lacking Hrd1 efficiently degraded the substrate, it was proposed that a saturated or inactive Hrd1 pathway causes shifting of the substrate to an alternative pathway. This alternative pathway requires transport through the Golgi. Substrate
degradation occurs in a proteasome-dependent and vacuolar protease-independent manner. Interestingly, Spear and Ng (2003) also observed efficient degradation of overexpressed CPY* proteins in yeast cells lacking the Hrd1 protein. They, however, concluded that vacuolar proteases were required for eliminating the overexpressed misfolded protein due to its stabilization in strains lacking the gene that activates those proteases. A connection of Rsp5p to vacuolar degradation was previously described by Galan and co-workes (1996) where Rsp5p was shown to be required for ubiquitination of cargo proteins for sorting and trafficking into the secretory pathway for eventual degradation in the vacuole. It is possible; however, that both degradation routes (vacuolar and ERAD) are used for eliminating overexpressed misfolded proteins as a mechanism to reduce toxicity in the cell. Either way, independently of the final degradation site, ER to Golgi transport has been observed for several ERAD substrates (Vashist et al., 2001, Taxis et al., 2002).

**ERAD substrate targeting**

Once some of the key components involved in ubiquitinating ER misfolded substrates through the use of yeast mutants had been identified, the next phase turned to understanding the transport of soluble misfolded proteins through the ER membrane for ubiquitination. Because the diameter of the Sec61 translocon is not large enough to allow the transport of globular proteins it was logical to hypothesize that molecular chaperones were involved in presenting misfolded substrates for retro-translocation. To address this hypothesis, temperature sensitive yeast cells for the Kar2 gene (BiP) were assayed for the accumulation of several known ERAD substrates under the restrictive temperature. The use of yeast cells mutant for Kar2/BiP showed that soluble misfolded substrates accumulate in cells under the restrictive temperature while membrane substrates were unaffected. Thus, it was concluded that this molecular chaperone is required in the degradation of soluble luminal but not membrane substrates (Plemper et al., 1997, Brodsky et al., 1999, Hill and Cooper 2000, Nishikawa et al., 2001).

The finding that BiP did not participate in the degradation of some substrates may reflect its location in the ER where it would not have contact with misfolded cytosolic domains. It would then be expected that ER proteins with misfolded cytosolic domains would require the involvement of cytosolic molecular chaperones such as Hsp40, Hsp70 and Hsp90.
The use of temperature sensitive yeast mutants for each of those genes showed that, in general, Hsp40 and Hsp70 are involved in aiding the degradation of membrane proteins with mutations in the cytosolic region while Hsp90 appears to prevent CFTR degradation by keeping it in its folded state (Huyer et al., 2004a, Huyer et al., 2004b, Youker et al., 2004). Indeed the requirement of molecular chaperones BiP and DNAJ-like proteins was shown for soluble and membrane proteins exhibiting mutations in their luminal but not in their cytosolic domains (Plemper et al., 1997, Brodsky et al., 1999, Nishikawa et al., 2001, Taxis et al., 2003, Huyer et al., 2004a, Huyer et al., 2004b).

Biochemical evidence for the involvement of soluble molecular chaperones in substrate targeting for degradation was obtained mostly by the studies of known ERAD substrates in animal cells. Sequential assistance from calnexin and BiP/PDI chaperones was observed in the targeting of a misfolded isoform of the human pancreatic β-secretase in vivo (Molinari et al., 2002). Immunoprecipitation from cells expressing the misfolded β-secretase proteins showed that the molecular chaperone calnexin briefly associates with fully oxidized forms of the misfolded protein while BiP and PDI can associate with complexes containing either oxidized or reduced forms of the protein. In a cell free system, immunoprecipitation with antibodies against ER resident molecules such as BiP, PDI and Erp57 showed increased association of the translation products of the MHC I glycoprotein while calnexin association with the substrate decreased over time (Wilson et al., 2000). However, despite the lack of a requirement for calnexin interaction with MHC I prior to degradation, treatments with glycosidase inhibitors alter the substrate half-life making it shorter when preventing calnexin association or longer when preventing calnexin dissociation from the substrate (Wilson et al., 2000, Molinari et al., 2002). The change in substrate half-life following treatment with glucosidase inhibitors suggests that trimming of mannose residues from oligosaccharides is critical for the degradation of glycosylated ERAD substrates even though the association of the substrate to the molecular chaperone calnexin decreased with time.

Also involved in the presentation of misfolded glycosylated proteins for degradation is the receptor for mannose-8 glycans, EDEM/HTM1. The Edem/Htm1 gene encodes a type II transmembrane protein with sequence homology to mannosidase I but lacking mannosidase function due to the absence of two cysteine residues (Hosokawa et al., 2001, Jakob et al., 2001). Edem mRNA is up-regulated by ER stress and EDEM overexpression leads to an
increased degradation rate of ERAD substrates, while down-regulation or knockout of Edem leads to stabilization of substrates in the ER (Hosokawa et al., 2001, Jakob et al., 2001). Immunoprecipitation assays showed that EDEM interacts with the transmembrane domain of calnexin and that upon release from the molecular chaperone the substrate, an α1-antitrypsin variant null Hong Kong (NHK) is transferred to EDEM for degradation (Oda et al., 2003). When associated with calnexin, the substrate is glycosylated, whereas most of the substrate associated with EDEM is non-glycosylated. This glycosylation requirement was shown by immunoprecipitation of calnexin or EDEM in animal cells expressing the pancreatic isoform of human β-secretase BACE457Δ, a type I membrane glycoprotein, followed by treatment with α mannosidase (Molinari et al., 2003).

Besides EDEM and calnexin two other soluble ER proteins have been described as being involved in the degradation of glycosylated proteins. One of those proteins is the yeast lectin-like receptor Yos9p, which is a member of the OS-9 protein family (Bhamidipati et al., 2005, Kim et al., 2005, Szathmary et al., 2005). Yos9p is involved in the degradation of lumenal but not cytosolic misfolded proteins and acts in the same pathway as EDEM (Bhamidipati et al., 2005, Kim et al., 2005, Szathmary et al., 2005). Pull-down assays with a functional Yos9p-ProteinA-His7 fusion protein expressed in cells lacking specific mannosyltransferase genes and CPY* showed that Yos9p specifically interacts with CPY* in cells that produce Man8GlcNAc2 and Man5GlcNAc2 N-glycans (Szathmary et al., 2005). The other gene was described in mouse and is called EDEM3. EDEM3, like EDEM, is a member of the alpha1,2 mannosidase family lacking two cysteine residues required for enzyme activity. Its expression is mildly up-regulated in some cell lines treated with tunicamycin. Cells co-transfected with EDEM3 and the ERAD substrate NHK showed reduced degradation rate in the presence of proteasome inhibitors (Hirao et al., 2006). It is however unclear if such reduction in the degradation rate of NHK would also be observed in the absence of EDEM3 making it hard to determine if EDEM3 in fact accelerates ER glycoprotein degradation.

Most of the progress made in ERAD substrate targeting involves glycoproteins, which led to the identification of new essential genes such as EDEM which functions as a receptor and is proposed to trap misfolded nonglycosylated proteins. Trapped substrates are prevented from continuously entering the calnexin cycle and instead are directed for degradation. It is
likely that the study of degradation of a greater variety of substrates will identify more genes involved in ERAD, highlighting even more the specificity and complexity of the pathway.

**ERAD retro-translocation pores**

Although it is not required for membrane proteins with misfolded cytosolic regions to be retro-translocated to the surface of the ER membrane, it is likely that soluble and luminal misfolded regions have to be transported to the cytosolic surface of the ER for ubiquitination and removal from the ER. The earliest candidate for functioning as a pore to allow misfolded proteins to reach the cytosol was the Sec61 complex. The Sec61 complex that is involved in the translocation of newly synthesized proteins into the ER would have its flux reversed and allow for retro-translocation of misfolded proteins from inside the ER to the cytosol. Genetic proof for the involvement of the Sec61 complex in the dislocation of substrates to the cytosol was obtained through the study of the known ERAD substrate CPY* (Plemper et al., 1997). Mutant yeast cells lacking components of the translocon such as Sec61, Sec63 and BiP exhibited a reduced degradation rate of CPY* when compared with wild type cells. Assays *in vitro* with microsomes from wild type and Sec61 yeast mutant cells showed that the unglycosylated substrate Δgpαf could be cross-linked to the Sec61 protein under conditions that limit dislocation. Under conditions that favor export and substrate degradation, Δgpαf released from the Sec61 complex in wild type but remained associated with Sec61 in mutant microsomes (Pilon et al., 1997). The Sec61 complex is not involved in the dislocation of all ERAD substrates, as is noted for the short-lived membrane protein Ubc6. The ubc6 protein is degraded by the proteasome independently of the Sec61 complex as observed by the lack of difference of the degradation rate in wild type and mutant cells (Walter et al., 2001).

In animal cells, biochemical evidence for the involvement of components of the Sec61 complex in the dislocation of ERAD targets for degradation in the cytosol was initially obtained in cells infected with cytomegalovirus (Wiertz et al., 1996). The virus encodes a protein, US2, which binds to newly synthesized MHC I and targets it for degradation. MHC I is a glycoprotein that when deglycosylated associates with US2 protein and the Sec61 beta subunit in cells treated with proteasome inhibitors. The glycosylated form of MHC I can also associate with the Sec61 complex in uninfected normal cells treated with the reducing agent DTT indicating that MHC I interacts with the translocon prior to degradation in the cytosol.
Besides the Sec61 translocon, another membrane protein has been proposed to act as a pore to allow the transport of ERAD substrates. This protein, named Der1p (yeast) or Derlin1 (animals), is located in the ER membrane and exhibits four transmembrane domains with both amino and carboxy terminal ends in the cytosol (Lilley and Ploegh, 2004, Hitt and Wolf, 2004). ERAD assays for a variety of substrates showed that yeast cells mutant for Der1p are deficient in degrading a subclass of substrates, mostly luminal misfolded targets (Taxis et al., 2003). There are three genes that encode Der1p-like (Derlin) proteins in animal cells (Lilley and Ploegh, 2004). Those genes encode proteins that have been named Derlin1, 2 and 3. As for the Sec61 complex, evidence that Derlin1 is a retro-translocation pore came from experiments with cells infected with cytomegalovirus. In virus-infected animal cells, Derlin1 is found in a complex containing the viral protein US11 and either the glycosylated or unglycosylated form of the ERAD substrate MHC I (Lilley and Ploegh, 2004). The association of Derlin1 with both classes of MHC I suggested to some that Derlin1 associates with the ERAD substrate prior and following translocation through the ER membrane (Lilley and Ploegh, 2004). Such a conclusion was also reached because Derlin1 associates with a small protein of unknown function present exclusively in vertebrates, VIMP. Immunoprecipitation assays showed that the Derlin1/VIMP complex transiently interacts with the glycosylated form of MHC I in virus-infected cells. The complex also interacts with ubiquitin modified proteins. This association with ubiquitinated proteins was shown in cells under ER stress which yielded increased amount of poly(GST-Ubiquitin) modified material associated with VIMP (Ye et al., 2004). Besides associating with substrates, Derlin1 was shown by immunoprecipitation assays to interact with other known ERAD components such as the ubiquitin ligase Hrd1 and Cdc48 AAA-ATPase and the putative homologue Derlin2 (Lilley and Ploegh, 2005, Ye et al., 2005). Interestingly, immunoprecipitation assays using solubilized dog pancreatic ER membranes showed that Derlin1 was mostly immunoprecipitated in a complex by itself suggesting that this protein can also be present independent of the complex containing the Hrd1 and Cdc48 proteins (Ye et al., 2005).

Additional immunoprecipitation assays using recombinant proteins expressed in vivo showed that Derlin1 can also be found in complexes with other proposed ERAD components such as Derlin2, the ubiquitin-binding like (UBL) domain containing proteins HERP or Ubx2, and the peptide:N-glycanase protein (Schulze et al., 2005, Schuberth and Buchberger,
2005, Katiyar et al., 2005). Immunoprecipitation assays in virus-infected cells transfected with recombinant proteins suggest that the other two mammalian Derlin proteins, Derlin2 and 3, also associate with the Cdc48 complex and form heterooligomers (Oda et al., 2006). The association of the mammalian Derlin proteins with the Cdc48 AAA-ATPase, and also of Derlin1 with polyubiquitinated substrates links the dislocation of targeted ERAD substrates to the energy dependent stage of ubiquitin tagging and removing targeted substrates for degradation.

Recombinant proteins expressing the carboxy terminus of the human Derlin1 protein were shown to interact both with the amino terminus of the peptide:N-glycanase protein as with the Cdc48 protein (Katiyar et al., 2005, Ye et al., 2005). These data in combination with the fact that Derlin1 can be immunoprecipitated independently of the ERAD complex would suggest that this protein has another function besides being a pore for retro-translocation and may serve as an adaptor protein, as suggested by Ye and co-workers (2005). Either way it will be interesting to learn if the association of Derlin1 proteins with other ERAD components such as the E3 ligase Hrd1 and the Cdc48 AAA-ATPase complex is conserved among organisms and which functions Derlin1 proteins are performing in the cell.

**ERAD substrate removal and degradation**

Degradation of misfolded ERAD substrates by the proteasome in the cytosol requires an energy dependent step for removal of the substrates from the ER membrane. A candidate for such role was the Cdc48 AAA-ATPase complex which functions in recognizing polyubiquitinated proteins and facilitates their presentation to the 26S proteasome (Ye et al., 2001). The Cdc48 AAA-ATPase complex was initially linked to ER degradation through yeast genetics where strains lacking a functional Cdc48 gene or any of its cofactors Ufd1 or Npl4 exhibit increased accumulation of the ERAD substrates MHC I and CPY* (Ye et al., 2001, Jarosch et al., 2002). Further evidence of the role of Cdc48 in removing degradation targets from the ER was obtained in animal cells expressing the US11 viral protein. Cells expressing the wild type Cdc48/p97 had little effect on the retro-translocation of MHC I, while cells expressing the mutant Cdc48/p97 showed strong inhibition of export of the substrate but no effect on the total amount of ubiquitinated molecules (Ye et al., 2001). The use of dominant negative forms of Cdc48/p97 that recognize the substrate but are deficient in
ATP hydrolysis inhibited the dislocation of MHC I to the cytosol and increased the amount of deglycosylated MHC I molecules associated with the ER membrane (Ye et al., 2003). An increase in the accumulation of polyubiquitinated MHC I chains in the ER membrane was also observed in the presence of a dominant negative mutant of Ufd1, a construct lacking the UT3 ubiquitin binding domain. This was not observed in the presence of a dominant negative mutant for the ubiquitin binding domain of Npl4 (ZFF) (Ye et al., 2003). Together these data indicate that both the ubiquitin binding domain from Ufd1 and the ATPase domain from Cdc48/p97 are important for the release of polyubiquitinated substrates into the cytosol for further degradation by the proteasome.

The final step in the degradation of ERAD substrates is their degradation by the proteasome. The experiments performed to determine the proteasome involvement in ERAD took advantage of yeast genetics. Yeast cells lacking genes that encoded subunits of the proteasome showed stabilization of the ERAD substrates indicating the requirement of this component for ERAD (Hiller et al., 1996, Jarosh et al., 2002). Even though the proteasome is widely involved in degrading proteins in a variety of cellular pathways, biochemical data for its function on ERAD is still missing.

Perspectives in the ERAD field

The field of ER associated degradation rapidly progressed in the last two decades. Most of the progress was due to the research of yeast geneticists who vigorously searched for mutants that abrogate the degradation of ER misfolded substrates, and often cause genetic diseases. Although several steps of the pathway have been identified through the use of mutant yeast cells, it appears that only the surface of this intricate pathway has been uncovered.

A still open question both in ERAD and in protein folding research is how target proteins are recognized and what the domains are that trigger their degradation or their folding. It is possible that additional components yet unidentified are involved in the selection for the folding or degradation route, as was the case for some of the ERAD components involved in the degradation of glycosylated proteins. It is also possible that additional ERAD components are yet to be identified or simply that some of those ERAD
components are specific to higher organisms and will not be identified through genetic mutations in yeast.

Adding to the unknowns about ERAD is the lack of research in plants. The study of ERAD has been neglected despite the fact that production of transgenic plants often requires the expression of heterologous proteins that could be targeted for ERAD if there is an imbalance in subunits or if the folding machinery does not recognize the domains for proper folding. Some effort has been put into identifying ERAD components in plants. Work with the mildew resistance O (MLO) protein from barley in Arabidopsis cells suggest that the ERAD pathway is conserved in plants. Point mutation isoforms of the MLO protein were degraded in Arabidopsis but accumulated in the presence of proteasome inhibitors or in the presence of a dominant negative mutant of the Arabidopsis Cdc48 homologue (Muller et al., 2005).

**Thesis Plan**

The major emphasis of this thesis concerns the characterization of putative homologues of components of ERAD pathway, ZmDerlins, in maize seeds. Using clones and antibodies provided by D.J. Meyer at Pioneer Hi-Bred International, A DuPont Company, I characterized the accumulation of the ZmDerlin proteins in maize. Also, access to the Pioneer Hi-Bred sequence database allowed us to identify four genes that encode the two classes of ZmDerlin proteins. I also characterized the ZmDerlin genes in response to ER stress caused by the synthesis of abnormal storage proteins in maize seeds. Out of the four genes that encode the ZmDerlin proteins, three are up-regulated in the endosperm mutants when compared to the normal isogenic line, W64A. In Chapter 2 we were able to correlate the expression and accumulation of ZmDerlin1 genes with the presence of ER stress in seeds and also determine that ZmDerlin1 and ZmDerlin2 proteins can functionally complement the yeast Der1 protein. This chapter was published in Plant Physiology (Kirst et al., 2005).

Chapter 3 is an overview of ERAD proteins that have putative counterparts in plants. To determine which putative ERAD components were present in plants I performed BLAST searches using yeast and/or human sequences against the Arabidopsis, rice and poplar genomes. I identified several putative counterparts for the yeast and/or human ERAD components in plants. However, the current plant genome databases do not contain predicted
sequences for all ERAD proteins. The lack of predicted sequences thus suggests differences in ERAD machinery in plants.

In Chapter 4 I extended the analysis of ZmDerlin proteins into other tissues and stages of seed development. My findings indicate that ZmDerlin1 and ZmDerlin2 proteins are not always present in the same tissues. Also, the use of different stringency buffers suggests different physical properties between ZmDerlin1 and ZmDerlin2.

Reference


IDENTIFICATION AND CHARACTERIZATION OF ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION PROTEINS DIFFERENTIALLY AFFECTED BY ENDOPLASMIC RETICULUM STRESS

Mariana E Kirst, David J Meyer, Bryan C Gibbon, Rudolf Jung and Rebecca S Boston

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Identification and Characterization of Endoplasmic Reticulum-Associated Degradation Proteins Differentially Affected by Endoplasmic Reticulum Stress

Mariana E. Kirst, David J. Meyer, Bryan C. Gibbon, Rudolf Jung, and Rebecca S. Boston

Department of Botany, North Carolina State University, Raleigh, North Carolina 27695–7612 (M.E.K., R.S.B.); Pioneer Hi-Bred International, Incorporated, a DuPont Company, Johnston, Iowa 50131 (D.J.M., R.J.); and Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721 (B.C.G.)

The disposal of misfolded proteins from the lumen of the endoplasmic reticulum (ER) is one of the quality control mechanisms present in the protein secretory pathway. Through ER-associated degradation, misfolded substrates are targeted to the cytosol where they are degraded by the proteasome. We have identified four maize (Zea mays) Derl-like genes (Zm Derlins) that encode homologs of Der1p, a yeast (Saccharomyces cerevisiae) protein implicated in ER-associated degradation. Zm Derlins are capable of functionally complementing a yeast Der1 deletion mutant. Such complementation indicates that the Der1p function is conserved among species. Zm Der1 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 Der1 genes increases during ER stress, with Zm Derlin-1 showing the strongest induction. Subcellular fractionation experiments localized Zm Der1 proteins to the membrane fraction of microsomes. In maize endosperm, Zm Derl proteins were found primarily associated with ER-derived protein bodies regardless of the presence of an ER stress response.

The endoplasmic reticulum (ER) serves as a versatile gatekeeper of the secretory pathway. It is not only the entry point for translocation of newly synthesized proteins, but also the site of quality control processes that discriminate between conformationally correct proteins in a native state and those that are terminally misfolded. In the latter case, continued protein accumulation could be toxic to the organism, possibly impairing its development. To cope with the accumulation of misfolded proteins and restore homeostasis, the cell initiates an ER stress response that has been linked to development of ER-rich tissues, up-regulation of molecular chaperones, selective translational attenuation, and activation of an ER-associated degradation (ERAD) process (Zhang and Kaufman, 2004). ERAD ensures that aberrant proteins do not transit through the secretory pathway. Instead, misfolded proteins are targeted for removal from this pathway by retrotranslocation through the ER membrane to the cytosol where they undergo ubiquitination and degradation by the proteasome (Brodsky and McCracken, 1999; Plummer and Wolf, 1999; McCracken and Brodsky, 2003).

Attempts to characterize the ERAD process have allowed identification of several components of the ERAD machinery, some of which are co-opted from their classical roles in importing proteins to additional functions in protein export. Genetic studies are consistent with a model whereby molecular chaperones could present ERAD substrates to the Sec61 complex (Nishikawa et al., 2001; Molinari et al., 2002). Upon association of ERAD components, the Sec61 complex would form a channel for retrotranslocation of the misfolded proteins (Plummer et al., 1999). Proteins emerging from the ER would be ubiquitinated by the combined action of ubiquitin-conjugating enzymes (ubc6, ubc1, and/or ubc7) and the ubiquitin-ligase complex Hrd1p/Der3. The ubiquitinated substrates could then be recognized by the Cdc48 AAA-ATPase complex and removed from the ER membrane for degradation by the proteasome (Ye et al., 2001; Jarosh et al., 2002; Muller et al., 2005).

In plants, little is known about the degradation mechanisms of the ER. A link between ER stress and ERAD was observed in a genome-wide analysis of Arabidopsis (Arabidopsis thaliana) cells treated with pharmacological agents to induce ER stress (Martinez and Chispeels, 2003). Use of ricin A as a reporter for degradation in transformed tobacco (Nicotiana tabacum) protoplasts revealed cytosolic accumulation after treatment with a proteasome inhibitor (di Cola et al., 2001). Expression of a dominant-negative form of the
AAA-ATPase Cdc48 in Arabidopsis plants stabilized the misfolded wheat protein, MLO-1, a finding consistent with this protein being a substrate for ERAD (Müller et al., 2005). In an effort to identify changes in gene expression that were associated with the ER stress response in seeds, we undertook an open-ended RNA-profiling study. This study took advantage of three maize (Zea mays) endosperm mutants that provide easily assayed models. The three mutants, feathery-2 (fl2), mucronate (Mc), and defective endosperm B30 (De*-B30) exhibit ER stress responses that are specific to the endosperm and coincident with the deposition of defective storage proteins in the seed (Boston et al., 1991; Coleman et al., 1995; Kim et al., 2004). The endosperm mutants exhibit different degrees of ER stress, depending on the deposition of defective storage proteins. The strongest phenotype is observed in the fl2 mutant, which produces a 24-kD a-zein that is blocked for cleavage of the signal peptide. As a result, the defective storage protein accumulates as a membrane-anchored protein in the ER and in ER-derived protein bodies (Coleman et al., 1995; Gillikin et al., 1997). The accumulation of this terminally misfolded protein causes an ER stress that leads to an increase in the accumulation of molecular chaperones such as binding protein (BiP). Mc and De*-B30 also have defective storage proteins (Kim et al., 2004). Mc is caused by a frameshift mutation (R. Jung and R.S. Boston, unpublished data), while De*-B30 is another zein signal peptide-processing mutant. Each of the mutations also leads to a decrease in the amount of the mutant protein along with other storage proteins in the seed (Soave and Salamini, 1984). This decrease, together with the presence of ER stress, is consistent with an attenuation of protein synthesis as a result of the ER stress response and degradation of the defective proteins by the ERAD pathway.

We hypothesized that the profiling of maize endosperm mutants would uncover changes not only associated with molecular chaperone gene expression but also associated with vesicle trafficking and ERAD. This was indeed the case as numerous genes showed changes in mRNA accumulation. Two of these were particularly interesting to us as they both showed homology to Der1p, a protein linked to ERAD almost 10 years ago but still lacking a functional characterization (Knop et al., 1996).

In yeast (Saccharomyces cerevisiae), Der1p has been implicated in ERAD by mutant analysis in which a Der1 deletion mutant accumulated a misfolded reporter protein in the lumen of the ER (Knop et al., 1996; Hill and Cooper, 2000; Taxis et al., 2003). A much more dramatic effect was seen in double mutants lacking both Der1p and Ire1p, the protein kinase/endonuclease responsible for signaling and transducing ER stress, as the double mutation was lethal at restrictive temperatures (Travers et al., 2000). Recently, homologs for the yeast Der1 protein have been identified in other organisms, including humans (Hitt and Wolf, 2004; Lilley and Ploegh, 2004; Ye et al., 2004). These proteins have been designated as Derlins (for Der1-like). Attempts to identify Der1p and Derlin activities to date have failed to provide a uniform picture with reports of involvement in degradation of soluble proteins in yeast and membrane reporter proteins in mammalian cells (Knop et al., 1996; Taxis et al., 2002, 2003; Lilley and Ploegh, 2004). Derlins have been linked to other ERAD-associated proteins by protein-protein interaction studies, but the mechanisms by which they contribute to the ERAD function remain unknown (Ito et al., 2001; Girot et al., 2003; Li et al., 2004; Ye et al., 2004). Here, we report the discovery of four Derlin genes in maize and provide a functional characterization of the gene family.

**RESULTS**

**Discovery of Maize Derlin Genes by RNA Profiling**

To identify genes associated with the ER stress response, we performed a high-throughput, open-architecture transcript-profiling assay (GeneCalling; Crete and Folkerts, 2003) with immature endosperm samples from the corn inbred line W64A (+) and its near-isogenic conversions Mc, fl2, and De*-B30 (Hunter et al., 2002). GeneCalling analysis measures, in a highly reproducible manner, differences in the abundance of individual cDNA fragments. In general, these differences are directly related to differences in the level of expression of specific transcripts (Shinkets et al., 1999; Bruce et al., 2000). Using 64 restriction enzyme pairs, we detected about 20,000 cDNA fragments from this analysis. From a comparison of electrophoretically obtained traces corresponding to cDNA fragments, we identified about 200 cDNA fragments that consistently showed differences between samples from the normal and mutant lines. The majority of these fragments were identified by either (1) competitive PCR confirmations (trace poisoning) based on their match to the Pioneer-DuPont corn gene database; or (2) cloning and sequencing. A subset of the identified differentially expressed genes matched genes that have previously been implicated in ER stress in other organisms (the comprehensive analysis is still in progress; B.C. Gibbon, R. Jung, and R.S. Boston, unpublished data). Two of the putatively up-regulated genes (Fig. 1) were of particular interest because they were members of the same gene family. One was nearly identical to, and the other a potential homolog of, a corn gene (GenBank accession no. CAB97035) that is annotated in public databases as NADH oxidoreductase. We found this annotation to be an error that probably originated because of its match to a Caenorhabditis elegans six-gene operon that, along with a homolog CAB97005, contained the coding sequence of an NADH oxidoreductase (Hough et al., 1999). BLAST searches with the deduced amino acid sequences from the two up-regulated transcripts indicated a clear homology to the yeast Derl protein, and we therefore refer to them as Zm Derlins.
hypothesize that the function, although unknown, of the Der1 and Zm Derlin proteins would also be conserved. This hypothesis was further supported by reports that appeared during the course of our study describing three mammalian Derlin proteins (Li and Ploegh, 2004; Ye et al., 2004).

Plant Derlin Genes Are Grouped into Two Subfamilies

BLAST searches of the Pioneer-DuPont gene databases identified two additional Derlin genes in corn and a search of the Arabidopsis and rice whole-genome databases identified three Arabidopsis Derlin genes and two rice Derlin genes (Table I). The plant Derlin genes fell into two groups, which we call Derlin1 and Derlin2, with deduced amino acid sequences having approximately 30% overall identity between subfamilies and >90% overall identity within each subfamily. The maize Derlin genes were mapped by RFLP mapping and each pair of paralogous sequences mapped to the same map position (Table I). Zm Derlin1-1 and Zm Derlin1-2 mapped to chromosome 8 (approximately 120 cM on the IBM map) and Zm Derlin2-1 and Zm Derlin2-2 both mapped to chromosome 1 (approximately 7 cM on the IBM map). Because of the indistinguishable map positions and sequence similarity between paralogous, we searched the maize genome survey sequences (GSS) section of GenBank and assembled sequences with identical nucleotide (nt) overlaps into GSS contigs (data not shown). GSS contigs that originated from the same maize inbred line B73 were identified with near-perfect matches to each of the four Zm Derlin cDNAs, thus verifying that they originated from distinct genes and not from alleles (Table I).

Table I. Putative Derlin homologs from maize, rice, and Arabidopsis

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<td>At Derlin-4</td>
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<td>Atg10360</td>
</tr>
</tbody>
</table>

*Full-length cDNA; related sequences reported in GenBank (accession no. CA87005) is likely a chimera between Zm Derlin-1 and Zm Derlin-3.  
*No full-length cDNA sequence available; predicted coding sequence (accession no. CMR000000346) 19 for the corresponding gene in Gramene (http://www.gramene.org) is probably incorrect.  
*Full-length cDNA; other predicted coding sequences for Atg29330, Atg21810, and Atg10360 in GenBank are probably incorrect.  
*Genbank accession numbers for Zm Derlin GSS contigs are Zm Derlin-1, AY854017; Zm Derlin-2, AY854018; Zm Derlin-2, AY854019; and Zm Derlin-2, AY854020.
Derlin Proteins Are Conserved across Kingdoms

Figure 2 shows an alignment of the deduced amino acid sequences of Zm Derlin1-1 and Zm Derlin2-1 as prototypes of the plant Derlins, along with Derl1p from yeast, and Derlin-1 and Derlin-2 from human and C. elegans. The maize proteins were similar in size to those of the other organisms, with the exception of Derl1p, which had only 211 amino acids. The plant Derlin1 and Derlin2 sequences each showed about 25% to 30% identity to Derlin proteins of the other species. The plant Derlin2 sequences, however, were distinguished by an 1-amino acid region that failed to align with sequences from other organisms. Despite the low overall identity observed among the aligned proteins, there was a clear overall homology with some amino acids being conserved across the entire group (Fig. 2). Analysis of other protein features in silico revealed similarities that are perhaps more important than the sequence conservation. For example, all Derlins, including the plant proteins, contained a domain that showed distant similarity to the Rhomboïd domain of intramembrane proteases (plam01694), and hydrophobicity analysis with the program TMHMM (Krog et al., 2001) predicted four and five transmembrane domains for Zm Derlin1 and Zm Derlin2, respectively (data not shown). Multiple transmembrane domains were also predicted for yeast Derl1p (underlined in Fig. 2) and for the mammalian Derlin proteins (Knop et al., 1996; Taxis et al., 2003; Hitt and Wolf, 2004; Lilley and Ploeg, 2004).

Zm Derlin Proteins Are Integral Membrane Proteins

We tested the predicted membrane association of the maize Zm Derlin proteins by immunoblot analysis of subcellular fractions from the fl2 endosperm mutant. Antibodies were raised against peptides from the deduced amino acid sequence of Zm Derlin1-1 in regions outside the predicted membrane-spanning regions (wavy lines in Fig. 2). Because of the high sequence homology between Zm Derlin1-1 and Zm Derlin2-1, the antibodies cross-reacted with both; however, they did not cross-react with Zm Derlin2 proteins. To evaluate the predicted integral membrane localization of Zm Derlin1 proteins, we performed alkaline fractionation and evaluated detergent extraction of microsomal samples from immature endosperm. The microsomal fraction did not include the protein bodies whose high content of insoluble zeins blocks efficient removal of luminal contents after alkaline lysis (I. Gillikin and R.S. Boston, unpublished data; in this work, we use the terms cisternal ER and protein bodies as operational designations for the endosperm fractions containing light and heavy ER subdomains, respectively). Figure 3A shows fractions probed by immunoblot analysis for Zm Derlin1 proteins, the ER luminal molecular chaperone calreticulin, and the related membrane protein calnexin. A Zm Derlin1 signal was detected in the microsomal membrane fraction but was undetectable in the soluble fraction. Alkaline treatment of microsomes followed by centrifugation to separate membrane and soluble luminal fractions showed little or no Zm Derlin1 in the soluble fraction,
Zm Derlin1 signals on immunoblots, but had no apparent effect on the calreticulin or calnexin signals.

To verify that the association of Zm Derlin1 with fractions of endomembranes reflected their localization in living cells, we examined their transient expression. Green fluorescent protein fusion constructs of Zm Derlin1 and Zm Derlin2-1 directed by a ubiquitin promoter were introduced into maize callus tissues by microprojectile bombardment. Fluorescence from both constructs was detected in a perinuclear and reticulate pattern around cells and was absent from the nucleus or plasma membrane (D.J. Meyer and W.J. Gordon-Kamm, unpublished data). This pattern was indicative of ER localization and was consistent with the pattern of Derlin-1 localization observed in animal cells (Lilley and Flogh, 2004).

Zm Derlin1-1 and Zm Derlin2-1 Genes Complement the Yeast Der1 Phenotype

To determine whether or not the Zm Derlin1-1 and Zm Derlin2-1 proteins have a conserved function between species, we performed a yeast complementation assay. The recipient strain harbored a double mutation for Der1 and Ire1, the kinase/endonuclease sensor responsible for initiating signal transduction of ER stress (Mori et al., 1993; Travers et al., 2000). The double mutant offers an easily-scored selection as it is viable at 25°C, but has a temperature-sensitive phenotype, rendering it unable to grow at 37°C (mutants of Der1 alone, in contrast, have no growth phenotype and must be assayed biochemically). If the Zm Derlin genes complemented the phenotype, we would expect to see growth at 37°C. Strains were constructed by transformation with the open reading frames (ORFs) of maize Zm Derlin1-1, Zm Derlin2-1, and Sc Der1 in expression plasmids with selectable markers. Individual transformants were grown overnight, inoculated onto complete media plates in 10-fold serial dilutions, and incubated at either 25°C or 37°C for 4 d. Strains containing either Zm Derlin1-1 or Zm Derlin2-1 sequences grew well even under the restrictive condi-

![Figure 3](image1.png)

Figure 3. Immunoblots showing association of Zm Derlin1 with microsomal membranes. Approximately equal fresh weight equivalents of endosperm (3 mg) were separated by SDS-PAGE (15%) prior to transfer and probing with antibodies against Zm Derlin1 (ZmD1), calreticulin/calnexin (CRT, CNX). A, Localization after alkaline lysis. A microsomal fraction from fl2 endosperm (SM) was further separated into membrane (P) and luminal (S) fractions by alkaline lysis and differential centrifugation. B, Localization after detergent treatments. A fl2 microsomal fraction (SM) was treated with the detergents shown above lanes and separated by centrifugation into soluble (S) and insoluble fractions (P). DOC, deoxycholate; NP-40, Nonidet P-40.

![Figure 4](image2.png)

Figure 4. Complementation of a yeast Ire1/Der1 double mutant with Zm Derlin1-1 (ZmD1-1) and Zm Derlin2-1 (ZmD2-1). Cells were grown to approximately OD600 = 2 (approximately 1 × 10^6 cells = 1 OD600), serially diluted in 10-fold increments (as shown from left to right) and inoculated onto solid media. Replica plates were incubated for 4 d at 25°C (permissive temperature) or 37°C (restrictive temperature). ScD1 shows complementation of the double mutant by the yeast Der1 gene.

as expected for an integral membrane localization (Fujiki et al., 1982; Shatters and Miernyk, 1991).

We further characterized the membrane association of Zm Derlin1 by subjecting microsomal samples to centrifugation after treatment with various detergents. Figure 3B shows an immunoblot analysis of the solubilization of Zm Derlin1 in comparison to calreticulin and calnexin controls. Deoxycholate, Nonidet P-40, and Triton X-100 all promoted nearly complete release of the calreticulin and calnexin marker proteins from the membrane fraction. In contrast, a portion of Zm Derlin1 remained in the pellet fraction after treatment with lower concentrations of the detergents. At high detergent concentrations, little Zm Derlin1 was visible by immunodetection in either fraction. Digitonin treatment produced a different result, with calreticulin and calnexin being detected in both fractions. Zm Derlin1 partitioned with the soluble fraction at low digitonin concentrations, but was found in both fractions when the detergent concentration was raised. In general, higher detergent concentrations led to weaker
tions (Fig 4), whereas cells containing an empty plasmid showed only minimal growth. Cells containing the yeast Der1 sequence (Sc Der1) grew well at 37°C as expected for complementation by the endogenous gene.

Zm Derlin mRNAs Are Expressed throughout the Plant

The functional data provided by the yeast complementation experiment led us to predict that the Zm Derlin proteins were involved in ERAD in maize not only during ER stress, but also under nonstress conditions. Because ERAD would be needed for eliminating misfolded proteins in most, if not all, cell types, we surveyed the transcription profiles of the four Zm Derlin genes from a variety of organs, tissues, and developmental stages of the maize inbred B73 by multiple parallel signature sequencing (MPSS; Brenner et al., 2000). During MPSS, more than 1 million 17-nt-long expressed sequence tags (ESTs) from each tissue are obtained, and this allows direct quantitative comparisons of mRNA abundance within and among different tissue samples (Meyers et al., 2004b). Table II shows the abundance of MPSS ESTs in representative tissue samples from a comprehensive gene expression data set of several hundred libraries. Zm Derlin-1, Zm Derlin-2a, and Zm Derlin-2b were expressed throughout the plant, although their relative levels differed with the tissue analyzed. Zm Derlin-1 mRNA was more abundant than transcripts of the other three Zm Derlins. Zm Derlin-2 transcripts were detected only in the endosperm and root tissues and generally were the least prevalent of the Zm Derlin genes. At 12 days after pollination (DAP), abundance of Zm Derlin-1 and 2-1 transcripts was reduced compared to other developmental stages. However, this combined decrease in expression was not observed at the protein level (data not shown).

Expression of Zm Derlin Genes Is Differentially Induced during ER Stress

We expanded our expression analysis of the Zm Derlin genes to compare their expression during ER stress. Semiquantitative reverse transcription (RT)-PCR and quantitative RT-PCR were carried out under nonsaturating conditions, with RNA isolated from developing endosperm tissue. Tissue was collected from the inbred line W64A (+), the near-isogenic ER-stress mutants f2, Mc, and De-350, and the endosperm opacity mutant opaque-2 (o2), which lacks a zein transcription factor but does not show an ER stress response (Schmidt et al., 1987; Boston et al., 1991). As shown in Figure 5A, the signal for the Zm Derlin-1 gene was dramatically increased in the maize mutants that exhibited an ER stress response compared to the normal line and was not increased in the o2 mutant. The signals of the other three Zm Derlin genes were weaker and showed only marginal changes across samples under our assay conditions. As an amplification and loading control, we used a maize catalase gene that is uniformly expressed during endosperm development (Redinbaugh et al., 1988; Basu et al., 1994).

To confirm that induction of Zm Derlin-1 expression was specific for ER stress, we compared endosperm and embryo tissues from normal and f2 lines. Because embryos do not synthesize zeins, they do not make mutant (or normal) zein storage proteins and thus do not exhibit the ER stress response seen in mutant endosperm. As shown in Figure 5B, only the endosperm sample from the f2 mutant showed an obvious increase in Zm Derlin-1 expression. The signal difference for the Zm Derlin-2 amplification product appeared insignificant between embryo and endosperm tissues of the f2 mutant, although perhaps increased compared to the normal endosperm sample.

To gain better resolution of the expression profiles of the Zm Derlin genes during ER stress, we performed real-time quantitative PCR (qPCR) assays of RNA from endosperm (Fig. 5C). qPCR allows accurate and real-time measurement of an amplified PCR product in independent samples (for review, see Gachon et al., 2004). Figure 5C shows a comparison of mRNA levels for the Zm Derlin genes normalized to 28S rRNA and calibrated against the normalized wild-type control. Zm Derlin-1 showed strong induction and high expression levels in the three mutants associated with ER stress and a slight induction in the o2 mutant. Zm Derlin-2 showed a qualitatively similar profile, except that ER stress induction was less pronounced. Expression of the Zm Derlin-2 genes did not correlate strictly with ER stress. Zm Derlin-2 expression was higher in all the mutants than in the normal control, while Zm Derlin 2-2 expression was relatively constant across all of the samples.

The differences in Zm Derlin expression profiles led us to search maize GSS contigs that extended into the 5' region of the Zm Derlin cDNAs (Table I) for upstream elements associated with ER stress (Roy and Lee, 1999; Kokame et al., 2001; Okada et al., 2002; Martinez and Chrispeels, 2003). This examination revealed two overlapping cis-acting elements in Zm Derlin-1 (CCACGTGTA and [CC][C]CtatecaggC-
that had strong homology to motifs 1 (CCACGCTNA) and 2 (CCCTCCACCG) previously identified on the coding strand beginning 266 nt upstream of the ORF in some Arabidopsis genes induced by ER stress (Martinez and Chrispeels, 2008). These elements, as well as one in the single Arabidopsis Derlin1 gene (Table I), share the common CCACG core sequence present in the ERSE-I and ERSE-II elements of induced mammalian genes (Roy and Lee, 1999; Kokame et al., 2001). The Arabidopsis sequence, however, is present in the reverse orientation 190 nt upstream of the ORF of At Derlin1-1. A sequence (CCaattcaaatCCACG) similar to the Arabidopsis motif 2 was present 1,910 nt upstream of the Zm Derlin2-1 ORF, but we were unable to find sequences resembling any of the ER stress elements upstream of the remaining two Zm Derlin genes (M.E. Kirst, unpublished data). Similarly, we found only
weak homology to a mammalian element 648 nt from the ORF of the At Derlin-2-1 gene and no cis-acting element in the At Derlin-2-2 sequence.

The RNA expression data and virtual promoter analysis suggested that the Zm Derlin1 genes were linked specifically to a function in the ER stress response. We extended our analysis to include protein accumulation for the Zm Derlins as judged by immunoblotting. We obtained antiserum against a Zm Derlin2-1 peptide (shared with Zm Derlin-2-2) and located within a domain predicted to be in the lumen of the ER. The peptide had 52% identity to the corresponding peptide in the Zm Derlin1 proteins (Fig. 2, wavy line above Zm Derlin2-1) and the antibody did not cross-react with Zm Derlin1. Figure 6 shows immunoblots probed for the Zm Derlins. Accumulation of Zm Derlin1 proteins reflected the pattern observed for mRNA with a strong up-regulation in the mutants associated with ER stress. The o2 mutant showed a slight increase of the immunoblot signal over the normal line even though both were much lower than in the mutants linked to ER stress. For Zm Derlin2, the relative signal strengths were reversed, with o2 being strongest, followed by the normal line, and then the mutants associated with ER stress having the weakest signals. We detected a strong induction of the well-characterized molecular chaperone, BiP, in endosperm of the ER stress mutants (Fig. 6), as shown in previous studies (Boston et al., 1991; Fontes et al., 1991; Marocco et al., 1991). Thus, on the protein level, Zm Derlin1 accumulation appears induced by the ER stress response, whereas Zm Derlin2 (at least the portion or polypeptide conformation that can be detected with the antibody) appears to decrease.

Zm Derlin1 and Zm Derlin2 Proteins Associate with Protein Bodies

In the mutants associated with ER stress, molecular chaperones have been found in association with protein bodies, which are the predominant location of the mutant zeins (Galante et al., 1983; Zhang and Boston, 1992). To determine whether Zm Derlins similarly localized preferentially to protein bodies in endosperm of ER stress mutants, we separated ER intc cisternal and protein body fractions (Gillikin et al., 1995). Figure 7 shows proteins from W64A+ and W64A/o2 endosperm loaded by equivalent fresh weights and probed for Zm Derlin1 or Zm Derlin2 by immunoblot analysis. Both Zm Derlin1 and Zm Derlin2 were detected primarily in the protein body fractions from the normal or o2 samples. Consistent with the data in Figure 6, we detected more Zm Derlin1 in the mutant, whereas Zm Derlin2 protein appeared lower in the mutant sample when compared to the protein body fraction isolated from normal endosperm. Calnexin and calreticulin controls are shown as references for the distribution of known molecular chaperones between membrane and luminal fractions of the two organelles, respectively.

In the experiment shown in Figure 7, the Zm Derlin1 signal was detected as a doublet. We frequently detected the migration of Zm Derlin1 bands as a doublet that can occur in both normal and mutant endosperm (e.g., sample from the normal line in Fig. 6). The doublet is unlikely the result of proteolytic processing of the protein as the smaller of the two bands is detected by antibodies against the NH-cap region shared between Zm Derlin1-1 and Zm Derlin1-2 (Fig. 2, wavy lines), as well as by an antibody developed against a COOH-terminal peptide (PPANGNCSGVFGRGSRRLN) of Zm Derlin1-1 (M.E. Kist and R.S. Boston, unpublished data).

DISCUSSION

The data presented here indicate that proteins associated with the ERAD machinery in yeast and animals are conserved in plants. Among putatively up-regulated genes in endosperm of maize ER stress
mutants, we detected two homologs of a yeast Der1 gene. This finding is of interest because, although very little is known about the ERAD pathway in plants, mammalian Derlins and yeast Der1p have been directly implicated in the retrotranslocation of misfolded proteins from the ER lumen into the cytosol (Lilley and Ploegh, 2004). Searches of gene databases identified two additional maize Derlin genes as well as multiple Derlin homologs in other plant species (Table 1). The four maize genes are members of two Derlin gene subfamilies, Derlin1 and Derlin2, each represented by two nearly identical maize paralogs. Based on RFLP mapping, we placed both Zm Derlin1 paralogs at the same locus on chromosome 8. Similarly, both Zm Derlin2 paralogs mapped to one locus on chromosome 1. The emergence of these two pairs of paralogs is therefore consistent with recent tandem gene duplications rather than duplications resulting from the allotetraploid event that occurred 5 to 10 million years ago in the progenitor of maize (Helenjaris et al., 1988; Gaut and Doebley, 1997; Svinganova et al., 2004). The putative Derlin proteins from other plant species, including mosses, gymnosperms, and eudicots, group in phylogenetic alignments in deeply rooted clades either with the Zm Derlin1 proteins or with the Zm Derlin2 proteins (R. Jung, unpublished data). Interestingly, Derlin proteins from animals also fall into two deeply rooted protein subfamilies. Moreover, this clustering extends beyond the plant kingdom, although the posterior probability values are much lower. In alignments of plant Derlins with animal Derlins (Lilley and Ploegh, 2004), one group of animal Derlins (e.g., Hs Derlin-1) appears to cluster with the plant Derlin1 subfamily, and the other group of animal Derlins (e.g., Hs Derlin-2 and Hs Derlin-3) appears to group with the plant Derlin2 proteins. In contrast, the single yeast Derlin protein, Der1p, has similar overall identity (approximately 15%) to both plant Derlin subfamilies. Together, these data are indicative of the emergence of the two Derlin gene subfamilies prior to the development of vascular plants and perhaps even earlier, during eukaryotic evolution, predating the split of plants and animals. The maintenance of two diverged Derlin protein subfamilies during evolution strongly suggests that, in addition to a core Derlin function, each subfamily has a nonredundant and important, if not essential, biological activity.

Zm Derlin1-1 and Zm Derlin2-1 were able to suppress the mutant phenotype of a yeast strain lacking Der1 and the capacity to sense ER stress (Aire1). This complementation confirmed that the heterologous maize proteins possess characteristics essential for the core function of a Derlin protein in yeast. At 37°C, the yeast growth was less robust than at 25°C. This difference, however, was observed for both the yeast and Zm Derlin transformants. A similar result was observed for the C. elegans Derlin gene (GenBank accession no. NM_056189), which reestablished growth of the yeast mutant in a complementation experiment (Hitt and Wolf, 2004). The C. elegans Derlin also partially restored the capacity of the yeast mutant to degrade a soluble misfolded substrate, thus confirming conservation of the ERAD function across widely diverged species.

The plant Derlin proteins have diverged considerably from the yeast Der1 protein (approximately 15% amino acid identity) during evolution. However, both Zm Derlin1-1 and Zm Derlin2-1 have a conserved Ser residue, suggested by Hitt and Wolf (2004) to be essential for Der1p function. Whether this residue is, in fact, important in preserving Der1 activity among species remains to be investigated. Because the maize proteins have little sequence homology to the yeast Der1p and members of both subfamilies complement the yeast mutant, members of both plant Derlin families may be considered orthologs of yeast Der1p.

The induction of Zm Derlin1-1 and Zm Derlin1-2 mRNAs in the f2, Mc, and De-B30 endosperm mutants (Fig. 5) was consistent with previous reports of expression of putative ERAD genes being upregulated during pharmacologically induced ER stress (Travers et al., 2000; Martinez and Chriseps, 2003). ER-stress induction has been linked to the presence of sequence motifs that can be found in either orientation upstream of many, but not all, target genes (Roy and Lee, 1999; Kokame et al., 2001; Martinez and Chriseps, 2003). Zm Derlin1-1, which shows a strong ER-stress response, has multiple cis-acting elements located in close proximity to the coding region. In contrast, Zm Derlin1-2 had no recognizable ER stress elements, even though it shows a similar, albeit attenuated, induction as Zm Derlin1-1. Zm Derlin2-1, which showed weak up-regulation during ER stress as well as in the o2 mutant (Figs. 1 and 5), has a single cis-acting element located 1,910 nt upstream of the protein coding region, while Zm Derlin2-2 lacks any recognizable ER-stress motifs. The upstream regions of Zm Derlin homologs in Arabidopsis show similar features, with the apparent ortholog of Zm Derlin1 having a more proximal element than the apparent orthologs of Zm Derlin2 (only one of which has an element). These putative regulatory features, along with the transcript and protein accumulation data, suggest that Zm Derlin1-1 and Zm Derlin1-2 have biological roles in maintaining and restoring ER homeostasis after perturbation of the protein secretory pathway. Zm Derlin2-1 may also participate in the ER stress response; however, its role would appear to be more general as it was also induced in the o2 mutant (Fig. 5C). Such a situation has precedent in the well-studied stress 70 family of molecular chaperones whose members show differential responses to stress. Hsp70 proteins are induced by heat stress, whereas BiPs are induced by ER stress, and some of the other Hsc70 members appear to be needed during normal growth and development (Guy and Li, 1998; Lin et al., 2001). Hsp70 expression is regulated by the heat stress transcription factors that bind to cis-acting elements, whereas elements upstream of BiP are recognized by different factors (Yoshida et al., 1998; Foti et al., 1999; Firkkala et al., 2001).
The induction of the Zm Derlin1-1 gene by ER stress led to increases in both RNA and protein. The defective zeins in the ER stress mutants are clearly responsible for initiating the induction of the ER stress response and they may also be the targets of Derlin-mediated degradation. Normal zeins have no Lys residues and thus lack the epsilon amino groups that serve as ubiquitin conjugation sites. Each of the mutations, however, leaves the affected zein with a Lys residue. The new Lys in the f2 and De*-B30 mutants lies in the uncleaved signal peptide at the NH₂ terminus, while the one in the Mc mutant lies very near the COOH terminus (J. Gillikin, R. Jung, and R.S. Boston, unpublished data). Mc has a dramatically lower accumulation of the 16-kD γ-zein and f2 has less of the 22-kD α-zein when compared to a normal maize line or other endosperm mutants (Hunter et al., 2002). The possible decrease of the 19-kD α-zein in the De*-B30 mutant cannot easily be assessed because the corresponding gene accounts for only 4% of the transcripts encoding members of the large 19-kD α-zein family. As a result, the protein encoded by the normal allele would be masked by the more abundant protein family members. In animals, ER stress can lead to an inhibition of translation mediated through the transmembrane kinase, PERK (for review, see Liu and Kaufman, 2003). Thus, it may be possible that the lower amounts of defective zeins are due to selective translational attenuation. Nevertheless, we think removal by ERAD is most likely because the lower accumulation appears rather specific for the defective zein in each respective mutant. In none of the other mutants associated with ER stress did we see a specific decrease in the accumulation of proteins encoded by the corresponding nondefective alleles.

Through subcellular fractionation, we observed that the Zm Derlin proteins predominantly associated with the protein bodies that are protein storage compartments derived from the ER. Although both types of Zm Derlin proteins were detected in the protein body fraction, Zm Derlin1 was more abundant in the f2 mutant compared to normal maize, whereas the converse pattern was found for Zm Derlin2, which accumulated to higher levels in the normal line. At present, we cannot explain this difference. It is plausible that the apparent decrease of Zm Derlin2 is the result of translational attenuation of mRNAs not needed during an ER stress response (Harding et al., 2002). Alternatively, it is also possible that, in response to the ER stress, the immunoelectrophoretic analysis of Zm Derlin2 epitope so far have been unsuccessful (D.J. Meyer, M.E. Kirst, and R. Jung, unpublished data), and we have not been able to test this hypothesis. In this context, it is noteworthy that Zm Derlin1 migrated electrophoretically as a doublet of approximately 23.5 and 25 kD, from which the faster migrating band was detected equally well with peptide antibodies regardless of whether they were made against the NH₂ or COOH terminus of the protein (Figs. 6 and 7). This observation rules out a simple proteolytic cleavage as an explanation for the size difference and leaves us favoring the possibility of another posttranslational modification of the Zm Derlin1 polypeptides. It is further remarkable that the prevalence of one polypeptide of the doublet over the other seemed to change in response to the presence of ER stress (Fig. 7).

The observations that Zm Derlin1-1 and Zm Derlin2-1 complemented the yeast Der1p function and associated with protein bodies, but that primarily Zm Derlin1 was strongly induced during ER stress, suggest that the two proteins have undergone some functional divergence in maize. One possibility is that both proteins serve a function in normal cellular metabolism, and possibly protein degradation, while only Zm Derlin1 is part of the ERAD pathway operating in response to ER stress. The Zm Derlin1s contain GxxG motifs that have been linked to interactions between transmembrane segments and oligomerization of transmembrane helices (Ubarr beetle-Belanda and Engelman, 2001; Langosch et al., 2002). They also contain weak homology to some elements of the Rhomboid family, which are multimembrane-spanning proteins with intramembrane Ser protease activity (Koonin et al., 2003).

Even though the exact function of Derlin1 remains unknown in any system, studies in mammalian cells have shown its requirement for dislocation of misfolded proteins from the ER (Lilley and Ploegh, 2004). In preliminary yeast two-hybrid experiments, Zm Derlin1-1 interacted with a putative ubiquitin-binding protein containing UBO and UBA domains suggested to recruit ubiquitinated substrates for release to the proteasome (M.E. Kirst and R. Jung, unpublished data; Buchberger, 2002). Several putative interacting partners for Der1 have been identified in high-throughput yeast two-hybrid screens (Ito et al., 2001; Giot et al., 2003; Li et al., 2004). For example, Drosophila and C. elegans putative Derlin1 homologs interacted with proteins encoded by genes induced during ER stress. The Derlin2 homolog in Drosophila also showed a possible link to ERAD, as both it and the Cdc48 AAA-ATPase that is associated with proteasomal targeting, bound to the same phosphatase. However, other interacting partners identified in the large-scale screens lacked an obvious link to ERAD or ER stress (Ito et al., 2001; Giot et al., 2003; Li et al., 2004). Clearly, further confirmation is needed to understand the protein-protein interactions that are functionally important for the Derlin1s during ERAD. A step toward this goal was made in an animal cell culture system in which immunoprecipitation assays placed Derlin-1 in a complex with the Cdc48 AAA-ATPase (Ye et al., 2004). Further investigation into the interacting partners of the various Zm Derlin1s will likely provide much-needed insight into the role of these proteins in ERAD or other cellular processes.
MATERIALS AND METHODS

Plant Material

The normal maize (Zea mays) inbred W64A (+) and its near-isogenic mutants, IL, MC, w2, and D7-96, were grown and self-pollinated at the Central Crops Research Station, Clayton, North Carolina, during summer field seasons for all experiments, except the GeneCalling experiment where plants were grown in the summer of 1998 in field plots at the Pioneer Fife-Red International genetic nursery in Johnston, Iowa (Hunter et al. 2002). Well-filled ears of each line were harvested 18 and 23 d after self-pollination between 7:30 and 9:30 am, immediately frozen in liquid nitrogen, and stored at −80°C until use.

GeneCalling Analysis

Endosperm was dissected from frozen seed from the middle portions of the ears. To minimize the effect of biological variation between ears on the gene expression analysis, equal numbers of endosperms from three ears were pooled and treated as one sample. Total RNA was isolated from ground endosperm tissues using the PUREscript kit (Centa Systems, Minneapolis), according to the manufacturer's instructions, and mRNA profiling was performed at Celeron (New Haven, CT) by GeneCalling, essentially as described by Shinketsu et al. (1999). In brief, cDNA was synthesized from three independently pooled endosperm samples per genotype (biological repeats). Each of the resulting 12 DNA preparations was further divided into three aliquots (technical repeats) to provide together nine repeats per genotype for the profiling analysis. Each of these cDNA aliquots was digested in parallel reactions with 64 different combinations of restriction enzyme pairs. Fragments from each digest were ligated to adapters; the fragments were amplified with primers that have unique tags (5′-tag on one end, fluorescent marker at the other). Labeled fragments were purified using streptavidin beads and were resolved by high-resolution gel electrophoresis to generate traces showing peaks whose position and height represented M, and abundance of DNA fragment(s), respectively. Trace data were used for qualitative (M) and quantitative (abundance) comparisons between the W64A+ and mutant samples. GeneCalling software compiled a list of differentially abundant fragments and assigned a ranking (significance) to each detected difference. The software further searched a nucleic acid database for fragments with the same length and sequence and predicted likely gene candidates. The identity of predicted fragments was confirmed by competitive amplification with an unlabeled gene-specific primer (positioning) or by cloning and sequencing the fragment (Shinketsu et al., 1999).

Sequence Alignment

Alignment of deduced amino acid sequences of two Zm Derlin proteins with putative homologs from other organisms was performed by ClustalW analysis in the software package Vector NTI suite 6 (Informax, Bethesda, MD).

Subcellular Fractionation

Endosperm was removed from kernels and ground (0.2 w/v) in buffer B (10 mM Tris-HCl pH 8.5, at 25°C, 10 mM KCl, 5 mM MgCl2, and 7.2% [v/v] Suc) by mortar and pestle (Shank et al., 2001). Homogenates were incubated on ice to allow the starch and cell debris to settle, and the remaining material was used as crude extracts. All extraction and cellular fractionation steps were carried out at 0°C to 4°C.

Membrane microsome isolation (Fig. 3) Shank et al., 2001). Crude extracts were subjected to centrifugation at 5,000 rpm for 10 min to remove protein bodies (pellet fraction), leaving a microsome fraction containing cisternal ER and other membranes. The supernatant was subjected to centrifugation at 100,000 rpm for 30 min in a fixed-angle rotor to collect the microsomes. For separation of the microsomal and luminal fractions (Fujita et al., 1992), samples were prepared as described by Shutters and Mierley (1991), with minor additional modifications. The microsomal pellet was resuspended in 1 mL of 100 mM Na2CO3 pH 11.5, and incubated under constant agitation at 4°C for 5 min before being placed on a Suc pad (100 mM NaHCO3 pH 8.3, 300 mM Suc) and subjected to centrifugation at 100,000 rpm for 30 min. This fractionation step leaves the luminal content in the supernatant while membranes form a pellet below the Suc pad. The membrane fraction was resuspended in buffer B.

For detergent solubilization, microsomal pellets were resuspended in 50 mM Tris-HCl pH 6.8, containing the nonionic detergent digitonin, Nonidet P-40, or Triton X-100, or theionic detergent deoxycholate at final concentration of either 0.2% or 1.0%, and incubated for 30 min at 4°C under constant agitation prior to centrifugation at 100,000 rpm for 30 min. Deoxycholate was further purified according to the method of Golich and Rapoport (1989) prior to use.

For total membrane isolation (Fig. 6), crude extracts were subjected to centrifugation at 100,000 rpm to yield a single pellet containing protein bodies, cisternal ER, and other membranes. Protein was quantified with a biocinchonic acid protein assay kit and bovine serum albumin standard (Pierce, Rockford, IL).

For fractionation by Suc density gradient centrifugation (Fig. 7), crude extract was filtered through two layers of miracloth, overlaid on a 2.0 M Suc solution in buffer B, and subjected to centrifugation for 10 min at 16,000 rpm in a swinging-bucket rotor. The supernatant fraction from the low-speed centrifugation was recovered and applied to a discontinuous Suc gradient prepared as 2 mL steps of 2.0, 1.5, 1.3, 1.2, and 0.5 M Suc in buffer B. Gradients were subjected to centrifugation at 100,000 rpm in a TLA-100.3 swinging-bucket rotor for 30 min at 4°C. The cisternal ER was collected from the 1.6-1.8 M Suc interface and the protein bodies were collected from the 1.5-2.0 M Suc interface (Gilkkin et al., 1999).

Immunoblot Analysis

Samples were adjusted to 2X SDS-PAGE sample buffer (Laemmli, 1970) and boiled for 5 min prior to fractionation through 15% SDS-polyacrylamide gels. Proteins were transferred by semidry blotting to Immobilon-P membranes (Millipore, Billerica, MA) in a semi-dry tank developed buffer (45 mA, 30 min, 30 g/L phosphoric acid) at 20°C. Membranes were blocked with 5% (w/v) nonfat dry milk for 1 h prior to incubation with primary antibody. For detection of the Zm Derlin protein, immunoblots were incubated with polyclonal antibodies raised in rabbits against peptide SPAYYKSLEPTPKAAG from the Zm Derlin proteins at a 1:3,000 dilution in Tris-buffere sodium phosphate buffer (TBSB) 20 mM Tris-HCl pH 7.5, at 25°C, 0.14 M NaCl, 0.1%, [v/v] Tween 20) or antibodies against peptide RYKCKLEISFRGTDG from the Zm Derlin2 proteins at a 1:3,000 dilution in TBSB. Antibodies against BIP ID9 (Stressgen, Victoria, British Columbia, Canada), calnexin and calreticulin (Pagny et al., 2001), and 19- 23K D Beta (Hunter et al., 2002), were used at 30,000 dilution in TBSB. Antibodies against the mitochondrial a-F0Pase (Lubberts et al., 1993) was used at 1:10,000 dilution in TBSB. Following incubation with primary antibodies, immunobots were incubated with the appropriate secondary antibody against rabbit or mouse couple to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) at 1:10,000 dilution in TBSB. Visualization of protein was made with chemiluminescent substrate (Pierce) by exposure to x-ray film (X-OMAX; EastmanKodak, Rochester, NY).

Quantitative Expression Analysis of Derlin Genes by MPSS

The mRNA from a variety of maize tissue samples (infertile line B73) was previously isolated and MPSS was performed by Lynx Therapeutics (Hayward, CA) as described (Brenner et al., 2000; Meyer et al., 2001). The resulting MPSS ESTs, here defined as the first 37 bp, including and following downstream of the most 3’ SauA site (GACT) of a gene transcript, are quantified and reported on a ppm basis (1-2 million sequencing reactions performed per sample) in a searchable database. The quantity of Derlin MPSS ESTs in each tissue sample was then obtained by queries of this database with the exact sequence of the conceptual MPSS ESTs identified for Zm Derlin1-1 (5’-CATCAGATGTCGGACGAG-3’) for Zm Derlin1-2 (5’-GATCTCAAGTTCTTCTT-3’) for Zm Derlin2-1 (5’-GATCTGAGATGGCGCAAC-3’) and for Zm Derlin2-2 (5’-GATCTGATGCGCAAC-3’).

RT-PCR Analysis

Total RNA was isolated from embryos (18 DAP) or endosperm (18 DAP) tissues (200 mg) using the TRIzol reagent (Invitrogen, Carlsbad, CA) by
Quantitative RT-PCR Analysis

Total RNA was isolated from endosperm (18 DAT) tissue from normal and mutant lines using the total RNA isolation mini kit (Agilent Technologies, Palo Alto, CA) following the manufacturer’s protocol. Two micrograms of total RNA were treated with 2 μg RNaseA (Benzonase, Harrods, MD). Twenty nanograms of treated RNA (2 μg for 25 μl RNA) were used for cDNA synthesis with a mix of random and oligo(dT) primers and RT-PCR amplification with sequence-specific primers using the Applied Biosystems 7500 PCR kit for probes (Bio-Rad) on an ABI 7900 thermocycler. Quantitative RT-PCR reactions were performed for 10 min at 50°C followed by 5 min at 95°C prior to amplification for 40 cycles of 15 s at 95°C and 1 min at 59°C with fluorescence reading during the annealing steps. All samples were assayed in triplicate or quadruplicate reactions. Samples were normalized to the 28S rRNA signal and calibrated to the normalized wild-type endosperm sample.

Yeast Complementation of Irel1/Der1 Mutants

The Irel1/Der1 double-mutant strain (MATa, trp1-1, leu2-3, ura3-1, can1-100, Δirel1Δder1ΔΔ::URA3) (Tavernier et al., 2003) was transformed with the plasmid vector pRS313 (Moskow and Firestein, 1999) containing the coding regions for either Zm Der1Δ1, Zm Der1Δ2, or the yeast Der1 gene (Y寺院sΔ101) inserted between H1 and S2 restriction enzyme cloning sites. Genes were amplified by PCR using the following primers: Zm Der1Δ1, 5’-CCGGATCCGAAGATCTCTGCT-3’ and 5’-CCCAAAGCTTCACTTAATCCATG-3’; Zm Der1Δ2, 5’-CCGGATCCGAGATCTCTGCT-3’ and 5’-CCCAAAGCTTCACTTAATCCATG-3’; and yeast der1 5’-CCGGATCCGAAGATCTCTGCT-3’ and 5’-CCCAAAGCTTCACTTAATCCATG-3’. PCR products were subcloned into the pGEM-T easy vector (Promega). Genes were transferred from pGEM-T to pRS313 vectors by digestion with BstI and SacI. Transforms were selected on synthetic complete-His-Ura media (QIBO-gene, Carlsbad, CA). A stable transformant from each complementation was grown overnight until the OD600 reached 2. Cells were spotted on replica plates in serial 10-fold dilutions and incubated at 25°C or 37°C for 4 d.
Cant BS, Doherty JF (1997) DNA sequence evidence for the segmental allotetraploid origin of maize. Proc Natl Acad Sci USA 94: 6809–6814


UNCOVERING PROTEINS FROM THE ENDOPLASMIC-RETICULUM-ASSOCIATED DEGRADATION PATHWAY IN PLANTS THROUGH COMPARATIVE GENOME ANALYSIS

Abstract

The removal of misfolded secretory proteins from the lumen of the endoplasmic reticulum (ER) into the cytosol for degradation is one of the quality control mechanisms present in the secretory pathway. Several proteins are part of the machinery involved in recognizing, transporting and degrading the misfolded substrates. Although the ER-associated degradation (ERAD) has been extensively studied in yeast, and to a lesser extent in animals, a couple of the proteins that function in the degradation machinery have been described in plants. We took advantage of the availability of the full genome sequences for Arabidopsis, rice and poplar to search for putative homologues in plants to the described ERAD proteins. Through our searches we found several predicted proteins that show homology to known ERAD proteins. We also found that not all ERAD proteins were identified in the plant genomes raising the possibility of differences between the ERAD pathway in plants and other organisms.

Introduction

Efficient synthesis and secretion of secretory proteins is necessary for the maintenance of many cellular functions and the proper development of organisms. Secretory proteins are important not only for proper cell to cell communication but also to maintain cell wall integrity (Romisch, 1999). The synthesis of abnormal proteins could be detrimental for development by leading to the accumulation of protein aggregates in the lumen of the endoplasmic reticulum (ER). To prevent both the transport of abnormal proteins through the secretory pathway and the accumulation of toxic aggregates the cell has developed a stringent quality control mechanism in the ER. One of the components of this ER quality control system is the ER-associated degradation pathway (ERAD) which removes terminally misfolded and orphan proteins.

Interestingly, ERAD co-opts components from previously described quality control mechanisms both in the lumen of the ER and in the cytosol. One such mechanism is the
unfolded protein response (UPR). The UPR triggers the up-regulation of molecular chaperones and modifying enzymes that are associated with helping newly synthesized proteins fold. Those molecular chaperones and modifying enzymes are co-opted by ERAD to present abnormal substrates for degradation (Wilson et al., 2000, Molinari et al., 2002). On the cytosolic surface of the ER, ERAD co-opts the Cdc48 complex and the proteasome, which are involved in the degradation of cytosolic proteins. Those two protein complexes are brought to proximity of the ER membrane to degrade secretory substrates (Ye et al., 2001). Some proteins involved in ERAD have so far only been reported as acting in this pathway, as is the case of Hrd1, an ER membrane localized ubiquitin ligase enzyme (Gardner et al., 2000).

Several reviews have addressed advances in identifying both ERAD machinery and substrates (Sayeed and Ng, 2005, Ahner and Brodsky, 2004, Romisch, 1999). The purpose of this review is to compare ERAD among eukaryotic systems with special focus on plants and offer insights on how some of the conserved components among yeast, plants and animals may have evolved.

**ERAD**

Several classes of secretory proteins that could be subjected to mistakes during their synthesis or assembly are synthesized at the ER. Because putative ERAD substrates can be either soluble or membrane-associated, and the site exhibiting the misfolded segment can be in the lumen or on the cytosolic side of the ER, ERAD must accommodate degradation routes from both locations. As a general rule, molecular chaperones present either in the cytosol or ER lumen recognize misfolded substrates (Huyer et al., 2004, Molinari et al., 2002). In the case of glycoproteins, ER-resident mannose receptors recognize the misfolded proteins by their mannose residue composition. Glycoproteins containing the Man₈GlcNAc₂ oligosaccharide are more rapidly degraded because they are poor substrates for UGGT-mediated reglycosylation that maintains the glycoproteins in a state that can be recognized by luminal molecular chaperones (Molinari et al., 2003, Oda et al, 2003, Szathmary et al., 2005, Bhamidipati et al., 2005, Kim et al., 2005, Hirao et al., 2006).
In yeast, substrates originating from the different ERAD routes have been shown to converge at a common point. From there either the E3 ligase protein Hrd1 and its associated factor Hrd3 or the E3 ligase protein Doa10 initiate the ubiquitination process required for proteasomal degradation (Gardner et al., 2000, Swanson et al., 2001). A third E3 ligase protein, Rsp5p, has been implicated in ERAD, but its requirement is restricted to soluble proteins that traffic to the Golgi apparatus prior to ERAD degradation (Haynes et al., 2002). Acting in conjunction with the E3 ligases are the E2 conjugating enzymes Ubc1, Ubc6 and Ubc7 (Friedlander et al., 2000, Swanson et al., 2001). Those proteins allow for proper ubiquitination of ERAD substrates by bringing ubiquitin molecules to the E3 ligases present in the ER membrane. However, for luminal misfolded substrates to be ubiquitinated on the cytosolic side of the ER they first have to be retro-translocated.

Both the retro-translocation channel and the requirements for substrate retro-translocation into the cytosol are controversial topics in the ERAD field. Initially, the Sec61 complex that is normally used to import newly synthesized proteins into the ER was proposed to function in a reverse flux allowing for the retro-translocation of misfolded proteins (Wiertz et al., 1996, Pilon et al., 1997). The use of temperature sensitive yeast cells lacking functional subunits of the Sec61 showed the requirement of a functional translocation complex for the proper transport of misfolded ERAD substrates to the cytosol (Plemper et al., 1997). Subsequently, another ERAD protein with unknown function was suggested to also form a retro-translocation channel. This protein Derlin1, is a putative homologue to the yeast Der1 protein. Derlin1 was shown to interact with other ERAD components and substrates in both glycosylated and unglycosylated forms. The glycoylated form is present prior to and the unglycoylated form after retro-translocation (Lilley and Ploegh, 2004, 2005, Ye et al., 2005). It has also been suggested that integral membrane proteins with cytosolic misfolded segments do not require a retro-translocation pore for proper degradation (Huyer et al., 2004). Instead, those misfolded proteins are recognized by molecular chaperones in the cytosol and presumably become ubiquitinated by the same ubiquitinating enzymes as soluble substrates. The ubiquitinated proteins would then be degraded by the machinery present in the cytosol.

The ERAD proteins in the cytosol that remove and degrade misfolded proteins are shared with other quality control pathways. The Cdc48 AAA-ATPase complex is involved in the energy dependent process that removes ubiquitinated substrates from the ER membrane.
and releases them into the cytosol where they are degraded by the proteasome. The proteasome, like the Cdc48 complex, is widely used by other pathways both to control the life span of short-lived proteins and to degrade abnormal proteins. In plants the proteasome is involved in several pathways including hormonal signaling, photosynthesis, circadian cycling and cell cycle division (reviewed in Vierstra, 2003).

**ER quality control in plants**

*Link between the unfolded protein response and ER degradation*

The accumulation of misfolded proteins in the lumen of the ER triggers the unfolded protein response (UPR) which is characterized among other things by an up-regulation of genes encoding molecular chaperones and ERAD components. In plants, very little is known about the sensing and signaling mechanisms used by the UPR, although the response has been observed in various plant systems (Boston et al., 1991, Koizumi et al., 2001). Arabidopsis plants treated with the pharmacological agent tunicamycin or with the reducing agent DTT, drugs that cause the accumulation of misfolded proteins in the ER, show up-regulation of molecular chaperones (Martinez and Chrispeels, 2003). Activation of UPR is also observed in maize mutants that synthesize defective storage proteins. Those mutants have an increased accumulation of the molecular chaperones BiP and protein disulphide isomerase (PDI, Fontes et al., 1991, Li and Larkins, 1996).

Work from Travers et al., (2000) to obtain an expression profile of genes induced by UPR in yeast cells showed that the UPR involves more than just the activation of ER molecular chaperones and protein folding activities. It also signals the up-regulation of genes involved in ERAD and vesicle trafficking. Similar genome-wide analyses with microarrays were performed for other organisms to study the effect of the UPR signaling pathway on gene transcription. Analysis in the metazoan *C. elegans* showed that different UPR sensors activate complementary sets of genes that act to protect the organism from the accumulation of toxic aggregates (Shen et al., 2001). Comprehensive genomic analysis to determine which genes are regulated by UPR was also performed in Arabidopsis plantlets treated with tunicamycin or DTT (Martinez and Chrispeels, 2003, Kamauchi et al., 2005). The outcome of the genomic experiments in Arabidopsis and *C. elegans* was that UPR activation leads to
the up-regulation of genes associated with protein folding activity, ER-associated
degradation and vesicle trafficking. Those were genes involved in the same cellular functions
as the genes identified in the genomic analysis in yeast (Travers et al, 2000). Among the
putative ERAD genes identified in the genomic analysis in Arabidopsis were the AAA-
ATPase complex, Hrd1, Derlin2-1 and Hrd3 genes.

**ERAD in plants**

The initial observation that a retrograde transport from the ER into the cytosol was
possible came from experiments with the A chain of the plant toxin protein ricin (RTA) in
tobacco protoplast cells (Frigerio et al., 1998). The RTA protein, which has two lysine
residues, is transported into the ER where it is glycosylated prior to retro-translocation into
the cytosol. Once in the cytosol it becomes deglycosylated and is degraded (Frigerio et al.,
1998, DiCola et al., 2005). Changing the RTA lysine content from 2 to 6 residues showed
that the mutated form of the protein is transported with equal efficiency through the
membrane into the cytosol but is rapidly eliminated by ERAD as judged by the stabilization
of the mutated protein in the presence of proteasome inhibitors (DiCola et al., 2005).
Additional indication that plants contain functional retro-translocation machinery was
obtained in BY2 tobacco cells for both endogenous and recombinant cell wall invertases
(Pagny et al., 2003). In those cells, non-glycosylated cell wall invertases were rapidly
degraded in a pre-Golgi compartment but possibly independently of the proteasome, as
treatment with proteasome inhibitors failed to prevent degradation.

To visualize the plant degradation route *in vivo*, protoplast and virus-infected tobacco
cells were transformed with a secreted derivative of GFP protein (sGFP-P). The sGFP-P was
constructed by the fusion of the signal peptide of calreticulin to the soluble GFP protein
(Brandizzi et al., 2003). This chimeric protein was efficiently synthesized in the ER prior to
export into the cytosol, however sGFP-P was not detected in the cytosol. Interestingly, the
reduction in sGFP-P was not caused by proteasomal degradation as indicated by the
inefficiency of proteasome inhibitors to stabilize the protein. Instead, it was observed that
with prolonged infection time the protein moved from the cytosol into the nucleoplasm. This
phenomenon, however, raises the question of how does ERAD work in plants? Is the
transport to the nucleoplasm an alternative route used by this unusual substrate or is this in
fact a specific disposal mechanism present only in plants? Either way it deserves further testing.

Although the use of proteasome inhibitors has been used both in animal and yeast cells to address the requirement of the proteasome for ERAD degradation the same straightforward results have not been observed in plant cells. In cells expressing the substrates RTA and mildew resistance O (MLO) the use of proteasome inhibitor drugs led to efficient blocking of degradation. However, when the proteasome inhibitors were used with cells expressing the putative substrates cell wall invertase and recombinant sGFP-P protein, substrate degradation was not impaired (Pagny et al., 2003, Brandizzi et al., 2003, Muller et al., 2005). The differences in these findings might reflect a variation in the uptake of those drugs by different types of plant cells.

Besides the proteasome only two other ERAD proteins have been reported as part of the ERAD pathway in plants. Those two proteins, Cdc48 and Derlin, were implicated on ERAD either by the use of misfolded substrates (Cdc48) or by functional complementation and sequence similarity to ERAD proteins from other organisms (Derlin; Muller et al., 2005, Kirst et al., 2005).

**Methodology**

Identification of putative plant homologues to ERAD proteins. The ERAD protein sequences from yeast and animals listed in Table I (Npl4, Rsp5, EDEM3, Yos9 and Cue1) were used as queries in the searches for Arabidopsis homologues, except for the following genes to which Arabidopsis homologues had been described: Ubc7, Ubc6, Ubc1, Hrd1, Hrd3 and Cdc48 (Muller et al., 2005).

Initial searches (TBLASTN) were performed using the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/) website. Once locus information was obtained for Arabidopsis genes, protein sequences were extracted from the Arabidopsis genome database at The Institute for Genome Research (TIGR; http://www.tigr.org/plantProjects.shtml). Arabidopsis protein sequences were used to search the both rice and the poplar genome databases (TIGR for rice sequences and
http://genome.jgi-psf.org/Poptr1/Poptr1.home.html for poplar). The yeast and animal protein sequences for which putative Arabidopsis counterparts had not been described were also used in searches with the position-specific iterative BLAST program (PSI-BLAST) with an $E$-value inclusion threshold of 0.005 and four iterations. Alignments were inspected visually to determine that motifs or domains specific to each family were conserved. For the ERAD proteins that we identified in this study an $E$-value threshold of $E<10^{-65}$ was used for sequences with similarity to EDEM, $E<10^{-45}$ for Ufd1 and $E<10^{-15}$ for Doa10.

For some of the ERAD proteins for which plant counterparts are not presented in Table I, initial hits were discarded after further analysis due to the lack of conserved motifs. That was the case for EDEM3 and Yos9 which have ER retention signals (K/HDEL), Npl4 which has conserved cysteine and histidine residues present in the amino-terminal end and Rsp5 which has a WW domain. For Cue1 no likely candidates emerged even after four iterations of PSI-BLAST with an $E<0.005$.

In addition to sequence similarity and conservation of predicted domains, putative plant ERAD sequences that matched ER membrane proteins were also analyzed for their predicted topological structure using TMHMM (Krogh et al., 2001). Sequences that met both the domain conservation and structural prediction requirements were considered as putative ERAD components in plants and are presented in Table I.

**ERAD proteins in plants**

ERAD in yeast and animal cells comprises a wide array of components that may or may not be shared with other previously described pathways. Interestingly, the number of genes encoding each of those components varies between animals and yeast with the latter commonly having a single gene encoding each protein. Like animals, plants frequently appear to have more than one gene encoding each of their ERAD components (Table I). Through sequence similarity searches we identified several protein sequences in plants that have homology to previously described ERAD proteins in yeast and animals. Among those proteins are the ubiquitin conjugating enzymes (Ubc) 1, 6 and 7; the ubiquitin ligases Doa10 and Hrd1; the Hrd1 co-factor Hrd3; the mannose receptor Htm1/EDEM; the putative
component of the translocation channel Derlin and the AAA-ATPase complex Cdc48 and its co-factor Ufd1.

**Cdc48**

The Cdc48 protein is conserved among organisms for which sequence data are available. It contains a highly conserved ATPase domain which is known as ATPases associated with diverse cellular activities (AAA, Kunau et al., 1993, Feiler et al., 1995). The Cdc48 protein contains two ATPase domains or cassettes (D1 and D2) and an additional N-terminal domain located upstream of the D1 domain (Ye et al., 2003). Each AAA-ATPase cassette contains two motifs called Walker A and B that mediate ATP binding and hydrolysis, respectively. In plants, the Cdc48 protein was initially identified in expanding cells of Arabidopsis was shown to be functional through complementation of the temperature sensitive phenotype of yeast cells mutant for the cdc48 gene (Feiler et al., 1995). The Arabidopsis genome has, however, three genes that encode Cdc48 protein isoforms, AtCdc48A, AtCdc48B and AtCdc48C (Rancour et al., 2002). The sequence originally characterized by Fieler and coworkers (1995) is the AtCdc48A isoform that shares 91% and 95% sequence similarity with isoforms AtCdc48B and AtCdc48C, respectively (Rancour et al., 2002). Using sequence similarity searches we identified several putative genes encoding the Cdc48 protein in the genome of other plant species with rice having two and poplar four predicted genes (Table I). Overall the Cdc48 protein sequences appear to be very conserved among organisms with 95% overall sequence similarity. A direct involvement of Cdc48 in ERAD in plants was shown by the use of a dominant-negative Cdc48 form (AtCdc48A QQ) which led to the accumulation of the misfolded substrate MLO in Arabidopsis cells (Muller et al., 2005). Because functional studies have been performed only for AtCdc48A and the overall sequence similarity of the different isoforms from Arabidopsis against other putative plant Cdc48 proteins is very similar sequence analysis alone does not provide the basis to determine if one isoform is more likely to be involved in ERAD than another.

**Ufd1**

In both yeast and mammalian cells, a trimeric complex containing Cdc48-Ufd1-Npl4 is involved in recognizing and extracting ubiquitinated substrates from the ER membrane and presenting them to the proteasome for degradation (Ye et al., 2003). The absence of either
co-factor, Ufd1 or Npl4, leads to reduced degradation of the substrate (Ye et al., 2001, Nowis et al., 2006). The yeast Ufd1 protein has a conserved ubiquitin-binding site or UT3 domain present in the amino-terminal region of the protein that has been proposed to recognize polyubiquitin chains (Ye et al., 2003). Besides this UT3 domain, the human Ufd1 sequences also contain a UT6 domain that binds both p97 (the Cdc48 homologue) and Npl4 through two distinct, short binding sites located between residues 215 and 241 (p97) and residues 258 and 275 (Nlp4; Meyer et al., 2002, Bruderer et al., 2004). Among eukaryotes UT3 domains are similar in sequence and a co-factor complex lacking this domain acts as a dominant-negative mutant in retro-translocation (Ye et al., 2003). These data are suggestive of the possibility that the Ufd1 proteins have a conserved function in retro-translocation. Using the yeast Ufd1 protein in sequence similarity searches we identified putative homologues in plants (Table I). The plant sequences appear conserved with the yeast and animal sequences mostly in the region between residues 1-200 which is associated with ubiquitin binding. The region suggested to bind Cdc48/p97 in the human sequence is more conserved among the plant and animal sequences than among the plant and yeast sequences (Figure 1). However, only experimental analysis will determine if this region in the Ufd1 plant sequences binds Cdc48 and if so which amino acids are required for the interaction.

**Hrd1**

Among the ubiquitin ligase enzymes involved in ERAD, Hrd1 was the first one to be described (Bordallo et al., 1998). Hrd1 is a transmembrane protein with a large hydrophobic region on its amino-terminal region which spans the membrane six times allowing for both the amino and carboxy-terminal ends to be on the cytosolic side of the ER membrane (Deak and Wolf, 2001). In the cytosolic carboxy-terminal region is the functional RING-H2 domain required to bind ubiquitin conjugating enzymes. Functional Hrd1 proteins have been described both in humans and yeast (Bordallo et al., 1998, Kaneko et al., 2002). Those sequences show approximately 26% overall sequence similarity and 45% identity in their conserved RING-H2 domains (Kaneko et al., 2002). Putative homologues to Hrd1 have also been identified in plants. Muller and co-workers (2005) initially presented a list of four genes that encode putative Hrd1 proteins in Arabidopsis (Muller et al., 2005). Although all four Arabidopsis genes encode proteins with the predicted amino-terminal transmembrane spanning regions and carboxy-terminal RING finger domains only two genes (At1g65040
and At3g16090) show high sequence similarity with predicted Hrd1 proteins from other organisms. In general, the amino-terminal region and the RING-H2 domain are more conserved among sequences of different species than the carboxy-terminal regions of the proteins.

The four predicted Arabidopsis Hrd1 proteins group in two classes. One class (At1g65040 and At3g16090) is highly conserved among organisms and the other appears to be present only in plants. Both classes have counterparts in other plant species such as rice, poplar and maize. The class that appears to be present only in plants is about 80 amino acids longer than the amino acid sequences that encode proteins from the other class and encodes a slightly different RING finger domain (Table I, Figure 2). The protein encoded by locus At1g65040 is smaller than the other predicted putative Hrd1 proteins. This protein also shows only four predicted transmembrane regions on its amino-terminal end. Because proteins containing RING finger domains are involved in a variety of cellular processes it is necessary to determine experimentally if the putative Hrd1 proteins in plants do in fact function in ERAD.

**Hrd3**

In yeast cells the Hrd3 mediates the stabilization of the Hrd1 protein. The luminal domain of Hrd3 (sequence 1 – 767) is proposed to be the required region for Hrd3 function due to its ability to restore the ERAD function in yeast cells lacking Hrd3 (Gardner et al., 2000). In animal cells, the interaction of Hrd1 and Hrd3/Sel-1 has been shown in cells expressing recombinant proteins (Ye et al., 2005). Hrd3 is a glycoprotein with a carboxy terminus in the cytosol and a large amino-terminal region in the lumen of the ER (Hampton et al., 1996). In plants, the predicted Hrd3 proteins are highly conserved and appear to have short cytosolic amino termini followed by large carboxy terminal luminal regions as predicted by the topology prediction program TMHMM (Krogh et al., 2001). Despite the differences in the locations of the transmembrane domain in the plant sequences compared to the yeast and animal sequences, all of the conserved amino acids are present in a sequence segment that is predicted to be present in lumen of the ER. Because the putative functional segment of the predicted plant Hrd3 proteins are located in the ER lumen it is possible that function may still be conserved. Specially conserved is a sequence of 28 aa
that may or may not represent the region of the yeast protein that is responsible for controlling Hrd1 function.

*Doa10*

In yeast, an additional E3 ligase enzyme has been experimentally identified as an ERAD component (Swanson et al., 2001). The Doa10 protein is a multispansing membrane protein that has a RING finger domain in its amino terminus and a WW domain (Swanson et al., 2001). The amino terminal region of the protein is fairly conserved among organisms while the consensus amino acids that form the WW domain appear to be present only in yeast. Swanson and co-workers (2001) indicated that the Doa10 protein in yeast was related to predicted proteins in several organisms including plants, nematodes and mammals. However, the sequence similarity among the human predicted sequence and the yeast sequences is mostly restricted to the RING finger domain and a second internal region of approximately 130 amino acids between residues 625 – 755 (position for yeast Doa10 protein). Like the human sequence, the predicted sequences from Arabidopsis, poplar and maize showed about 14% overall identity to the yeast Doa10 protein sequence. The overall identity to the human sequences is around 25% suggesting that those could also be considered Doa10 proteins. Interestingly, rice has two genes predicted to match the amino-terminal end of Doa10 and a different gene predicted to encode the carboxy-terminal end. One of the genes, encoded by locus LOC_06g43200, aligns with the carboxy terminal end of the predicted Doa10 protein of Arabidopsis. The other gene, encoded by locus LOC_06g43210.2, encodes a portion of the protein that aligns with the amino terminal region of the predicted Arabidopsis sequence. However, a 37 amino acid gap is observed between the proteins encoded by each gene in relation to the Arabidopsis sequence. The second gene that encodes the amino-terminal region (LOC_Os08g01040) shows also a conserved RING finger domain although its overall sequence similarity to the predicted Arabidopsis sequences is lower than that of the other predicted gene (LOC_06g43210.2). No gap is observed between the protein encoded by this gene and the protein encoded by the LOC_06g43200 locus. Because no expression data is available for locus LOC_06g43210.2 we do not know if this sequence extends through the predicted 37 aa gap. Thus, it is not clear if the rice genome has a single gene encoding a full-length Doa10 protein.
Among the E2 conjugating enzymes required for ERAD are the soluble enzymes Ubc1 and Ubc7 and the membrane associated enzyme Ubc6. All the ubiquitin conjugating enzymes have a conserved cysteine residue required for their function. In yeast cells, Ubc7 is the principal E2 conjugating enzyme involved in Hrd1-dependent degradation (Bays et al., 2001). This Ubc enzyme associates with Hrd1 through the RING finger domain present in the E3 ligase (Bays et al., 2001). In plants several sequences with similarity to the yeast Ubc7 sequence have been identified. Initially, three protein sequences were proposed to represent Ubc7 enzymes in the Arabidopsis genome (Muller et al., 2005). Two of those Ubc7 sequences have been annotated as Ubc13 and Ubc14 and only one as Ubc7. However, when sequence similarity, size and topology are taken into consideration the sequence annotated as Ubc7 is the least likely to encode a homologue of the yeast Ubc7 protein. The protein encoded by the At5g55380 locus is about 200 amino acids larger than the other predicted Ubc7 proteins in plants and encodes a transmembrane protein. Because Ubc7 proteins involved in ERAD are soluble proteins it is unlikely that this protein (At5g55380) is a Ubc7 enzyme. Nonetheless, it may be a new component of ERAD if localized in the ER since it encodes an integral membrane E2 conjugating enzyme. The sequence encoded by locus At5g59300 is a little larger than other predicted Ubc7 sequences and less conserved in its amino terminus (1-64 aa) but it has high overall similarity with the other predicted sequences and shows the conserved domains (Table I). In our sequence similarity searches at least two sequences with high similarity with the predicted Ubc7 proteins were identified in rice, maize and poplar. In poplar at least two sequences have been identified that show similarity to the larger transmembrane E2 conjugating enzyme. Interestingly, sequence similarity searches using the protein encoded by locus At5g55380 against the yeast and human databases yielded no sequences with a high degree of similarity. Thus, this protein may be a plant specific protein.

Like Ubc7, Ubc1 proteins are primarily involved in the degradation of ERAD substrates associated with the E3 ligase Hrd1 (Bays et al., 2001, Swanson et al., 2001). Yeast cells lacking either Ubc7, Ubc1 or both show increased accumulation of misfolded substrates (Friedlander et al., 2000). Ubc1 was initially described as essential in the turnover of proteins in yeast during early stages of growth after germination of spores (Seufert et al., 1990).
protein was suggested to be part of the same subfamily of ubiquitin-conjugating enzymes as Ubc4 and Ubc5 (Seufert et al., 1990). Although slower degradation of an ERAD substrate has been observed in yeast cells mutant for both Ubc1 and Ubc4, the latter has otherwise not been implicated in ERAD (Hill and Cooper, 2000). Several of the conserved identical amino acids between Ubc1, Ubc4 and Ubc5 are present in the Ubc1 sequences from plants and human. Specifically conserved is the region containing the single cysteine residue required for Ubc1 function. Alignment of the putative plant ubc1 sequences listed on Table I showed that those proteins are highly conserved with an overall 97% consensus in amino acid positioning (data not shown).

The Ubc6 protein is the only member of the family of ubiquitin-conjugating enzymes in yeast reported to be associated with the ER membrane (Yang et al., 1997). Ubc6 has its functional domain located on the cytosolic side of the ER and is anchored to the ER membrane through its carboxy-terminal region (Lenk et al., 2002). In yeast cells, this ubc enzyme functions preferentially with the Doa10 E3 ligase enzyme rather than with the Hrd1 E3 ligase (Swanson et al., 2001, Bays et al., 2001). Two distinct families of Ubc6 proteins were described in mammals. Although neither class was capable of functionally complementing the yeast Ubc6 protein in vivo, both exhibit significant sequence similarity, structural organization and function in degradation of mammalian ERAD substrates (Lenk et al., 2002). One of the classes (Ubc6) shows an overall high degree of sequence identity and calculated molecular weight to the yeast ubc6p. The other class (Ubc6e), which is present only in higher eukaryotes, shows lower sequence similarity and contains a longer carboxy-terminus with the transmembrane segment (Lenk et al., 2002). In mammalian cells, this longer ubc6e form is phosphorylated, in response to ER stress, at serine residue 184 which resides within the uncharacterized region of the protein linking the active site and the transmembrane region (Oh et al., 2006). Although ubc6 sequences homologous to the ubc6e mammalian sequence are present in plants and nematodes, the region containing serine 184 appears to be conserved only among vertebrates (Figure 3). Despite the lack of similarity on the regions surrounding serine residue 184, the predicted sequences from Arabidopsis and poplar show this conserved residue. Because the enzyme that phosphorylates the serine residue 184 has not yet been described in plants the significance of this conserved amino acid
is still unclear. It is possible that the plant predicted ubc6e enzymes act on ERAD or that they are involved in another unrelated cellular process which also requires ubiquitination.

**EDEM/Htm1**

The glycoprotein known as EDEM (animals) and Htm1 (yeast) is a receptor for ER glycoproteins targeted for degradation. EDEM encodes an approximately 69 kDa membrane protein prior to glycosylation (Hosokawa et al., 2001). The yeast Htm1 protein shows 40% sequence similarity to mannosidase enzymes, but proteins from this family group are inactive mannosidases due to the lack of two cysteine residues that form disulfide bonds (cysteine residues 340 and 385, position for the yeast sequence, Jakob et al., 2001). Putative homologues to EDEM/Htm1 are present in the plant, insect and worm genomes (Jakob et al., 2001). Despite being shorter than the human and yeast sequences, the plant putative EDEM/Htm1 proteins have an alanine residue at the position of cysteine 340 (in the yeast sequence) conserved in 1,2-α-mannosidases.

**Der1/Derlin**

The yeast Der1 protein and its animal homologues Derlin (Der1-like) proteins are multispansing membrane proteins that have four membrane segments with both amino and the carboxy termini located on the cytosolic side of the ER (Hitt and Wolf, 2004, Ye et al., 2004). In mammals, there are three encoded proteins that show reasonable sequence similarity to the yeast Der1 protein. Those proteins are called Derlin1, Derlin2 and Derlin3. Two isoforms of Derlin3 are present, a short and a long isoform (Oda et al., 2006). In plants there are also several genes that encode proteins with sequence similarity to the yeast Der1 protein. The plant proteins separate into two classes called Derlin1 and Derlin2 (Kirst et al., 2005). The Derlin2 sequences have a 9 amino acid insert that is specific to this class of proteins in plants and is also absent from the animal and yeast sequences. The plant Derlin2 class also exhibits closer overall sequence similarity to the three Derlin proteins in mammals than does plant Derlin1.

A phylogenetic tree derived from analysis of the full-length Derlin protein sequences from animals, plants and yeast using the Bayesian method (Huelsenbeck and Ronquist, 2001) shows that the plant Derlin1 sequences group in a separate strongly supported subclade (100% posterior probability, Figure 4). The plant Derlin1 subclade appears to have originated
from a common ancestor to the major subclade which contains Derlin sequences from both animals and plants. That the plant Derlin2 sequences are grouped in the major subclade suggests that the predicted plant Derlin2 proteins are more closely related to the human Derlins than to the plant Derlin1 proteins.

**Conclusions and perspectives**

In general, the vast diversity of substrates that traffic through the secretory pathway and are subject to synthesis and assembly mistakes makes the study of ERAD both challenging and exciting. Although most of the progress in ERAD to date has occurred through the use of recombinant substrates and yeast genetics, much is still to be learned in other organisms such as plants and animals where gene knockouts are not so easily obtained.

Here we showed a preliminary assessment of putative plant homologues to the known ERAD components. It is interestingly to note that although plants have multiple genes that encode ERAD proteins, not all ERAD proteins described to function in the yeast or animal ERAD have putative homologues in the plant genome. The lack of those proteins raises interesting questions as to how is ERAD conserved among organisms and which other genes are present in plants to compensate for the lack of such “required” proteins since at least one protein has been identified for every function.

It is possible that the multicellular composition of plants and their sessile behavior leads to a requirement of different proteins that would work in eliminating misfolded proteins that occur not only due to synthesis mistakes but are also caused by environmental conditions. The knowledge of ERAD in plants would then allow for manipulating plants to be better suited to adapt to adverse environments and/or be more economically suitable.

**References**


Table I: ERAD proteins in yeast, humans and plants. Accession numbers, predicted protein size, conserved domain, function and predicted subcellular location are shown.

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<tr>
<th>Protein (Other names)</th>
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<td>Cdc48 (p97 or VCP)</td>
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<td>Derlin3</td>
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1Location for poplar sequences and their predicted size are based on information from the preliminary draft of the poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html).


3The presence of domain and subcellular location were described in human cells.
Figure 1: Amino acid alignment of Ufd1 sequences. Domains proposed to bind Cdc48 and Npl4 are underlined in the human sequence (Bruderer et al., 2004). Accession numbers or chromosomal locations for sequences provided in Table I. Amino acids shown in red are identical in all sequences.
yeast (1) MFSGFSSFGGNGFNMPQT FEEFRCYPIAMMNDRIRKDDANFGGKIFLPPSALSKLSM
arabidopsis (1) MF--FDG--GYH-----HGTGFEQTYRCYFASFIEKPQ----IESGDKIIMPFPSSDLRAS
poplar (1) MF--FDG--GYH-----HGTGFEQTYRCYFASFIEKPQ----IESGDKVIMPFPSSDLRAS
rice (1) MF--FDG--FYH-----HGTGFEQTYRCYFASFIEKPQ----IESGDKIIMPFPSSDLRAS
mouse (1) MF--FDG--FYH-----HGTGFEQTYRCYFASFIEKPQ----IESGDKIIMPFPSSDLQ13SR
human (1) MF--FDG--FYH-----HGTGFEQTYRCYFASFIEKPQ----IESGDKIIMPFPSSDLQ13SR

yeast (61) LNIRYPMFLKLTANETGRVTHGGVLEFIAEEGRVYLPQWMMETLGIQPGSLLQISSTDVP
arabidopsis (49) LHIDYPMLFELRNAGIERVTHCGVLEFIAEEGMIYMPWMLLQEGDIVVRNVTLP
poplar (49) LHIDYPMLFELQNDAAERVSHCGVLEFIAEEGMIYMPWMLLQEGDIVVRNVTLP
rice (49) LHIEYPMLFELHNDATQRISHCGVLEFVAEEGMIYMPWMLLQEGDIVVRNVTLP
mouse (59) LNITYPMFLKTNKNSDRMTHCGVLEFVAEEGICLYPLHWMNLLLEEGDIVVRNVTLP
human (59) LNITYPMFLKTNKNSDRMTHCGVLEFVAEEGICLYPLHWMNLLLEEGDIVVRNVTLP

yeast (121) LGQFVKLEFQSVDLDISDPKAVLENVLRNFSLTVDVDVIESYNGKTFKIKILEVKG
arabidopsis (109) KGTYVKKQPHTTDFLDISNPKAILETALRNYSCLTTGSDIMAYVNNKYYKIDIVETKBP--
poplar (109) KGKYVKKQPHTTDFLDISNPKAILETTLRNYSCLTTGSDIMAYVNNKYYQIDIVETKBP--
rice (109) KGTYVKKQPHTTDFLDISNPKAILEKTLLRNKSLICTTGGSDIMAYVNNKYYQIDIVETKBP--
mouse (119) VATYKSFQPQPSDFDLTNPVKALENRNFACTMTGDIATIYNKEIYELRVEK\nhuman (119) VATYKSFQPQPSDFDLTNPVKALENRNFACTMTGDIATIYNKEIYELRVEK--

yeast (181) SSISCVETTLVTFDPAPPVFGYVFPYPDK-ALKAQQDVKNSFGKQVLDPSVGQGQMS
arabidopsis (167) -ANAISIIECTEVDFAPLDNYKEPR-TAPS-AKGGGAAEEVQGQPEKFPNPGPS
poplar (167) -SNAISIIECTEVDFAPLDNYKEPR-VASVVEPKATSQAEEVPAEKEFKPNPGPS
rice (167) -SAVSIIECTEVDFAPLDNYKEPR-VASVVEPKATSQAEEVPAEKEFKPNPGPS
mouse (177) -DKAVSIIECMNFVDAFPLGYKEPRQ-VQHEESIEG--EADHSGYAGEVGFRAFSS
human (177) -DKAVSIIECMNFVDAFPLGYKEPRQ-VQHEESIEG--EADHSGYAGEVGFRAFSS

Cdc48 binding domain

yeast (240) TRIDYAGIANSSRNKLSK---------FVGQGQNISGKAPKAEPKQDIKDMKITFDGEPA
arabidopsis (224) RRDLGDRPLAYEPA PASSS--KGKQP-VVANGNGQSVSASSSEKATR-AQQLVFGAN-G
poplar (225) RRDLGKPLSYPQPPALSSVSDKQKAP--AVVAGSQRPSLGSQSSNQARKSGKLVFGSNGT
rice (226) RRDLGKASQKPLYEAPQVPSAASPSGSDNKNQETLQAPASSQASSTRQSKGKLVFGSANA
mouse (233) NRRDGKQKVEPSPSPIK-----------------PDGKIGKIPN--YEYFKLGKTFIRN-S
human (233) NRRDGKQKVEPSPSPIK-----------------PDGKIGKIPN--YEYFKLGKTFIRN-S

Npl4 binding domain

yeast (291) KKLDFEPQGQFFGMPNPCKEDEEESAAAGSNSQFQGQGIKLNSKRRKTKSDDSHSDSKK
arabidopsis (278) NRRAPKEAPAKGVAEKKEEKEKQDPKQFAFSQKSYSLRQ-------------
poplar (283) GRTPKETQ-REESGKEEQPEGKKEPKQFQFTGKSYSLKQ-------------
rice (255) NNNKEQPK------------------KAVSKDEXPCKDKEPKQFAFSQKSYSLKQ-------------
mouse (275) RPLVKKVE-------------------EDEAGGGFVAFSGEQGSLKKGKGRK
human (275) RPLVKKVE-------------------EDEAGGGFVAFSGEQGSLKKGKGRK

yeast (351) APKSPEVIEID
arabidopsis (320) ----------------
poplar (324) ----------------
rice (321) ----------------
mouse (308) ----------------
human (308) ----------------
**Figure 2:** Amino acid alignment of putative Arabidopsis, human and yeast Hrd1 sequences. Arabidopsis sequences are used as an example for the differences among the predicted Hrd1 proteins in plants. The predicted RING finger domain is underlined in the yeast sequence. Amino acids shown in red are identical in all sequences.
At5g51450    (1) MGITYLHISVATTALSFVGLQWTELSLDRADGIITKNISLGNSENTLELLLS-SHTT
At4g25230    (1) MGITYLHISVATTALSFVGLQWTELSLDRADGIITKNISLGNSENTLELLLS-SHTT
At3g16090    (1) ------------------MIRLRTYAGLSFMATLAVIYHAFSSRGQFYPATVYLSTKIS
At1g65040    (1) ------------------MPRTAVMAASLALTGAVVAHVAYLHQQFYPATVYLSTKIS
human    (1) ------------------MVQVENRRKALAIFFVVFYLLYLYCYSATKTSVSLQVTLEEG

At5g51450    (60) IALLASFVLNIYILLVLSLKTLFFGDLYAIETRKLVERLANYIIYKGTFLPFVVPRTVFQ
At4g25230    (60) IALLTNFVLNYILLVLSLKTLFFGDLYDVETKKLVERLANYIIYKGTFLPLVIPPTIFQ
At3g16090    (43) LVLLLNLCLVLMLSLWHLVKFVFLGSLREAEVERLNEQANRELMELAIFFIQDFESS
At1g65040    (43) ----------------------LF-------------------
human    (43) MAVLYIQAFVLVFLLGKVMGKVFFGQLRAAEMEHLLFSNVLAVLAVDQYI

At5g51450    (120) GVLWTIWLTVLCTLKMFQALARDRLDRLNA----SPSSTPWTYFRVYSALFMVLSTDLCW
At4g25230    (120) GVLWTVWLTVLCTLKMFQALARDRLERLNA----SPSSTPWTYFRVYSLFVLVSDLW
At3g16090    (103) ---FLPLVVTLLLIKALHWLAQKRVEYIET----TPSVKLHFRIVSFMPFLLVVDSLE
At1g65040    (45) ----------------------LF-------------------
human    (103) ---FVALFTLLLFLKCFHWLAEDRVDFMER----SPNISLWFCRIVSLFGLFLDLPF

At5g51450    (176) IKLSLMIYN------TVGSSVYLLLFPCIGAETFQALLHGQFDWLMNHLAVKNS
At4g25230    (176) IKLSLMTYN------TIGASYLLLLLFFPCIGAETFQALLHGQFDWLMNHLAVKNS
At3g16090    (156) MYSSIRHLIQ-----SRQASVQLVFGFEAYLMTMVLTIFIKVLYHSLQDS--
At1g65040    (85) ----------------------LF-------------------
human    (156) VS-HAYHSLIL---------------TRGASVQLVFGFEAYLMTMVLTIFIKVLYHSLQDS--

At5g51450    (230) DQCRSKFDSMTAGSLLEW-KGLLN------------------RNLGFFLDMATLVMALG
At4g25230    (230) DQCRSKFDSMTAGSLLEW-KGLLN------------------RNLGFFLDMATLVMALG
At3g16090    (203) ----------------------LF-------------------
At1g65040    (132) ----------------------LF-------------------
human    (202) ----------------------LF-------------------

At5g51450    (271) HYLHIWWLHGMAFHLVDAVLFLNIRALLSSILKRIGYIKLRIALGALHAALDATSEEL
At4g25230    (271) HYLHIWWLHGIAFHLVDAVLFLNIRALLSSILKRIGYIKLRIALGALHAALDATSEEL
At3g16090    (231) FFFVIFMNYGVPLHLLRELYET-----FRNFQIRVSDLYRKYRITSGNDRPFDFAPPE
At1g65040    (160) FFFVIFMNYGLPHLHRELYET-----FRNFQIRVSDLYRKYRITSGNDRPFDFAPPE
human    (230) FMTIMIKVHTFPLFAIRPMYLA-----MRQFQKAVTDAIMSRRAINNTLYDAPFDE

At5g51450    (331) R---DYDDECAICREPMAK-------------AKRLHNHLFHLGCRLSLWLDQGINVEVS
At4g25230    (331) R---AYDDECAICREPMAK-------------AKRLHNHLFHLGCRLSLWLDQGINVEVS
At3g16090    (286) ----------------------LF-------------------
At1g65040    (215) ----------------------LF-------------------
human    (285) Q---AMDNVCIICREEMVTG------------AKRLPCGNKSLKLMNKLWLDQGINVEVS

At5g51450    (375) CPTCRKPLFVGRTENAEPSREGEVSDEHLARQF-------------------
At4g25230    (375) CPTCRKPLFVGRTENAEPSREGEVSDEHLARQF-------------------
At3g16090    (326) ----------------------LF-------------------
At1g65040    (255) ----------------------LF-------------------
human    (326) CPTCRMDVLASLPQSPSFPEPADGQPPPHPPPLQPPFPQPLFPLFPMLFM

At5g51450    (396) CPTCRKFVDEKGNVQTTFTSNSDITQTIVDTSTG-------------------
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<tr>
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<td>-----------</td>
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**Figure 3:** Comparison of amino acid sequences for animal ubc6e and predicted plant ubc6e proteins. The invariant active-site cysteine in the conserved catalytic core is indicated with a number sign. Sequence surrounding the serine residue 184 in the human ubc6e human sequence is underlined and the serine residue is indicated by a star. Amino acids shown in red are identical in all sequences.
Hsubc6e (1) --METRYNLKSPAVKRLMKEAAELK-DPTDHYHAQPLEDNLEFWHFTVTGPPDSDFDGGV
mouseubc6e (1) --METRYNLKSPAVKRLMKEAAELK-DPTDHYHAQPLEDNLEFWHFTVTGPPDSDFDGGV
galusubc6e (1) --MEARYNLKSPAVKRLMKEAAELK-DPTDHYHAQPLEDNLFEWHFTVRGPPDSDFDGGV
xenoubc6e (1) --MEARYNLKSPAVKRLMKEAAELK-DPTDHYHAQPLEDNLFEWHFTVRGPPDSDFDGGV
Celegubc6e (1) --MEARYNLKSPAVKRLMKEAAELK-DPTDHYHAQPLEDNLFEWHFTVRGPPDSDFDGGV
LG_VIIII0439 (1) --MAEDKYNPVARLQKVEMQNSPSSDFFMPLPLEEFNQFA1RGCPGETEFEGGI
LG_X1296 (1) --MAEDKYNPVARLQKVEMQNSPSSDFFMPLPLEEFNQFA1RGCPGETEFEGGI
At3g17000 (1) --MAEDKYNPVARLQKVEMQNSPSSDFFMPLPLEEFNQFA1RGCPGETEFEGGI
OS03g19500 (1) --MAEDKYNPVARLQKVEMQNSPSSDFFMPLPLEEFNQFA1RGCPGETEFEGGI

Haubc6e (58) YHGRIVLPPEYPMKPPSIILLTANGRFEVGKKICLSISGHHPETWQPSWSIRTALLAIIG
mouseubc6e (58) YHGRIVLPPEYPMKPPSIILLTANGRFEVGKKICLSISGHHPETWQPSWSIRTALLAIIG
galusubc6e (58) YHGRIVLPPEYPMKPPSIILLTANGRFEVGKKICLSISGHHPETWQPSWSIRTALLAIIG
xenoubc6e (43) YHGRIVLPPEYPMKPPSIILLTANGRFEVGKKICLSISGHHPETWQPSWSIRTALLAIIG
Celegubc6e (58) YHGRIIFPADYPMKPPNLILLTPNGRFELNKKVCLSISGYHPETWLPSWSIRTLALLIG
LG_VIII0439 (60) YHGRIQLPAEYPFKPPSFMLLTPNGRFETQTKICLSISNYHPHQSWSVRVTALVALIA
LG_X1296 (60) YHGRIQLPAEYPFKPPSFMLLTPNGRFETQTKICLSISNYHPHQSWSVRVTALVALIA
At3g17000 (60) YHGRIQLPAEYPFKPPSFMLLTPNGRFETQTKICLSISNYHPHQSWSVRVTALVALIA
OS03g19500 (61) YHGRIQLPSDYPFKPPSFMLLTPSGRFEIQKKICLSISNYHPHQSWSVRVTALVALIA

Haubc6e (118) FMPTKGEGAIGSLDYTPEERRALAKKSQDFCCEGCGSAMKDVLLPLKSQSGDSSQADQEAK
mouseubc6e (118) FMPTKGEGAIGSLDYTPEERRALAKKSQDFCCEGCGSAMKDVLLPLKSQSGDSSQADQEAK
galusubc6e (118) FMPTKGEGAIGSLDYTPEERRALAKKSQDFCCEGCGSAMKDVLLPLKSQSGDSSQADQEAK
xenoubc6e (103) FMPTKGEGAIGSLDYTPEERKALAKRSQDYFCEVCMKTALLPLTSGSGSSQADKEAK
Celegubc6e (118) FLPSTPGGALGSLDYPPKERQRLAKLSCEWKCKECGCVMKTALLPITE-DGQLKQTEEAK
LG_VIII0439 (120) FMPTSPNGALGSLDYKKEERRVLAVKSREAAPRFGTPERQKLIDEIHQYMLSKAPSVPQ
LG_X1296 (120) FMPTSPNGALGSLDYKKEERRVLAVKSREAAPRFGTPERQKLIDEIHQYMLGKAPSVPQ
At3g17000 (120) FMPTSPNGALGSLDYKKEERRVLAVKSREAAPRFGTPERQKLIDEIHQYILSKATVVPK
OS03g19500 (121) FMPTPGGGALGSLDFKKEDRRALAIKSRETPPKFGSAERQKVIDEIQEVGSLRAPPVPQ

Haubc6e (178) ELARQISFKAEVNSSGKTISE------SDLNQCSLSNDQSDLPTTFQGATASTSYGLNQNSS
mouseubc6e (178) ELARQISFKAEVNSSGKTISE------SDLNQCSLSNDQSDLPTTFQGATASTSYGLNQNSS
galusubc6e (178) ELARQISFKAEVNSSGKTISE------SDLNQCSLSNDQSDLPTTFQGATASTSYGLNQNSS
xenoubc6e (161) ELARQISFKAEVNSSGKTISE------SDLNQCSLSNDQSDLPTTFQGATASTSYGLNQNSS
Celegubc6e (177) TLAAQLKFQDESVVKKEVEAA----NNQKNPTETPSETSTSVVTVESESDEADERE
LG_VIIII0439 (179) QNPAQGSEEH------PNNSVC------ETQESSPDAEAATVDLNPANGQIGEVQGVFTVE
LG_X1296 (179) QNSVQGSEEH------PNNSVC------ETQESSPDAEAATVDLNPANGQIGEVQGVFTVE
At3g17000 (179) PLPLECSQAP------SIVSEA------HSQVEPQEAITVEESSATTTIDEVDQIIETEAE
OS03g19500 (180) LLTNETNEETNQPASDASDE------HAHKAVGVNTAGLSDNSVNDLPRDSEIEQVVE

Haubc6e (234) AASFHQPTQPVAKNTSMSPRQRRAQQQSQRRLSTSPDVIQGHQPRDNHTDHGS---AVL
mouseubc6e (234) GAPLPQPTQPAPKNTSMSPRQRRAQQQSQRRLSTSPDVIQGHQPRDNHTDHGS---AVL
galusubc6e (238) TAPGQERAPSVPTNTSLSPRQRRAQQHSQRWAPASTDFNQHQQPRDNHTDHGS---AVL
xenoubc6e (218) PTP--------STNVSRSPRQRRAQQ---RRIPASTGLIQVQQH-AVNGSNTGS---AVL
Celegubc6e (233) GTT--------VNVNSSEVPDVAQPVQPRQDRPSLQFQHAARLASTNFYDLKIPV
LG_VIIII0439 (230) QVH--------EAHITNPSPAGTSAEVPAKCSQDQLQRQVTRQVKPPADRFLETFWA
LG_X1296 (228) EVH--------EAPVWNNSPSTGTSVREIRAPGTDQLPVRQVTRQKFAPADRFLETFWAA
At3g17000 (230) AVN--------TAASVPPAAPLPVESVVKASVESREMQARAA--QKPVDDRLFTWAA
OS03g19500 (236) IVE--------GRTEGVSNHSRNLSERNIPRAVTPQ------NPVVAIQKPKHDLRLTLAA

Haubc6e (291) IVILTLALAALIFRRIYLANEYIFD---FEL
mouseubc6e (291) IIILTLALAALIFRRIYLANEYIFD---FEL
galusubc6e (294) IVLLTFALALALIFRRIYLANEYIFD---FEL
xenoubc6e (263) IVVILTLALAALIFRRIYLANEYIFD---FEL
Celegubc6e (285) IALCFIFITLALARRFLIVDLNTPSIEG
LG_VIIII0439 (282) VGMLATAVLLKKFMKSSGYSQFMGMDGS---AVL
LG_X1296 (280) VGLTIAVMVLLKKFMKSSGYSQFMGMDGS---AVL
At3g17000 (280) VGLTIAVMVLLKKFMKSSGYSQFMGMDGS---AVL
OS03g19500 (285) FGLTLAIMALVIFKFKKINGL-AGYIEGK
Figure 4: Bayesian consensus tree of Derlin proteins resulting from analysis of protein sequences using MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Posterior probability values are shown above internodes along with maximum parsimony values in parentheses. The tree was rooted with the yeast Dfm1 protein sequence (accession number NP_010699) which shows both sequence and topology similarity to Der1p (Hitt ad Wolf, 2004). Protein sequences were aligned using the ClustalX program. For analysis using Bayesian inference 400 trees were discarded as the burn-in, based on the stationary phase. For maximum-likelihood analysis the program PHYLIP was used (Felsenstein, 1989). Abbreviations for species shown in the tree are as follows: Hs, Homo sapiens (accession numbers Derlin1 NP_077271, Derlin2 Q9GZP9, Derlin3a NP_940842, Derlin3b NP_001002862); Mm, Mus musculus (accession numbers Derlin1 Q99J56, Derlin2 Q8BN14); Os, Oriza sativa (accession numbers OsDerlin1 LOC_Os05g09550, OsDerlin2 LOC_Os03g63520); At, Arabidopsis thaliana (accession numbers AtDerlin2-1 At4g21810, AtDerlin2-2 At4g21810); Sc, Saccharomyces cerevisiae (accession number NP_009760); Zm, Zea mays (accession numbers ZmDerlin1-1 AY854013, ZmDerlin1-2 854014, ZmDerlin2-1 854015, ZmDerlin2-2 854016) and Pt, Populus trichocarpa (accession numbers PtDerlin1-1 scaffold_86000046, PtDerlin1-2 LG_VI0994, PtDerlin2-1 LG_IV1734, PtDerlin2-2 LG_XII1160). The nomenclature used for the predicted Derlin sequences in poplar and Arabidopsis do not represent their similarity to the previous annotated maize proteins (Kirst et al., 2005).
CHARACTERIZATION OF DERLIN1 AND -2 IN MAIZE TISSUES AND THROUGHOUT SEED DEVELOPMENT

Abstract

The degradation machinery associated with the ER has been reported in several organisms although little is known about the proteins of this pathway in plants. Here we report the characterization of two putative ERAD proteins in maize, ZmDerlin1 and ZmDerlin2.

Even though the genes encoding the ZmDerlin proteins are transcribed at low levels in several tissues and organs, ZmDerlin1 and ZmDerlin2 proteins are not necessarily detected in those tissues. ZmDerlin1 is detected in ear shoots while ZmDerlin2 is not, although both proteins are detected in the kernels. In seeds ZmDerlin1 and ZmDerlin2 appear to have distinctive properties that are reflected in their extraction efficiency at late stages of seed development.

Introduction

A fully functional quality control system in the ER is required to facilitate the folding and assembly of newly synthesized secretory proteins (Vitale and Denecke, 1999, Ellgaard and Helenius, 2003). In the case of synthesis of defective proteins the ER quality control system responds to the accumulation of misfolded proteins by eliciting the unfolded protein response (Hampton, 2000, Patil and Walter, 2001). Among the downstream pathways that are affected by the accumulation of unfolded proteins is the ER-associated degradation (ERAD) which is up-regulated to aid in the elimination of misfolded proteins that saturate the ER machinery.

There are several proteins that participate in ERAD. Some of those proteins are co-opted from other pathways while others have so far only been implicated on ERAD (Pilon et al., 1997, Plemper et al., 1997, Bordallo et al., 1998, Molinari et al., 2002). Although the ERAD pathway has been identified in plants, very little is known about the proteins that are part of this pathway. To date, the few players implicated in ERAD in plants are the proteasome, Cdc48 and Derlin proteins. The proteasome was implicated in ERAD mostly by the use of proteasome inhibitors that led to the accumulation of putative ERAD substrates in the cytosol (DiCola et al., 2001, Muller et al., 2005). Another protein whose ERAD function
was inferred by the accumulation of misfolded substrate is Cdc48. Cdc48 is one member of a protein complex that removes ERAD substrates from the ER membrane in an ATP-dependent fashion. In Arabidopsis cells expressing a dominant-negative form of Cdc48, which is inactive in its ATPase domain, there was an increased accumulation of the mildew resistance O (MLO) substrate in the cell (Muller et al., 2005). The Derlin protein was one of the elements of the ERAD pathway in plants to be identified through its sequence homology to ERAD proteins from other organisms. In plants, in contrast to yeast which has only one gene encoding Der1p, there are four genes that encode Der1-like or Derlin proteins. Those genes can be separated in two classes of proteins called Derlin1 and Derlin2 (Kirst et al., 2005). Genes encoding a protein from each of the classes were shown to be functionally conserved by complementing a yeast Der1 mutant. In humans, three Derlin proteins were described which suggests that higher eukaryotes have several genes that encode putative Derlin proteins. Like the plant and yeast proteins, the human Derlins are transmembrane proteins whose genes are induced by ER stress (Ye et al., 2004, Oda et al., 2006). Derlin proteins are possibly the only ERAD associated protein still lacking a proven function. To address the role of the Derlin proteins, research in animal cells has focused on the protein interactions. Immunoprecipitation assays for the Derlin1 protein from virus-infected cells recovered complexes containing both glycosylated and unglycosylated forms of the ERAD substrate MHC I (Lilley and Ploegh, 2004). Because those forms of the substrate are found in the ER lumen and cytosol, respectively, these findings showed that Derlin1 protein can associate both with luminal and cytosolic substrates (Lilley and Ploegh, 2004). Besides associating with ERAD substrates, Derlin1 also is present in complexes with other ERAD components such as Cdc48, Hrd1 and Hrd3 (Lilley and Ploegh, 2005, Ye et al., 2005). Whenever the Derlin proteins were expressed with a GFP or a c-myc epitope tag associations in vivo between Derlin1 and Derlin2 proteins were observed. These associations, however, were not always observed when endogenous levels of the two proteins were assayed (Lilley and Ploegh., 2005, Oda et al., 2006). Because the ERAD substrates reported to associate with the Derlin proteins are glycoproteins, Oda and co-workers (2006) asked if the Derlin proteins could also associate with the ER degradation enhancing mannosidase-like protein (EDEM) which is also involved in ERAD. Immunoprecipitation from cells transfected with tagged EDEM and Derlin1 or Derlin2 recovered both Derlin proteins in complexes containing
Cdc48 but only Derlin2 was recovered in complexes containing EDEM (Oda et al., 2006). In our previous work we observed that the maize Derlin1 was more abundant in tissues under ER stress than unstressed tissues while Derlin2 was the opposite (Kirst et al., 2005). To further investigate the differences between ZmDerlin1 and ZmDerlin2 we extended our analysis to other tissues and stages of seed development. Here we describe our observations that suggest that ZmDerlin1 and ZmDerlin2 proteins have different physical properties and/or are present in independent complexes.

**Results**

**ZmDerlin proteins tissue distribution**

As required components for ERAD, ZmDerlin proteins would be expected to accumulate in all plant tissues. We previously showed through massively parallel signature sequencing (MPSS) that transcripts from ZmDerlin1-1, ZmDerlin2-1 and ZmDerlin2-2 are widely detected in several plant tissues, although their relative levels differed from tissue to tissue (Kirst et al., 2005). For example, ZmDerlin1-1 was the most abundant transcript in all analyzed tissues while ZmDerlin1-2 was detected only in roots and endosperm. ZmDerlin2-2 was most abundant in ear shoots and endosperm from seeds at mid-stages of development (Kirst et al., 2005). Interestingly, when some of the tissues for which gene expression had been analyzed were assayed for the accumulation of ZmDerlin proteins their distribution was not coincident with gene expression. Following electrophoresis and immunoblot detection with specific antibodies we could place ZmDerlin1 protein in ear shoot, leaf, hypocotyls and endosperm (Figure 1). ZmDerlin2 protein was not detected in any of the analyzed samples when a total of 10 μg of protein was loaded onto the gel. The discrepancy between the accumulation of the ZmDerlin proteins and the expression of the encoding genes was very intriguing. This was the case especially for the ZmDerlin1-1 gene in roots which exhibit the higher transcript level but had no detectable ZmDerlin1 protein.

**ZmDerlin1 and 2 proteins are detected in seeds but only ZmDerlin1 is detected in ear shoots**

We previously showed that ZmDerlin proteins accumulate to different levels in normal (+) and ER stressed (fl2) maize endosperm. Because the tissue distribution of ZmDerlin2 suggested that this protein was not present in ear shoots, even though RNA for
the ZmDerlin2 genes was loaded onto polysomes (Figure 2), we assayed for its accumulation in seeds as early as 6 DAP. Total proteins from ear shoots and kernels of normal and fl2 samples were extracted with 1:2 (w:v) 2x SDS sample buffer (Laemmli, 1970) and fractionated through polyacrylamide gels (Figure 3). Immunoblot detection of ZmDerlin1 protein showed that the protein is present in ear shoots and kernels although a higher molecular weight band that cross-reacted with the antibody was observed both in normal and fl2 samples in the endosperm tissue compared to the ear shoot and kernels at later developmental stages. Although the ZmDerlin2 protein was not detected in ear shoot tissue, the protein was detected in seeds as early as 6 DAP which is a few days prior to the detection of storage proteins. In general the molecular chaperones BiP, calnexin and calreticulin produced equivalent signals in normal unstressed tissues and stronger signals following synthesis of defective storage proteins during ER stress (12 and 18 DAP, fl2 samples).

**ZmDerlin proteins are initially associated with the ER**

At 18 DAP both ZmDerlin1 and ZmDerlin2 proteins from normal and fl2 endosperm tissue are found preferentially associated with the protein body fraction instead of the ER while the molecular chaperone calnexin is equally distributed between the two organelles (Kirst et al., 2005). Because leaves, ear shoots and very young seeds (6 and 8 DAP) lack protein bodies we asked if in the absence of protein bodies ZmDerlin proteins were associated with the ER. To determine where the ZmDerlin proteins were localized we separated ER into cisternal and protein body or denser fractions (Gillikin et al., 1995). Figure 4 shows proteins from leaves, ear shoots and kernels at 6, 8 and 12 DAP loaded by equivalent fresh weights and probed for ZmDerlin1, ZmDerlin2, the molecular chaperones BiP and calnexin/calreticulin and the α-zein storage proteins. ZmDerlin1, calnexin and BiP were detected in the ER fractions from all tissues but not in the denser fraction from leaf tissue which could contain thylakoid membranes. Those proteins were also detected in the protein body fraction of kernels at 12 DAP. At this initial stage of storage protein deposition ZmDerlin1 was observed in a distribution similar to that of the molecular chaperones used as references for the distribution between ER and protein bodies. Although an upper band of approximately 37kDa cross-reacts with the ZmDerlin1 antibody in most samples this band did not compete with the antibody (data not shown), thus it is not ZmDerlin1. ZmDerlin2,
however, appears to be preferentially associated with the protein body fractions. The immunoblot probed for the $\alpha$-zein proteins showed that these proteins start to accumulate in the ER of seeds at 8 DAP and are distributed between the ER and protein body fractions at 12 DAP. Also, bands that cross-reacted with the anti-$\alpha$-zein antibody were observed in both leaf samples.

**ZmDerlin proteins are differently extracted at later stages of seed maturation**

Although we had previously demonstrated that ZmDerlin1 and ZmDerlin2 proteins exhibit distinct accumulation patterns at 18 DAP in response to ER stress, the accumulation of those proteins as starch was deposited in the cell and seeds started to dry was unknown. To characterize the two ZmDerlin proteins in ER-stressed or unstressed seeds we extracted protein from endosperm tissue of normal and $fl2$ samples. Figure 5A shows proteins extracted from seeds prior to the synthesis of storage proteins up to stages where the seeds start to dry. Samples from endosperm tissues at 10, 18, 24, 27, 30, 33 and 36 DAP were extracted with an aqueous buffer containing 7.2% (w:v) sucrose and fractioned through SDS polyacrylamide gels on an equal fresh weight equivalent basis. Immunoblot detection with ZmDerlin1, ZmDerlin2 and various molecular chaperone antibodies suggested a decrease in the accumulation of ZmDerlin1 but not ZmDerlin2 as the seed matured. However, despite the fact that the samples were loaded by equal fresh weight equivalents, Coomassie-stained gels of samples from the normal inbred line indicated that at later developmental stages when the seed is starting to desiccate less protein had been extracted. To address this problem we performed protein extraction from the same tissues using a more stringent buffer containing detergent and reducing agent that were not present in the original extraction buffer (Wallace et al., 1990). Proteins extracted with this buffer were also loaded on an equal fresh weight basis and immunoblots probed for ZmDerlin1, ZmDerlin2, BiP, calnexin and calreticulin. Figure 5B shows less fluctuation in signal for ZmDerlin1 throughout development and for the molecular chaperone controls in the normal sample at later stages. Coomassie-stained gels also showed a more even loading of the samples indicating a more efficient protein extraction than when an aqueous buffer was used.

**ZmDerlin1 protein is efficiently immunoprecipitated from 18 DAP $fl2$ endosperm**
The different solubility of the ZmDerlin1 protein in the buffers containing different amounts of detergent and reducing agent led us to question if the antibodies would recognize the proteins in their native states. Figure 5 shows a peptide competition assay performed during immunoprecipitation of ZmDerlin1 from 18 DAP fl2 membranes followed by immunoblot detection with ZmDerlin1 antibody. Because the ZmDerlin1 antibodies were produced to a peptide region in the carboxy terminal end of the protein we were able to use the same peptide region to compete for the binding of the antibody. To perform the competition and show that the immunoprecipitated band was ZmDerlin1 we pre-incubated the ZmDerlin1 antibody coupled to protein agarose A with either its specific peptide or the peptide for an unrelated protein. The specificity of the ZmDerlin1 band is observed by the immunoprecipitation of a band of approximately 25kDa either with the ZmDerlin1 antibody alone or in the presence of the unrelated peptide, but not for samples where the ZmDerlin1 peptide was present. The ability to immunoprecipitate ZmDerlin1 proteins from endosperm extracts also indicate to us that at least some ZmDerlin1 may not be present in complexes or resides in complexes that do not restrict its detection by the antibody.

Discussion

The data presented here suggested a difference in the accumulation of ZmDerlin1 and ZmDerlin2 proteins. This is of interest because human Derlin proteins were reported to form hetereocomplexes in steady-state labeled cells (Lilley and Ploegh, 2005). If we assume that the plant and human homologues have the same proposed function and interaction partners we would hypothesize that the plant Derlin proteins would also be present in a common complex. However, ZmDerlin1 protein is detected at quite different levels in the various tissues and organs analyzed while ZmDerlin2 protein was often not detected at all (Figure 1). Despite the presence of ZmDerlin transcripts in the analyzed tissues and organs, in general, an expression level of less than 100 parts per million was observed for the ZmDerlin2 and ZmDerlin1-2 genes while for ZmDerlin1-1 genes the expression level ranged from 80 to 333 parts per million (Kirst et al., 2005). The relatively low gene expression could in part explain the lack of detection if the ZmDerlin proteins were present in amounts below the detection limits of the immunoblots. It is also possible that the ZmDerlin proteins are modified in the different tissues in a way that would hinder their detection by our antibodies. Despite the low
abundance of gene expression for ZmDerlin2 in ear shoots we observed that the RNA was loaded onto polysomes but the protein was not detected (Figure 2). Because ear shoots are the maternal tissue that will develop into kernels the absence of ZmDerlin2 proteins could also indicate that those proteins are preferentially present in paternal tissues over maternal ones and once the plant has been fertilized they are present in the kernels. Interestingly, in seeds from the normal line there is a slight and gradual increase in the accumulation of the ZmDerlin proteins but not the molecular chaperones used as reference once the storage proteins start to accumulate (Figure 3). In the fl2 samples, despite the presence of ZmDerlin proteins prior to the detection of storage proteins, an increase in the accumulation of the ZmDerlin proteins was observed which is coincident with the detection of the storage proteins (10 DAP for normal sample and 12 DAP for fl2 sample).

Because our previous work had shown that both ZmDerlin proteins were preferentially associated with protein body fractions during mid-stages of seed maturation we asked if in tissues that lack protein bodies would have ZmDerlin proteins associated with the ER. In fact, that was the case for both proteins (Figure 4). Like the molecular chaperones, ZmDerlin1 protein was detected in the ER fractions from all analyzed tissues but not in the denser fraction from leaf which perhaps contained thylakoid membranes. Interestingly, although ZmDerlin2 protein was previously detected in an 8 DAP kernel extract, this protein was not present at levels detected by the antibody in the ER fraction. This protein was though preferentially detected in the denser fraction of the 12 DAP sample which corresponds to the protein body fraction in kernels. This fraction was also enriched for the storage protein compared to the molecular chaperones and ZmDerlin1 proteins.

The shift to and preferential association of ZmDerlin proteins with the protein body fraction in seeds is intriguing since protein bodies serve as terminal storage compartments for accumulation of insoluble storage protein aggregates. In Figure 5 we observed an interesting difference in the physical properties of ZmDerlin1 and ZmDerlin2 proteins which corroborates the fact that in seeds they could be present in different complexes. A decrease in the amount of ZmDerlin1 protein extracted using an aqueous buffer lacking any detergent or reducing agent was observed earlier than for other molecular chaperones and ZmDerlin2 in fl2 samples. However, when a buffer containing both reducing agent and detergent was used ZmDerlin1 proteins were efficiently extracted. Also, an even extraction was obtained with
the more stringent buffer as observed when comparing the Coomassie-stained gels from both extraction methods. Since the only difference between those two extraction methods was the buffer used we speculate that the aqueous buffer did not disrupt the complexes containing ZmDerlin1. As a result, most of the protein was removed during the very low speed centrifugation step to remove cell debris.

Although the aqueous buffer is not as efficient in extracting ZmDerlin1, it does allow for the protein to remain in its native state. To address if a peptide antibody against ZmDerlin1 would recognize the ZmDerlin1 protein in solution we performed an immunoprecipitation assay with the antibody specific to the carboxy-terminal end of the protein. Figure 6 shows that ZmDerlin1 can be immunoprecipitated from fl2 endosperm at 18 DAP. Although ZmDerlin1 is specifically immunoprecipitated we do not know if the proteins from the immunoprecipitated pool are present in complexes or not. Immunoprecipitation assays for HsDerlin1 and HsDerlin2 proteins in tissue culture cells indicated the association of the HsDerlin proteins with other ERAD proteins (Lilley and Ploegh, 2005, Ye et al., 2005). In future studies to characterize ZmDerlin1 and ZmDerlin2 proteins it would be interesting to identify to which proteins interact with the ZmDerlin proteins and if whether or not they interact with each other. The ability to identify complexes containing the ZmDerlin proteins should help in understanding the role of these proteins both in ERAD and in seed development.

**Materials and Methods**

*Plant material*

Normal and fl2 maize (Zea mays) plants were grown and self-pollinated during the summer field seasons from 2003 to 2006 at the Central Crops Research Station, Clayton, NC. Whole ears were harvested at 6, 8, 10, 12, 18, 24, 27, 30, 33 and 36 days after pollination between 7:00 and 9:00 am and immediately frozen in liquid nitrogen. Kernels were removed and stored at −80°C until use. Ear shoots and silks were collected and stored on ice prior to being frozen in liquid nitrogen. Seeds were germinated and leaves collected from 7 days old plants, frozen in liquid nitrogen than stored at −80°C until use.
Protein extraction

Modified Wallace buffer extraction (Fig. 5B): Total protein was extracted from normal and fl2 endosperm using buffer containing 12.5 mM sodium borate pH10.0, 2% β-mercaptoethanol (v:v), 1% SDS (w:v). Endosperm tissue was ground at 1 g/2 mL of buffer using a PRO Scientific (Oxford, CT) probe for 30 seconds or until a homogeneous solution was obtained. Samples were subjected to centrifugation for 30 seconds at 300xg (4°C) to remove cell debris. Equal fresh weight equivalents of 0.8 mg of tissue were loaded for each of the developmental stages.

Protein extraction using buffer B+S (Fig. 5A): Protein was extracted as previously described (Kirst et al., 2005). Briefly, endosperm tissues were ground in buffer B+S (10 mM Tris-HCl pH 8.5 (25°C); 10 mM KCl; 5 mM MgCl2; 7.2% (w:v) sucrose), cleared from cell debris prior to separation through polyacrylamide gels. Equal fresh weight equivalents (1.25 mg tissue) were loaded for each of the developmental stages.

Total protein extraction using SDS samples buffer (Laemmli, 1970): Total protein was extracted using 2x SDS sample buffer. Tissues were ground (1 g/2mL of buffer) using mortar and pestle, boiled for 5 min then subjected to centrifugation for 3 min at 1,500xg (room temperature). Supernatants were loaded as equal fresh weight equivalents of 1.25 mg of tissue for ear shoots and endosperm tissue from 6 to 18 days after pollination (Fig. 3) or as equal protein amounts of 10 µg of protein per lane for the different tissues (Fig. 1). Total protein extracts were quantified using the Bradford protein assay (BioRad Laboratories, Hercules, CA) according to manufacturer’s instructions.

Discontinuous Sucrose Gradient

Ear shoots (2-3 cm in diameter), leaves (7 days old) and kernels at 6, 8, and 12 days after pollination were ground in 1 gram/2 mL of buffer B+S using mortar and pestle as previously described (Kirst et al., 2005). After being strained through two layers of miracloth, samples were overlaid onto a 2M sucrose pad and centrifuged at 184xg in a swinging-bucket rotor. Two mL of supernatant was overlaid on a discontinuous sucrose gradient composed of four 2 mL layers of 0.5, 1.0, 1.5 and 2.0 M sucrose in buffer B. Gradients were subjected to centrifugation at 80,000xg for 30 min in a TFT41.14 rotor. Visible layers at 1.0/1.5 (ER) and 1.5/2.0 (protein body) were collected and diluted to a sucrose concentration of less than 8%
prior to centrifugation at 40,000xg for 1 hour. Pellets were resuspended in 200 µL of 2x SDS sample buffer, boiled for 5 min then loaded onto polyacrylamide gels as equal fresh weight equivalents of 1.25 mg of tissue per lane (Fig. 4).

**Immunoprecipitation Assays**

Endosperm from normal and fl2 seeds was ground in a 1:2 (w:v) ratio of buffer B+S, centrifuged for 30 seconds at 300xg (4°C). Supernatant was centrifuged at 100,000xg in a TL100.3 rotor for 30 minutes (4°C). Pellets were resuspended in buffer containing 50 mM Tris-HCl, pH 8.0 and 1% NP-40 (v:v) and incubated on ice for 30 minutes prior to a second 100,000xg centrifugation step for 30 minutes (4°C). Supernatant was incubated with protein A agarose for 40 minutes under constant agitation (4°C), then subjected to centrifugation at 300xg for 1 minute to pellet the protein A agarose beads and any cross-reacting components. Supernatant was then incubated with anti-Derlin1 antibody coupled to protein A agarose beads overnight under constant agitation at 4°C. Protein A beads coupled to the antibody were subjected to centrifugation at 300xg for 1 minute. Supernatant was removed and beads were washed 3 times with buffer (50 mM Tris-HCl, pH 8.0 at pH 25°C and 1% (v:v) NP-40) to remove non-specific interactions. Protein A beads were resuspended in 2x SDS loading buffer (Laemmli, 1970), boiled for 5 minutes and centrifuged at 1,500xg for 1 minute. Supernatants were separated by SDS polyacrylamide gels (Fig. 6).

**Immunoblot Analysis**

Following separation through SDS polyacrylamide gels, proteins were transferred to PVDF membrane using a semi-dry transfer blotter as previously described (Kirst et al., 2005). Membranes were blocked in 1x PBS + 5% (w:v) milk for 1 hour and incubated in the presence of primary antibody (anti-derlin1, anti-derlin2, anti-alpha zeins, anti-CRT and anti-BiP) overnight at room temperature. Antibodies were diluted to 1:15,000 (anti-derlins) and 1:10,000 (alpha-zeins, CRT and BiP) in 1x PBST (1% Tween-20 (v:v)) + 5% milk (w:v). Membranes were washed several times with 1x PBST prior to incubation with secondary antibodies [goat anti-mouse (BiP) or anti-rabbit (derlins, alpha-zeins and CRT)] coupled to fluoro 680 nm (Molecular Probes, Eugene, OR). Secondary antibodies were diluted 1:10,000 in 1x PBST and incubated in the dark with membranes. Following extensive washes with 1x
PBST membranes were scanned with a Licor-Odyssey instrument according to manufacturer’s instructions (http://biosupport.licor.com/docs/whatsnew/Western-08328.pdf).

**Polysomal fractionation and RT-PCR reactions**

Polyribosomes were isolated from ear shoots and kernels at 6 and 8 DAP as described (Davis and Abe, 1995). Kernels were ground in 3 volumes of buffer containing 200mM Tris-HCl, pH 8.5 at 25°C; 60mM KCl; 50mM MgCl₂; 5mM EGTA; 0.1 mg/ml heparin; 5mM DTT; 50 μg/ml cycloheximide; PTE 3% (v:v); DOC 0.75% (v:v). After homogenization, extracts were strained through cheesecloth and centrifuged at 11,500 xg for 10 minutes. Eight hundred uL of extract was loaded onto a 15 to 60% linear sucrose gradient and subjected to centrifugation for 2 1/2 hours at 245,000 xg in a TFT41.14 rotor. Gradients were analyzed with gradient fractionator linked to an UV detector and monitored for absorbance at 254 nm. RNA from polysomal fractions was extracted by phenol:chloroform clean up followed by ethanol precipitation in the presence of 0.4 M ammonium acetate pH 5.4. cDNA was produced using Imprompt-II reverse transcriptase (Promega, Madison, WI) according to manufacturer’s instructions. One μg of RNA from each sample was incubated with reverse transcriptase at 42°C for 1 hour. PCR reactions were performed with 1 µl of cDNA for the number of cycles pre-determined for each primer set to prevent band saturation. Primers and PCR conditions for the four Derlin genes were as described (Kirst et al., 2005).

**References**


**Figure 1:** Immunoblots showing accumulation of ZmDerlin1 protein in several maize tissues from a normal W64A inbred line. The molecular chaperones BiP, calnexin and calreticulin (CNX/CRT) are shown as controls. Samples were loaded on an equal protein amount of 10 μg/lane. At this protein amount ZmDerlin2 was not detected in any tissue (data not shown).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>BiP</th>
<th>CNX/CRT</th>
<th>ZmDerlin1</th>
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<tr>
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<td>ear shoot</td>
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**Figure 2:** Association of ZmDerlin RNAs with polysomes. First-strand cDNA was synthesized from polysomal RNA fractions and amplified by PCR. Samples (ear shoots and kernels at 6 and 8 DAP from normal and *fl2* lines) are not below lanes.
Figure 3: Immunoblots showing accumulation of ZmDerlin1 and ZmDerlin2 proteins in normal and fl2 samples from ear shoots and seeds at mid-stages of maturation. Samples contained equal fresh weight equivalents (1.25 mg). Coomassie-stained gels are shown at bottom. Molecular weights (MW) of standards are shown on the side of the gels in kDa. The predicted sizes for the molecular chaperone BiP and calnexin/calreticulin (CNX/CRT) are 70 and 55-50kDa respectively. ZmDerlin1, ZmDerlin2 and alpha-zein run around 25 kDa, total zeins are 19-27 kDa in coomassie stained gels.
Figure 4: Distribution of ZmDerlin1 and ZmDerlin2 proteins in subcellular membrane fractions of leaf, ear shoot and kernel tissues from a normal W64A maize line. Fractions 1.0/1.5M (cisternal ER) and 1.5/2.0M (denser fraction or PB) were analyzed by immunobloting based on equal fresh weight equivalents (1.25 mg). The molecular chaperones BiP, calnexin and calreticulin and alpha zeins are shown as controls. Samples are: 1-leaf ER, 2-leaf PB, 3- ear shoot ER, 4- 6 DAP ER, 5- 8 DAP ER, 6- 12 DAP ER and 7- 12 DAP PB. Arrows indicate ZmDerlin1 and ZmDerlin2 proteins.
**Figure 5**: Distribution of ZmDerlin proteins across developmental stages in normal and fl2 seeds. (A) Samples were extracted using buffer B+S as described in materials and methods and loaded as equal fresh weight equivalents (1.25 mg). The molecular chaperones BiP, calnexin and calreticulin are shown as controls. Coomassie-stained gels are shown at bottom as references for the amount of protein loaded in each lane. The zein storage proteins are seen as shadows in the ZmDerlin blots. The ZmDerlins and zein proteins migrate at around 25kDa. (B) Samples were extracted using buffer containing 12.5mM Sodium Borate pH10.0, 2% (v:v) β-ME and 1% (w:v) SDS.
### Figure 5A

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- **BiP**
- **CNX/CRT**
- **ZmDerlin1**
- **ZmDerlin2**

<table>
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**Figure 5A**

- The figure shows a gel electrophoresis image with samples labeled + DAP and $\beta 2$ DAP. The samples are arranged in lanes with molecular weight markers (MW) at 75, 50, 37, 25, and 20.
Figure 5B
Figure 6: Immunoblots showing peptide competition assay for ZmDerlin1 protein. Samples were loaded as: 1- total extract; 2- protein agarose A; 3- protein agarose A coupled to ZmDerlin1 antibody; 4- protein agarose A coupled to ZmDerlin1 antibody and incubated with ZmDerlin1 peptide; 5- protein agarose A coupled to ZmDerlin1 antibody and incubated with unrelated peptide.
CONCLUSION AND PERSPECTIVES

The previous chapters have described what is known about ERAD in yeast and animal cells as well as provided a preliminary description of the ERAD proteins in plants. Also emphasis was given to our work which focused primarily on the study of expression and accumulation of ZmDerlin proteins in maize seeds. This chapter will focus on what I see as important points for the study of ERAD in plants and how we have contributed to the field.

Although the ERAD mechanism had been suggested in plants through the transport of ricin A toxins across the ER membrane into the cytosol, none of the proteins involved in the pathway had been described (DiCola et al., 2001). Our contribution comes primarily by being the first work to identify and characterize a putative ERAD protein in plants. Through the use of RNA profiling studies in maize endosperms exhibiting ER stress we identified two genes that had sequence homology to the yeast Der1 protein and were functionally conserved. Those two genes encoded proteins that we called ZmDerlin1 and ZmDerlin2. Besides our work, only one other group has attempted to describe ERAD components in plants. In their work Muller and co-workers (2005) focused primarily in the degradation of the recombinant MLO protein in Arabidopsis cells harboring a dominant-negative Cdc48 form. Although they provided a list of putative counterparts for some of the ERAD components with known function their work built on the Cdc48 protein which is one of the ERAD components shared with other pathways.

In our characterization of the ZmDerlin proteins we uncovered two more genes that also encoded proteins of the ZmDerlin1 and ZmDerlin2 classes. Expression analysis of those four genes in maize tissue exhibiting ER stress showed that not all ZmDerlin genes were up-regulated. We also observed that ZmDerlin1 and ZmDerlin2 proteins have different accumulation patterns in response to ER stress although both are preferentially associated with the protein body fraction in seeds. ZmDerlin1 shows increased accumulation in the endosperm of seeds that produce defective storage proteins while ZmDerlin2 is more abundant in the endosperm tissue of seeds that synthesize normal proteins.

The difference in the accumulation of those two classes of ZmDerlin proteins was also observed in several maize tissues and throughout seed development. Despite the fact that
gene expression was observed in the tissues analyzed for protein accumulation the
detection of the protein was not coincident with gene expression for both ZmDerlin
classes. The lack of signal for the ZmDerlin proteins in some of the tissues analyzed
could indicate either the occurrence of post-translational modifications that make the
protein undetectable by the antibody or that the overall level of those proteins in the
analyzed tissue is below the antibody detection level.

Despite the overall difference in the distribution of the two classes of ZmDerlin proteins
in the assayed tissues both classes are present in the maize seeds. Protein extraction using
different stringency buffers showed that the ZmDerlin proteins are differentially extracted
from seeds at later developmental stages. This corroborates our previous observations and
is suggestive that even though ZmDerlin1 and ZmDerlin2 proteins are functionally
conserved they could be in different complexes.

The differential extraction of the plant Derlin proteins is specially interesting because
human orthologues for the maize Derlins were proposed to form heteroligomers that
would act as a pore to allow the transport of misfolded proteins from the lumen of the ER
into the cytosol (Lilley and Ploegh, 2005). Also the human Derlin1 and Derlin2 proteins
have been shown through independent immunoprecipitation assays to be in a common
complex with other ERAD proteins such as the Cdc48 and Hrd1, as well as ubiquitinated
substrates (Ye et al., 2005, Lilley and Ploegh, 2005). The differences observed in plants
would suggest that the Derlin proteins act independently of each other. Nonetheless, it
would be interesting to address if some of the same complexes described in the animal
system are present in plants. We have taken a first step in that direction by performing
immunoprecipitation assays that efficiently recover ZmDerlin1. As part of our work we
have also identified several putative ERAD proteins in plants to which antibodies could
be developed and used for further analysis.

Besides using antibodies to determine interactions among the ZmDerlin proteins, one
could consider using mass-spectrometry to determine putative interacting partners and/or
substrates for the ZmDerlin proteins. Also because ERAD is turning out to be such a
complex machinery with more proteins being discovered as part of the complexes that
compose this pathway, extending the analysis to other proteins of the pathway would enrich our knowledge.

An interesting point that we did not attempt to address is the degradation of the defective storage proteins by ERAD. Considering that the storage proteins are the triggers of the unfolded protein response and the primary candidates for being degraded it also would be interesting to address if those are normal ERAD substrates by taking advantage of other systems. This could be done by following the accumulation of the candidate substrates in the widely described yeast ERAD mutants versus their wild-type counterpart. Although such experiment would not directly answer questions related to ERAD in plants it would provide a solid base for addressing ERAD in seeds by showing that a storage protein can be an ERAD substrate.

References


