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The ER Folding Helpers: A Connection Between Protein Maturation, Stress Responses and Plant Development

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Abstract The main resident proteins of the endoplasmic reticulum (ER) collaborate to ensure that newly synthesized secretory proteins acquire their correct conformation. Most ER residents are therefore, directly or indirectly, folding helpers and controllers of the quality of newly synthesized secretory polypeptides. Genetic approaches have revealed that these helpers are necessary for virtually any major aspect of plant life, from differentiation to reproduction to interactions with the environment. Detailed biochemical analysis on the protein–protein interactions that occur during folding in the ER has been performed on a number of model secretory proteins, and the integration between genetics and biochemistry is a major future goal of this field of plant cell biology.

1 Introduction

Structural genomics data indicate that several thousand plant proteins start their life in the endoplasmic reticulum (ER), representing well over 10% of all expressed genes (The Arabidopsis Genome Initiative 2000; Kikuchi et al. 2003). Proteins that enter the ER, collectively termed secretory proteins (because the pathway of protein delivery to the different compartments of the endomembrane system and to the cell surface is called the secretory pathway), have a wide array of functions that cover all the aspects of plant life: primary and secondary metabolism, cell division, organ development, plant reproduction, interactions with the environment, and defence and stress responses. This mass of proteins enters, mostly co-translationally, a narrow labyrinth of connected cisternae and tubules where the newly synthesized polypeptides must fold and assemble as efficiently as possible. The high protein concentration in the ER lumen could easily lead to unspecific hydrophobic interactions that would lead to permanent misfolding and consequent loss of function. To avoid this, a sophisticated system of folding helpers has evolved to operate within the ER. These comprise a set of enzymes, molecu-

lar chaperones and lectins, which form a major group of resident proteins of this compartment. It is thus expected that folding helpers of the ER are fundamental to plant life, as they are to the life of other eukaryotes. Detailed analyses of the specific interactions of helpers with their ligands, and of the effects of changes in their expression (due to mutations or overexpression), are giving exciting insights into how complex and finely regulated are both the direct molecular interactions within the ER and the effects that they have on plant life.

2 The ER Folding Helpers, Protein Quality Control and the Unfolded Protein Response

Collectively, the ER folding helpers perform a function that is termed protein quality control. (The term was coined by Hurtley and Helenius 1989; see Ellgaard and Helenius 2003 for a recent general update. See also Vitale and Denecke 1999; Vitale and Ceriotti 2004; Ceriotti and Roberts 2006, this volume, for plant-relevant aspects of this process.) Quality control probably exists anywhere protein synthesis occurs, but the peculiar functional position of the ER within the plant endomembrane system allows ER quality control to perform wider functions. All secretory proteins must first pass through the ER and, because of the topological aspects of vesicle traffic, they do not have to cross any other membrane to reach the cell surface or vacuoles. Because proteins usually cross membranes in an unfolded state, the ER is the only compartment of the cell that takes care once and for all of the maturation of a large number of proteins destined for other locations. Thus, the ER has the possibility of performing downstream regulation of gene expression by allowing only correctly folded and assembled proteins to progress along the secretory pathway to their compartment of action. ER quality control therefore performs three related functions: (1) to help newly synthesized proteins to acquire their correct conformation, (2) to retain in the ER proteins that are not yet matured, and (3) to target for degradation proteins that cannot mature properly due to genetic defects or environmental conditions. The latter two effectively ensure that malfolded or unassembled proteins do not reach their final destination where they normally carry out their function and could be harmful if present in a defective form.

ER folding helpers are expressed in all cell types investigated, even if tissue-specific differences in mRNA levels exist, and most likely reflect variability in secretory activity between different tissues (Kalinski et al. 1995; Shimoni et al. 1995; Muench et al. 1997). Even in developing bean cotyledons, where a massive proliferation of the ER occurs to support the very high levels of synthesis of storage proteins, the synthesis of the binding protein (BiP, the ER-located member of the HSP70 chaperone family) is not very

high, as estimated by its relative abundance within the SDS-PAGE pattern of total secretory radioactive polypeptides synthesized during pulse labelling (D'Amico et al. 1992). Nevertheless, an analysis of steady-state levels of microsomal proteins indicates that BiP is a major ER protein in that tissue (D'Amico et al. 1992). This can be explained by the very low turnover rate of BiP, a characteristic that is shared by calreticulin and possibly other ER helpers as well (D'Amico et al. 1992; Crofts et al 1998; Crofts et al. 1999). However, the synthesis of these ER residents is greatly increased (up to more than one order of magnitude) by imposed stresses or genetic defects that negatively affect the folding of newly synthesized secretory proteins (Vitale and Denecke 1999). This induction is termed the unfolded protein response (UPR; Rutkowski and Kaufman 2004) and is regulated not only by stress but also by developmental programmes. As mentioned above, in the absence of imposed stress it most probably parallels the normal workload of the ER in a given tissue.

A detailed description of the signal transduction mechanisms that regulate UPR is given in Ceriotti and Roberts 2006, this volume. However, for our purposes, we wish to underline that the UPR mainly, or exclusively, senses the amount of BiP that at a given moment is not active in assisting protein folding. Certain membrane proteins of the ER work as sensors that sequester BiP molecules; when the folding workload of the ER increases, BiP is released by these sensors to perform its chaperone functions (Rutkowski and Kaufman 2004). Once freed of BiP, the sensors start a cascade of events that in the end lead to the activation of specific promoter sequences that are common to the genes of ER helpers, thus increasing their transcription. Conversely, an excess of free helpers within the ER leads to an inhibition of transcription. The whole system thus guarantees that the number of ER helpers is not too low or too high.

In plants, not all genes of ER helpers respond equally to any UPR inducing agent (Denecke et al. 1995). These differences are present even within a single gene family; for example, *Arabidopsis* has three BiP genes, but one is much more highly induced than the others by the antibiotic tunicamycin, an inhibitor of glycosylation and the most widely used UPR inducer (Noh et al. 2003). In spite of these differences, the UPR often treats the whole population of helpers as a unit, even if not all members are equally involved in the folding of each secretory protein, as we will detail below. This means that the UPR is not very sophisticated in discriminating what is going on in the ER; for example, ectopic overexpression of BiP leads to an inhibition of transcription not only of endogenous BiP genes, but also of genes encoding other ER helpers (Leborgne-Castel et al. 1999). The presence of structural defects in the *floury-2* zein polypeptide, which is not a glycoprotein, also increases the expression of calreticulin, a helper that specifically acts on glycoproteins (see below) and is therefore unlikely to be involved in zein folding (Hunter et al. 2002).

3
The Individual Members

3.1
Signal Peptidase and the Removal of Signal Peptides

The enzyme signal peptidase is possibly the first member of the ER helper community encountered by nascent secretory polypeptides when entering the ER. The enzyme removes co-translationally the signal peptide present at the *N*-terminus of soluble and type I membrane secretory proteins. The reasonable hypothesis that signal peptide removal is essential for correct protein folding is supported by studies on two natural mutations in the signal peptide cleavage site of zein genes (Gillikin et al. 1997; Kim et al. 2004). Zeins are the major storage proteins of the maize seed and normally accumulate within the ER lumen as protein bodies (large electron-dense protein granules). For one of these mutants, *floury-2*, it has been shown that the mutated polypeptide remains attached to the ER membrane because of the uncleaved signal peptide (Gillikin et al. 1997). The final effect is pleiotropic: the kernels have an opaque appearance, the accumulation of storage protein is reduced, the protein bodies have deformed shapes and the UPR is activated (Marocco et al. 1991; Zhang and Boston 1992; Hunter et al. 2002). The storage proteins of maize are synthesized in the endosperm, which is anyway destined to die at the end of maize seed maturation, and therefore it is not known whether the mutations (or similar lack of cleavage of the signal peptide in other proteins) would compromise plant ER functions to a level incompatible with plant life. However, the data clearly indicate that cleavage of the signal peptide is very important for protein structural maturation within the ER.

3.2
Oligosaccharyltransferase and *N*-glycosylation

Many secretory proteins are *N*-glycosylated by oligosaccharyltransferase, a protein complex present at each protein translocation channel of the ER membrane. The enzyme transfers co-translationally the structure $Glc_3Man_9GlacNAc_2$ (termed high-mannose glycan), from a lipid-linked precursor to the N of Asn residues present in the consensus Asn-X-Ser/Thr, where X is any amino acid but proline. It is thought that *N*-glycosylation originally evolved because it has a general property of increasing the solubility of folding intermediates, thus allowing the production of a wider variety of protein structures (Helenius and Aebi 2004). Additional functions, probably evolved later, are related to quality control of folding within the ER (as detailed in the paragraph below) and protection from proteolysis. Finally, in vertebrates, processing in the Golgi apparatus allowed the formation of glycan structures that play roles in intracellular protein sorting, and in protein

clearance from intracellular fluids and in cell recognition events (Helenius and Aebi 2004). These latter functions have never been detected in plants, in spite of various efforts (Lerouge et al. 1998). The plant Golgi apparatus also modifies *N*-linked glycans, but the modifications are partly different to those of vertebrates, and are more similar to those of invertebrates. Apart from being allergenic to several animals including humans, their role is still not clear (Lerouge et al. 1998).

The bacterial antibiotic tunicamycin inhibits one of the first steps in the biosynthesis of high-mannose glycans. In the presence of tunicamycin, *Arabidopsis* does not germinate (Koizumi et al. 1999), and sycamore (*Acer pseudoplatanus* L.) cell cultures undergo apoptosis (Crosti et al. 2001), indicating that *N*-linked glycosylation is necessary for plant development and metabolism. Treatments with tunicamycin can nevertheless be performed on plant cells and tissues for several hours before cell survival is compromised, and this has allowed the role of *N*-glycosylation in many tissues and on many natural or recombinant proteins to be established. Lack of glycosylation can have a variety of effects that are not the same for each glycoprotein, and range from aggregation and inhibition of traffic (Sparvoli et al. 2000) to rapid degradation (Pagny et al. 2003), or to no consequence at all (Bollini et al. 1985). Point mutagenesis that inactivates potential glycosylation sites, followed by expression in transgenic plants has also allowed one to determine that on certain proteins the lack of glycans can still allow nearly normal folding and assembly and intracellular traffic, but may reduce the long-term stability in the compartment of accumulation, most probably because of the exposure of proteolysis-sensitive sequences (Bustos et al. 1991).

Oligosaccharyltransferase is a heteropolymer that is best characterized in *Saccharomyces cerevisiae*, where it is composed of eight different transmembrane polypeptides. Five of them are essential and six have homologues in mammals (Helenius and Aebi 2004). One of the essential genes, STT3, has two homologues in humans, STT3-A and STT3-B (Kelleher et al. 2003). Oligosaccharyltransferase containing the STT3-A isoform has high preference towards the complete $Glc_3Man_9GlacNAc_2$ structure versus intermediates of assembly of the high-mannose glycan, whereas STT3-B confers less selectivity but a higher V_{max}. The two isoforms also have tissue- and cell-type differences in expression, and it has been speculated that this may be related to different requirements for high selectivity or high efficiency of glycan transfer, depending on tissue-specific differences in the load of glycoprotein biosynthesis (Kelleher et al. 2003). *Arabidopsis* has at least two STT3 isoforms, STT3a and STT3b (Koiwa et al. 2003). T-DNA insertion mutants of STT3a have been isolated in a screen for salt/osmotic stress-sensitive mutants. *stt3a* plants are hypersensitive to NaCl, KCl and mannitol; the plants are viable, but under hyperosmotic stress conditions that do not affect wild-type plants, *stt3a* root cells swell, root tip growth is arrested, lateral roots are induced and general mitotic activity is reduced (Table 1). The plants also show unusu-

Table 1 Effects of altered expression of ER folding helpers in plants

Protein	Transformed or mutated plant	Type of transformation or mutation	Phenotype	Refs.
Oligosaccharyltransferase subunit SST3a	Arabidopsis	T-DNA insertion	Salt/osmotic stress sensitive; under stress, mitotic activity is reduced	Koiwa et al. 2003
Oligosaccharyltransferase subunit SST3b	Arabidopsis	T-DNA insertion	No phenotype	Koiwa et al. 2003
Oligosaccharyltransferase subunits SST3a and SST3b	Arabidospsis	T-DNA insertions (double mutation)	Gametophytic lethal	Koiwa et al. 2003
Oligosaccharyltransferase subunit OST48	Arabidopsis	T-DNA insertion	Seedling lethal; altered matrix polysaccharides in cell wall	Lerouxel et al. 2005
Glucosidase I	Arabidopsis	Point mutations at *knopf* locus (EMS treatment)	Seedling lethal; reduced content of cellulose	Mayer et al. 1991; Gillmor et al. 2002
α Subunit of glucosidase II	Arabidopsis	Point mutation at *rsw3* locus (EMS treatment)	Temperature-sensitive; reduced content of cellulose; sterile	Burn et al. 2002
α Subunit of glucosidase II	Arabidopsis	Ds insertion	Embryo lethal (pollen affected more than ovules)	Parinov et al. 1999; Burn et al. 2002
α Subunit of glucosidase II	Potato	Antisense inhibition (partial) of MAL 1 gene	No phenotype in greenhouse. In open field: plasmolysis altered cell walls; increased accumulation of BiP; reduced growth; reduced tuber production	Taylor et al. 2000
Calreticulin (from maize)	Tobacco cell culture	Overexpression or antisense suppression (partial), under heat-shock control	Increased (overexpression) or decreased (antisense) accumulation of calcium within membranes	Persson et al. 2001

Table 1 (continued)

Protein	Transformed or mutated plant	Type of transformation or mutation	Phenotype	Refs.
Calreticulin (from maize)	Arabidopsis	Overexpression or antisense suppression (partial), under heat-shock control	Resistance (overexpression) or higher sensitivity (antisense) to calcium depletion	Persson et al. 2001
BiP	Tobacco	Overexpression or antisense suppression (partial), under constitutive promoter	Downregulation of endogenous BiP mRNA levels (overexpression); tolerance to tunicamycin stress (overexpression); retarded root formation (especially antisense, but also overexpression)	Leborgne-Castel et al. 1999
BiP	Tobacco	Overexpression or antisense suppression (partial), under constitutive promoter	Tolerance (overexpression) or higher sensitivity (antisense) to water deficit; tolerance to tunicamycin stress (overexpression)	Alvim et al. 2001
BiP2	Arabidopsis	T-DNA insertion	Reduced secretion of pathogenesis-related proteins; reduced resistance to *Pseudomonas syringae*; hypersensitivity to salicylic acid analogues and to tunicamycin; hyperactivation of endoplasmin, calnexin and protein disulphide isomerase genes upon treatment with salicylic acid analogues	Wang et al. 2005
Endoplasmin	Arabidopsis	T-DNA insertion immediately upstream of the gene	*clavata*-like phenotypes; inhibition of pollen tube elongation and pollen fertility	Ishiguro et al. 2002
Protein disulphide isomerase	Rice (*esp2* mutation)	*N*-methyl-*N*-nitrosourea mutagenesis	Absence of detectable PDI mRNA and protein; abnormal morphology of the ER-derived protein bodies; high accumulation of proglutelin within ER-derived protein bodies	Takemoto et al. 2002

ally high water loss, indicating that the response to lower water potential is impaired. T-DNA insertion mutants of STT3b instead do not show salt sensitivity or other abnormal phenotypes, but the double *stt3a sst3b* mutants are gametophytic lethal, indicating that STT3b is less important but can in part compensate STT3a functions when this is mutated (Table 1). Transcript analysis suggests that STT3a is indeed the major isoform.

RNA interference of STT3a, but not of STT3b, elicits the UPR, again pointing to a major role of the former. The fact that the *STT3a* defect becomes evident under osmotic stress suggests that adaptation to this stress requires correct synthesis, traffic and stability of glycosylated secretory proteins. The *Arabidopsis* mutant *defective glycosylation 1-1* (*dgl1-1*) was isolated during a screen of T-DNA insertion mutants defective in growth and cell wall composition (Table 1; Lerouxel et al. 2005). The insertion affects the orthologue of the OST48 subunit of human oligosaccharyltransferase (the essential subunit WBP1 in *S. cerevisiae*). All homozygous mutated alleles of *dgl1* are lethal at the seedling stage and one severe allele, *dgl1-2*, is embryo-lethal. *dgl1-1* is a leaky allele (the T-DNA insertion is 62 bp upstream of the start ATG codon) that is still able to *N*-glycosylate proteins, albeit at a much reduced level compared to wild-type. Matrix polysaccharides, but not cellulose, are affected with increased callose deposition and arabinose content (Lerouxel et al. 2005), possibly because one or more *N*-glycosylated proteins necessary for the biosynthesis of non-cellulosic polysaccharides are defective.

3.3
Glucosidases and Glucosyltransferase, and the Calnexin/Calreticulin Cycle

The $Glc_3Man_9GlacNAc_2$ high-mannose glycan undergoes removal of the Glc residues within minutes after transfer of the glycan to the growing polypeptides (Helenius and Aebi 2004). This processing is due to the action of two ER-located enzymes: glucosidase I, which is a type II membrane protein, and glucosidase II, a soluble heterodimer. Glucosidase I removes the terminal α1,2-linked Glc residue, and glucosidase II removes the two α1,3-linked residues. A single Glc residue is then transiently re-added by UDP-Glc:glycoprotein glucosyltransferase, another ER enzyme. The resulting $Glc_1Man_9GlacNAc_2$ is again a substrate for glucosidase II. The newly synthesized glycopolypeptides thus enter a cycle of re- and de-glycosylation that puzzled cell biologists for years until it was discovered that $Glc_1Man_9GlacNAc_2$, but not $Glc_{2-3}Man_9GlacNAc_2$ or $Man_9GlacNAc_2$, are recognized and bound by two ER-located lectins, calnexin and calreticulin, and that the cycle is a form of ER quality control acting on glycoproteins (Helenius and Aebi 2004). All forms of quality control discriminate between folded and unfolded polypeptides. In this particular case the key player is the glucosyltransferase, which acts only on unfolded polypeptides. In this way, polypeptides that are not yet structurally mature undergo cycles of binding and release by the two ER-resident

lectins, and the cycle is interrupted when the polypeptide is correctly folded. Binding to the lectins avoids possible aggregation and prevents export from the ER; thus, the newly synthesized glycopolypeptides are retained in this compartment as long as they are recognized by the glucosyltransferase.

Permanently misfolded glycoproteins must also exit the cycle, to be targeted for degradation by quality control. However, obviously, folding intermediates must not be degraded. How does the system discriminate between intermediates of folding and misfolded defective polypeptides? Because of the characteristics of the cycle, permanently misfolded polypeptides reside in the ER much longer than intermediates of folding, and thus have more chances to be trimmed by an ER mannosidase, which specifically removes one of the terminal Man residues of the *N*-linked glycan. The presence of Man_8 instead of Man_9 makes the glycan a less efficient substrate for both glucosidase II and the glucosyltransferase. As a result, the cycle is slowed down and the protein is dislocated into the cytosol, probably through the help of other proteins, where it is degraded (more details on this ER-associated degradation are in the chapter by Ceriotti and Roberts 2006, this volume). Thus, the sensing mechanism is based on the length of time that a given polypeptide spends in the cycle (Helenius and Aebi 2004).

Co-immunoprecipitation experiments provided direct evidence for the association of calnexin with oat vacuolar H^+-ATPase (v-ATPase) or with plasma membrane H^+-ATPase, present in microsomes, which presumably represent the newly synthesized proteins still located in the ER (Li et al. 1998). In the case of the v-ATPase, an association occurred with a precomplex that was slightly smaller than the fully assembled pump but already contained several subunits, which demonstrated that the lectin also associates with partially assembled proteins (Li et al. 1998). Some of the ATPase subunits are glycoproteins, but which of them interacts directly with the calnexin is not known.

Evidence that the calnexin/calreticulin cycle modulates the rate of assembly of an oligomeric plant protein has been obtained by studying the synthesis of the glycoprotein phaseolin, the major storage protein of common bean seeds (Lupattelli et al. 1997). Phaseolin is the product of a small gene family that codes for the very similar α- and β- polypeptides. Phaseolin assembles into a homotrimeric glycoprotein in the ER which is then transported through the Golgi complex to storage vacuoles, where it accumulates. In an in vitro translation–translocation system, treatment with the ER glucosidase inhibitors castanospermine and *N*-methyldeoxynojirimycin increases the rate of trimerization of phaseolin polypeptides (Lupattelli et al. 1997). An increase in the rate of folding of glycoproteins when the ER glucosidases are inhibited has also been observed for a number of non-plant glycoproteins, and is probably due to a failure to enter the calnexin/calreticulin cycle. The hypothesis is that proteins do fold faster if they do not enter the cycle, but the efficiency of their folding is decreased, with a net loss of production of a structurally correct protein (Helenius and Aebi 2004).

The effects of gene inactivation of glucosidase I and II indicate that these enzymes are necessary for plant development. A T-DNA insertion into the *Arabidopsis* gene encoding glucosidase I is embryo-lethal (Boisson et al. 2001). Homozygous mutant seeds have a shrunken appearance and do not germinate. Embryo development is blocked at the heart stage, accumulation of protein (notably of storage protein) is severely inhibited and cell wall disruptions are occasionally observed. Several years before, screening of ethyl methane sulphonate-mutagenized *Arabidopsis* plants (which have point mutations) for altered embryogenesis led to the identification of several mutated alleles of the KNOPF locus (Mayer et al. 1991), which was later established to be the glucosidase I gene (Gillmor et al. 2002). A number of *knopf* point mutations allow seeds to germinate, but seedlings are not able to elongate or grow to any extent (Table 1; Gillmor et al. 2002). These mutants have strongly reduced cellulose content, and it was concluded that there is a requirement for correct removal of Glc residues in one or more glycosylated components of the cellulose synthase complex rather than the catalytic subunit itself (because this subunit does not appear to be glycosylated and its abundance is not affected in the mutants).

During another search for mutations causing defects in *Arabidopsis* seedling development, several non-allelic mutants with abnormal root radial swelling (*rsw* mutants) were isolated. One of them, *rsw3*, is a temperature-sensitive mutant with a point mutation in what is most probably the α subunit of ER glucosidase II, which contains the catalytic site of the enzyme (Table 1; Burn et al. 2002). At the restrictive temperature, *rsw3* plants have reduced cell division and produce few flowers and no seeds. The seedlings have a 50% reduction in the amount of cellulose, whereas non-cellulosic polysaccharides show little alteration; secretion of Golgi-derived seed mucilage during imbibition is also strongly reduced. A null mutant of the same allele, due to insertion of a transposable element, is embryo-lethal (Table 1; Parinov et al. 1999; Burn et al. 2002). Antisense-mediated, partial inactivation of the α subunit of potato glucosidase II (MAL 1 gene) does not result in any phenotype when plants are grown in the greenhouse, but in open-field cell plasmolysis, alterations in the cell wall and an increase in BiP accumulation (suggesting induction of UPR) occur, accompanied at the macroscopic level by reduced plant growth and tuber production (Table 1; Taylor et al. 2000). Probably, the remaining activity after antisense inhibition allows normal cell functions in the controlled greenhouse environment, but does not allow the plants to tolerate the common stresses occurring in the open field. These genetic studies point to a fundamental role of the calnexin/calreticulin cycle in the synthesis of a functional cell wall, probably because one or more of the glycoproteins necessary for cellulose production require an efficient ER quality control mechanism for their synthesis (see also Vitale 2001).

As we mentioned above, during traffic through the Golgi complex the *N*-linked glycans can be further processed by the action of glycosidases

and glycosyltransferases. These events can generate several different structures, collectively termed complex glycans, which have the common core $Man_3GlcNAc_2$ and in plants also contain to variable extents (depending on the glycoprotein) additional GlcNAc residues as well as Gal, Fuc and Xyl residues (Lerouge et al. 1998). Not all glycans become complex, probably because in certain glycoproteins the accessibility of Golgi enzymes to the high-mannose glycan is inhibited by the conformation of the glycoprotein, but complex glycans are very frequent. The first Golgi enzyme acting on the $Man_9GlcNAc_2$ high-mannose glycan is mannosidase I, which produces the structure $Man_5GlacNAc_2$, a substrate that is converted by Golgi GlcNAc-transferase I into $GlcNAc_1Man_5GlcNAc_2$. This is necessary for further processing leading to the formation of complex glycans. The *N*-linked glycans present in the glycoproteins of the *Arabidopsis* ER glucosidase I knockout have the structure $Glc_3Man_{7-8}GlacNAc_2$ and therefore no complex glycans are formed, indicating that the removal of Glc residues in the ER is also a prerequisite for the Golgi processing events (Boisson et al. 2001). However, the severe defects of the ER glucosidase I and II mutants cannot be ascribed to the absence of complex glycans on glycoproteins, because a mutant that lacks Golgi GlcNAc-transferase I activity does not show any obvious phenotype (von Schaewen et al. 1993). Therefore, the removal of Glc residues is much more critical to plant life than the extensive glycan modifications occurring in the Golgi apparatus, underlining the key role played by protein quality control in the ER.

Calreticulin also has other roles (Mariani et al. 2003). As we mentioned above, the ER chaperone BiP is a very abundant protein, and at any given moment not all BiP molecules are involved in helping the folding of newly synthesized proteins. At least in tobacco, a large fraction of these "unemployed" BiP polypeptides are associated to calreticulin, through interactions that are distinct from those between the chaperone and its folding substrates (Crofts et al. 1998). Thus, the lectin seems also to act as a store of inactive BiP polypeptides, whereas BiP polypeptides actively acting on the substrates that are in the process of folding are not associated to calreticulin (Crofts et al. 1998). A number of observations suggest that in general the ER folding helpers are in very close contact with each other, forming a sort of matrix containing one or more chaperone complexes, where newly synthesized polypeptides would be delivered from one helper to the other until correct maturation has occurred (Kleizen and Braakman 2004). If the observed interactions between plant BiP and calreticulin are within a putative larger complex, this would mean that the complex is locally disrupted when the newly synthesized polypeptides traffic though it and interact with the helpers, but this needs to be demonstrated. Furthermore, there seems to be a contradiction between the observed storage of BiP with calreticulin and the model in which unemployed BiP would be mainly associated to ER membrane proteins that function as the UPR sensors (see above).

Finally, calreticulin is a calcium-binding protein and may be a key player in the regulation of calcium storage in the ER (Mariani et al. 2003; see also Persson and Harper 2006, this volume). Calcium is certainly important for the action of ER folding helpers, because calcium ionophores are also UPR inducers, BiP is also a calcium-binding protein and calreticulin binds protein disulphide isomerase in a calcium-dependent process. However, because of the important role of calcium in signal transduction, this would also involve the lectin in the plant response to a wide range of stimuli, similar to that which occurs in animal cells (for more details see the chapter by Persson and Harper 2006, this volume). Calreticulin overexpression or partial antisense suppression in tobacco cell cultures, under the control of a heat shock promoter, lead to heat shock-induced increased or decreased accumulation of calcium in the ER, respectively (Table 1; Persson et al. 2001). The same constructs expressed in transgenic *Arabidopsis* lead to increased or decreased resistance to the stress induced by calcium depletion, respectively, as measured visually by the occurrence of chlorosis, suggesting that calcium homeostasis in the plant can be modulated by the levels of calreticulin (Table 1; Persson et al. 2001). It cannot, however, be excluded that the ER quality control role of calreticulin also contributed to this induced resistance to abiotic stress, as has been suggested for BiP overexpression induced by drought tolerance (Alvim et al. 2001, and see below).

3.4
The Luminal Binding Protein

The luminal binding protein (BiP) is one of many hsp70 proteins spread over all organismal kingdoms, including prokaryotes, as illustrated by the DnaK gene product in *Escherichia coli*. It is an ER-resident heat shock 70 cognate that is essential for the folding and maturation of newly synthesized secretory proteins (Gething 1999; Kleizen and Braakman 2004). Hsp70 proteins bind to intermediates of protein folding and assembly, misfolded proteins and peptides displaying hydrophobic regions (Blond-Elguindi et al. 1993b; Gething 1999). The classic view on the biological significance of this interaction is to prevent aggregation which could lead to permanent misfolding (Gething et al. 1986; Hurtley et al. 1989; Gething and Sambrook 1992; Hendershot et al. 1996). However, this may only be the most basic function of the interaction and much remains to be discovered.

Within the hsp70 family, BiP is extremely conserved among eukaryotic kingdoms. It was first identified as the glucose regulated protein GRP78 (Pouyssegur et al. 1977) and then as the immunoglobulin heavy chain binding protein (Haas and Wabl 1983). Both were then found to be identical and classified as a major hsp70 cognate (Munro and Pelham 1986). It carries a typical *N*-terminal ATPase domain and a *C*-terminal polypeptide binding domain (Gething 1999), but the coding region is supplemented by an additional sig-

nal peptide for entry into the ER lumen and an ER retention motif (HDEL) to allow recycling from the Golgi apparatus. The latter remains attached permanently whilst the signal peptide is cleaved after ER entry.

In plants, BiP is encoded by several genes, including eight or more isoforms in *Nicotiana tabacum* and three in *Arabidopsis thaliana*. The possible reason for such redundancy has not yet been established. The degree of conservation among different BiP genes is so high that tobacco BiP can functionally complement yeast BiP (Denecke et al. 1991). Two BiP sequences from different kingdoms have a higher sequence similarity than any other hsp70 member within the same species. This suggests that hsp70 members have evolved early in the evolution of eukaryotic cells to accommodate different cellular compartments with this group of protein folding helpers. This chaperone is one of the best studied proteins of the ER and will thus be discussed in more detail in the following subsections.

3.4.1
BiP: The Midwife of the Secretory Pathway

It was discovered over a decade ago that reticuloplasmins (proteins residing in the ER lumen) were required for the completion of translocation (Nicchitta and Blobel 1993). One of these is BiP, shown to be essential for translocation of nascent proteins across the ER membrane in vivo (Vogel et al. 1990). BiP was later shown to carry out a crucial role in Sec61-mediated protein translocation (Brodsky et al. 1993; Brodsky and Schekman 1993; Lyman and Schekman 1995, 1997; Young et al. 2001). This chaperone is also believed to seal the luminal end of the translocation pore when it is not in use (Hamman et al. 1998; Alder et al. 2005), and is thus ready to interact with any newly synthesized molecule when it arrives in the ER. Nascent polypeptides, emerging in the lumen of the ER during co-translational translocation, are still unfolded and interact with BiP which acts as a molecular ratchet in the translocation process (Matlack et al. 1999). This is thought to help in displacing the equilibrium during the transfer and encouraging the nascent chain to proceed into the lumen of the ER. Therefore, BiP can be regarded as the midwife of the secretory pathway, and any secretory protein is a potential BiP ligand during synthesis and translocation. Ironically, BiP is also a protein and its de novo synthesis, translocation and folding would theoretically require pre-existing BiP in the ER lumen. This may have interesting implications for the UPR response (see above).

3.4.2
What is a BiP Ligand?

BiP will not only associate to nascent polypeptides but also may retain affinity for certain folding intermediates that have been fully translocated, although

it may yet have to acquire their final conformation or possibly assemble with other subunits to form complexes. How BiP binds to its ligands has been investigated with synthetic peptides, and a role of hydrophobic residues has been established (Blond-Elguindi et al. 1993a,b). This has led to the working hypothesis that BiP binds to exposed hydrophobic regions of proteins. Correctly folded and assembled molecules usually only expose hydrophilic residues at the surface facing the aqueous solutions within cells, and BiP will therefore no longer bind. However, it should be noted that this concept explaining the (lack of) affinity of BiP is currently merely a working hypothesis. It may be well-founded and makes sense, but misfolded proteins are difficult to crystallize and there are no structural data to back up the hypothesis. Currently, the only definition of BiP ligands is that they can be co-immunoprecipitated with BiP after extraction from the cells, and subsequently released from the pellet by addition of ATP in vitro (Munro and Pelham 1986; Vitale et al. 1995) and, reciprocally, that BiP can also be co-precipitated and released when the ligand is precipitated first.

3.4.3 Energy Consumption by BiP

ATP-mediated release suggests an energy-dependent release mechanism, and consistently, dominant negative BiP ATPase mutants that fail to release their ligands compromise proper protein folding (Hendershot et al. 1996). Ligand binding is thought to be regulated by a communication between the ATPase domain and the peptide binding domain; the presence of ATP in the *N*-terminal binding site produces rapid low-affinity binding to ligands, whereas the ADP-bound form exhibits high-affinity binding with slow exchange. The rate of ATP hydrolysis is increased by the binding of ligands (Gething 1999) and by the interaction with HSP40 DnaJ-like co-chaperones (Cyr et al. 1994). In the yeast *S. cerevisiae*, BiP (Kar2p) has been shown to interact with three ER-resident HSP40s: Sec63p, Scj1p and Jem1p.

Experiments with the bacterial hsp70 protein DnaK have demonstrated that this chaperone can actively dissolve protein aggregates formed from malfolded proteins in an ATP-fuelled manner (Ben-Zvi et al. 2004). This suggests a more active role beyond merely preventing aggregation, but it remains to be shown if this principle is valid for all members of the hsp70 family including BiP. Thus, a model explaining the exact purpose of this energy-dependent step remains to be formulated and generally accepted, but regardless of this, ATP hydrolysis-mediated ligand release strongly suggests that the N domain influences the C domain to regulate peptide binding and release.

3.4.4 Examples of BiP Ligands

In contrast to investigations with synthetic peptides, BiP ligand-interaction studies with real proteins have been much less frequent. In vivo studies of protein folding using immunoglobulin light chains have suggested that the rate at which a protein folds and the energetic stability of the fold determine whether BiP will bind. The chaperone binds preferentially to slow-folding and unstable conformations (Hellman et al. 1999), supporting its proposed function in quality control. In the plant kingdom, several model BiP ligands are now available and can be used for analysis in vivo (Vitale et al. 1995; Pedrazzini et al. 1997; Nuttall et al. 2002; Brandizzi et al. 2003; Foresti et al. 2003; Mainieri et al. 2004; Randall et al. 2005). The individual BiP ligands used by the plant community have completely different properties and will form very useful tools to study the fate of such molecules or the way in which they interact with BiP. For instance, a secreted GFP fusion construct was shown to first enter the ER, to undergo signal peptide processing and BiP association, and then to re-localise to the cytosol until it accumulates in the nucleoplasm (Brandizzi et al. 2003). The most studied plant BiP ligand is assembly-defective phaseolin, a bean storage protein that fails to assemble into trimers and shows prolonged interactions with BiP in the ER until it is degraded (Pedrazzini et al. 1997; Foresti et al. 2003). In neither of these cases was it established how the decision is made that BiP association is no longer productive, and that instead a disposal mechanism should be initiated.

3.4.5 Moving from Chaperoning to Disposal

When folding and assembly is complete, hydrophobic regions are no longer displayed and BiP will cease to bind. This is the normal scenario when proteins acquire a range of intermediate folding states until the final structure is established. Permanently malfolded proteins can emerge when physiological conditions are unfavourable or when erroneous proteins are synthesized. In these cases, quality control mechanisms exist to dispose of these proteins. However, it is currently unknown in any eukaryotic model system how the quality control machinery discriminates between folding intermediates and permanently malfolded proteins.

Continuous binding of BiP is thought to result in ER-associated protein degradation (ERAD). The current pathway for this event leads via the translocation pore back to the cytosolic proteasome (McCracken and Brodsky 2003) and is discussed at length in Ceriotti and Roberts 2006, this volume. Evidence for the ERAD pathway in plants arose from studies with soluble proteins (Di Cola et al. 2001, 2005; Brandizzi et al. 2003) and membrane-spanning proteins (Muller et al. 2005). However, it has become clear that alternative

degradation routes in addition to the ERAD pathway exist (Ellgaard and Helenius 2003; Schmitz and Herzog 2004).

BiP is constitutively expressed under normal growth conditions, but transcription can be induced by UPR upon the accumulation of malfolded proteins in the ER (Kozutsumi et al. 1988; Denecke et al. 1991; Fontes et al. 1991; Nuttall et al. 2002). In addition, plant BiP can be induced by UPR-independent signal transduction pathways (Kalinski et al. 1995; Jelitto-Van Dooren et al. 1999) and during systemic acquired resistance (Wang et al. 2005). However, transcriptional induction of BiP seldom leads to increased BiP protein levels, even though mRNA concentrations and pulse labelling demonstrate a higher synthesis rate (Leborgne-Castel et al. 1999). This suggests that under ER stress, BiP turnover is increased, but it remains to be shown where BiP degradation occurs.

Recently, it was proposed that ER folding helpers can reach the vacuole in a constitutive manner, possibly via autophagy (Tamura et al. 2004). This was proposed to occur in a Golgi-independent manner whereby ER-resident proteins, such as protein disulphide isomerase, BiP and the HDEL-tagged GFP, would be deposited non-discriminatively into the vacuole (Tamura et al. 2004). It is unlikely that such a mechanism could be suitable for quality control, because it would be unclear how correctly folded proteins and folding intermediates would be distinguished from permanently malfolded proteins that must be disposed of. In *S. cerevisiae*, misfolded proteins, which are not retained in the ER, can be degraded in the vacuole (Ellgaard et al. 1999), but are targeted to the vacuole via the Golgi apparatus and are dependent on the yeast vacuolar sorting receptor Vps10p (Hong et al. 1996). This receptor is also responsible for the transport of correctly folded vacuolar enzymes. It was therefore suggested that Vps10p could serve in the disposal of soluble malfolded proteins via direct interaction with exposed hydrophobic regions (Hong et al. 1996; Jorgensen et al. 1999). Vps10p would then act as a general folding sensor within the process of quality control, but it is unclear how such multitasking would be accomplished.

It has also been shown that the ER chaperone calreticulin is a constituent of COPI vesicles (Pimpl et al. 2000), and that it leaves the ER in a COPII-dependent manner (Phillipson et al. 2001). This is consistent with the accepted model for the action of the KDEL receptor, which allows accumulation within the ER of its residents, and that ER residents, being ER export competent, could be degraded in a post-ER compartment such as the vacuole. Evidence for a possible degradation of BiP in the vacuole can also be derived from work on *S. cerevisiae* where inhibition of COPII-dependent ER export led to the formation of dilated ER cisternae that contained large quantities of BiP together with vacuolar proteins (Nishikawa et al. 1994). Moreover, ER retention defective (*erd2*) mutants were shown to contain induced transcription of the BiP gene (Semenza et al. 1990), possibly to compensate for the loss of BiP from the ER when recycling from the Golgi fails. In plants, deletion of the

HDEL signal (BiPΔHDEL) does not induce significant BiP secretion, and the truncated molecule is only present at low steady-state protein levels compared to those of plants overexpressing wild-type BiP (Crofts et al. 1999; Leborgne-Castel et al. 1999). Evidence has been presented that suggests a higher BiP turnover during ER stress or when BiP is overproduced (Table 1; Leborgne-Castel et al. 1999). This suggests that BiP could be degraded in a post-ER compartment, such as the vacuole, and may contain a vacuolar sorting signal. If this was proved to be true, then BiP could act as a universal adaptor to detect misfolding on one side and bind to a vacuolar sorting receptor specifically. Possibly such a system could act as a backup for the ERAD pathway, but this remains to be demonstrated.

3.4.6
BiP and Pathogen Stress

A number of conditions have been reported in the plant kingdom that lead to increased BiP synthesis without any obvious link to ER stress or misfolding, but instead to various forms of abiotic or biotic stress (Anderson et al. 1994; Kalinski et al. 1995; Jelitto-Van Dooren et al. 1999; Cascardo et al. 2000; Alvim et al. 2001; Wang et al. 2005; Table 1). It could be argued that in these cases, secretion or vacuolar deposition of defence-related proteins represent such an increase in the secretory protein synthesis that it would be accompanied by a higher error rate, and defective proteins would thus be created with a higher frequency compared to that under normal physiological conditions. If this were the sole reason, one would expect that BiP induction should occur via the normal UPR as part of a feedback mechanism, when the problem of ER stress has already manifested itself. However, evidence was obtained suggesting that BiP induction can occur independently of the classic unfolded protein response, and occur prior to a noticeable increase in the synthesis of secretory and vacuolar defence-related proteins (Jellitto-Van Dooren et al. 1999). Consistently with this notion, it has now been shown through partial gene knockout that a 50% reduction in the steady-state BiP levels renders the plants hypersensitive to the drug tunicamycin and leads to a defective pathogen response (Wang et al. 2005). This clearly shows that an up-regulation of BiP levels and possibly the entire secretory pathway machinery is required for the normal pathogen response, and that plants regulate BiP synthesis not merely via a feedback mechanism, but can actively anticipate ER stress and prepare for this. Evidence for this was also obtained by showing that plant pathogen stress can lead to rapid induction of a plasma membrane SNARE (SNARE proteins are regulators of vesicle fusion and thus of traffic within the secretory pathway), independent of the normal route downstream of the signalling molecule salicylic acid (Wick et al. 2003). This is an exciting feature of the plant kingdom that may be exploited in the future generation of crops with increased resistance to pathogen attack. Using the plant's natural

defence mechanisms rather than pesticides should yield real consumer benefits, but for this purpose it will be necessary to reveal the secrets about the UPR-independent signal transduction mechanisms that regulate BiP expression in plants.

3.5 Endoplasmin

Endoplasmin, also termed glucose regulated protein 94 (GRP94), is the ER-located member of the heat shock 90 family of chaperones. Unlike BiP, endoplasmin is a single gene in all eukaryotes in which it has been characterized, which include multicellular species and the parasitic protozoan *Leishmania infantum* (Argon and Simen 1999; Larreta et al. 2000; Krishna and Gloor 2001). A gene encoding endoplasmin has not been found in the genome of *S. cerevisiae*, indicating that this ER chaperone is not necessary for the life of a eukaryotic cell out of the context of a multicellular tissue. The name endoplasmin derives from the observation that in certain mammalian cells this is the most abundant protein of the ER. The name GRP94 instead recalls its first identification, together with BiP/GRP78, as a polypeptide of 94 kDa whose accumulation is induced by glucose starvation. HSP90 proteins are soluble homodimers and the polypeptide is composed of *N*-terminal regulatory and ligand binding domains, a charged region, a middle domain, and a *C*-terminal dimerization domain (Argon and Simen 1999). The *N*-terminal domain of GRP94 is highly similar to that of cytosolic HSP90, but the mechanisms of action seem to be different. Cytosolic HSP90 is an ATPase and requires cofactors for its activity. Endoplasmin also binds ATP, but the affinity is very low, the conformational changes induced by binding are different, and neither ATP hydrolysis nor accessory proteins seem to be necessary for endoplasmin action (Immormino et al. 2004; Rosser et al. 2004). Endoplasmin binds the unassembled chains of immunoglobulins after BiP, indicating that the in vivo recognition properties of the two chaperones are different and that endoplasmin may in general operate later than BiP during protein maturation in the ER (Melnick et al. 1994). However, a much lower number of ligands has been identified to date for endoplasmin than for BiP (Argon and Simen 1999), which could simply reflect weaker or more transient interactions or could indicate that endoplasmin acts on a limited number of proteins, consistent with the above mentioned failure to find an endoplasmin gene in the yeast genome.

Vertebrate endoplasmin also binds peptides, in particular those from foreign or altered proteins present in cells infected by viruses or affected by tumours, and it is involved in presenting such peptides as antigens to T-cells and therefore in the immune response (Lee 2001). This antigen presentation occurs extracellularly and it is not clear if the release of endoplasmin from cells occurs because of necrosis of the affected tissues, or whether it is an active, regulated secretion process (Brunati et al. 2000; Lee 2001).

In developing cotyledons of common bean, the synthesis of a resident polypeptide of the endomembrane system, with apparent molecular mass of 97 kDa, was induced upon tunicamycin treatment, suggesting that it could be bean endoplasmin (D'Amico et al. 1992). The first plant nucleotide sequence encoding endoplasmin was isolated by screening a barley cDNA library prepared from a pathogen-induced RNA population (Walther-Larsen et al. 1993), indicating that, like other plant ER folding helpers (see above), endoplasmin is involved in supporting the pathogen response. T-DNA insertion in the region immediately upstream of the *Arabidopsis* endoplasmin gene decreases mRNA accumulation to levels undetectable by RNA blot (although a very low level of protein is still synthesized—Klein and Vitale, unpublished observation) and causes enlargement of shoot apical meristems, an increased number of carpels and additional whorls in flowers (Table 1; Ishiguro et al. 2002). These phenotypes are similar to those of the weak alleles of the *clavata* (*clv*) mutations. Clavata proteins are two integral membrane proteins and one soluble, secreted protein that interact with each other in a receptor/ligand fashion, to promote the differentiation of meristem stem cells towards initiation of plant organs (Doerner 2000). Clavata proteins have antagonistic functions with respect to the transcription factor Wuschel, which supports meristem formation. In agreement with this, ectopic overexpression of CLV3 in wild-type *Arabidopsis* causes suppression of shoot meristems similarly to *wuschel (wus)* mutations. However, overexpression of CLV3 in the endoplasmin mutant has no effect, and the *wus* mutation is fully epistatic to the endoplasmin mutation (Ishiguro et al. 2002). These genetic relationships indicate that endoplasmin is necessary for the CLV functions, possibly because the chaperone is involved in promoting the correct folding of one or more CLV proteins or their complex formation (Ishiguro et al. 2002). The mutant was thus termed *shepherd* (*shd*). In addition to the *clv*-like phenotypes, *shd* also has low pollen fertility, and pollen tubes poorly elongate into the styles, suggesting that endoplasmin is also important for the high secretory activity that leads to pollen tube elongation (Ishiguro et al. 2002).

On the whole, these observations support a role for endoplasmin in the synthesis of proteins that are necessary for key processes of plant development, reproduction and pathogen response. It is interesting to note that the secretory proteins involved in these processes do not seem to have counterparts in animals and, conversely, the mechanism of antigen presentation in animals does not exist in plants. Thus, endoplasmin seems particularly important for functions that in different kingdoms are kingdom-specific. This would be a puzzling phenomenon from an evolutionary point of view, unless it more trivially reflects a higher requirement in tissues with high activity of the secretory pathway, regardless of the passenger proteins. The fact that the *shd* mutation is not lethal would also be consistent with the hypothesis that the fundamental metabolic functions of a eukaryotic cell do not require this

chaperone, even if more independent mutants were to be analyzed, because, as we mentioned, the T-DNA insertion in *shd* does not completely suppress gene expression.

3.6 Protein Disulphide Isomerase

As in other eukaryotes, the plant ER is an oxidizing environment that permits the formation of disulphide bridges. Not all secretory proteins carry such covalent bonds, but they are found very often in vacuolar or secreted proteins. Proteins destined for secretion in bacteria can also acquire disulphide bonds in the periplasm, through which they must pass to reach the extracellular environment.

Disulphide bridges are thought to stabilize the proteins outside the cells or in lytic compartments within the cells, and sometimes they are needed to hold together multimeric protein complexes. Because these bonds are of covalent nature, folding and re-folding is not a trivial task. This is illustrated by the fact that spontaneous folding in vitro is particularly inefficient for proteins carrying disulphide bonds, and can take from hours to an infinitely long time. Therefore, in vivo, this process is not left to chance and depends on ER-resident proteins that act as catalysts of oxidative protein folding.

In vitro, the ratio between oxidized and reduced glutathione $[GSH]_2/[GSSG]$ determines the redox potential and influences the rate of oxidative re-folding. A redox buffer that contains both oxidizing and reducing equivalents supplemented by an enzymatic catalyst for thiol–disulphide exchange seems to be the minimal requirement for this process. Four decades ago, the search for enzymatic catalysts within microsomal preparations led to the identification of protein disulphide isomerase (PDI, Goldberger et al. 1963).

Like many other abundant ER residents, PDI is a soluble protein carrying a typical ER retention motif. PDI can catalyse either the formation, reduction or isomerization of disulphide bonds. These mutually exclusive reactions depend on the redox conditions of the medium in which the protein folds, and on the folding status of the protein itself. PDI contains two copies of the thioredoxin motif (Cys – X – X – Cys) that can be found in the reduced or oxidized form to support the different types of reactions (disulphide bond transfer to substrate proteins or disulphide re-shuffling).

The ER lumen was initially thought to contain relatively high concentrations of reduced equivalents (GSSG) which would facilitate the formation of disulphide bonds within and between proteins. In addition to PDI, an ER-resident membrane-spanning protein was defined by a temperature mutant (*ERO1* = ER oxidation deficient 1) to be essential to replenish the oxidative capacity of the ER lumen. In its absence, secretory proteins remain in the reduced state when they normally acquire disulphide bridges, and ERO1p-deficient cells are hypersensitive to reducing agents such as DTT. The current

model of oxidative protein folding in the ER implies that oxidative equivalents are transferred from ERO1p via PDI to protein substrates (Fig. 1). In contrast to initial speculation, oxidized ERO1p, not oxidized glutathione (GSSG), serves as the primary source of oxidizing equivalents in the ER lumen (Frand et al. 2000). In vivo, most of the microsomal PDI is present in the oxidized form, ready to act as a protein dithiol oxidase.

Disulphide bonds are important key features of most storage proteins of the albumin and prolamin families (Shewry et al. 1997; Kawagoe et al. 2005). For instance, glutelin is one of the main storage proteins of wheat and forms large polymers held together by disulphide bonds. The physicochemical properties of the polymers crucially influence the properties of wheat flours in the manufacturing of bread, pizza and pasta. Consequently, it has been demonstrated that stable expression of high molecular weight glutenin subunit genes in transgenic rye drastically increases the polymeric glutelin fraction in rye flour and may improve its bread-making properties (Altpeter et al. 2004).

The plant vacuolar sorting receptor BP80 contains a very large number of cysteines at conserved positions throughout the luminal ligand binding domain (Hadlington and Denecke 2001), but it is far from understood which of those are engaged in the formation of disulphide bridges. Whether any of these are necessary for its function in binding to ligands or possibly binding to other membrane proteins, which may assist in the complex process of ligand delivery, release and specific recycling of unloaded empty receptors back to the Golgi apparatus, still remains a mystery today.

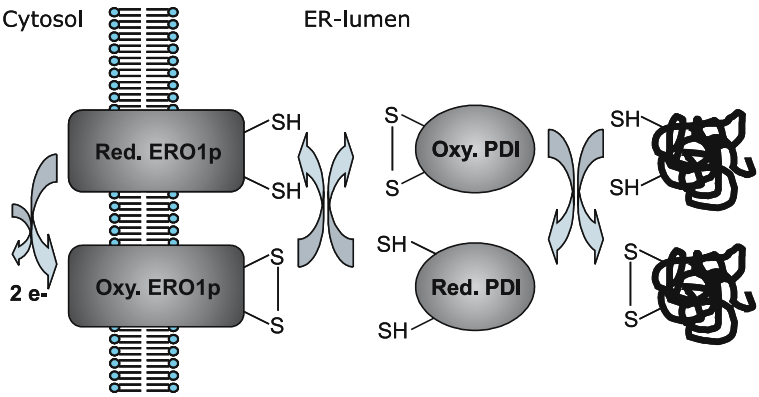

Fig. 1 Illustration of the role of the ER membrane protein ERO1 in the transfer of oxidative equivalents. The ER lumen is separated from the reducing cytosol via a membrane. Oxidized ERO1p can transfer oxidizing equivalents to the soluble ER luminal protein PDI, which can then be handed over to a folding intermediate that is yet to acquire disulphide bonds. Possibly, the two-step procedure allows better access to folding intermediates by using the soluble mediator PDI

It is fair to assume that oxidation and isomerization of disulphide bonds is necessary for the growth of all organisms, and plants will be no exception to this rule. A PDI homologue was cloned more than a decade ago, but initial investigations were restricted to expression analysis (Shorrosh and Dixon 1991). A few years later, a glycosylated wheat PDI was purified and characterized (Shimoni et al. 1995). PDI was also found to be rapidly induced upon fungal attack, which could be an indication that some defence-related proteins require an up-regulated machinery for the catalysis of disulphide bond formation in the plant ER (Ray et al. 2003). Curiously, PDI appears to be targeted to the ER and also the chloroplasts in protists (Levitan et al. 2005), but it is not known how important plastid-borne disulphide bond formation is for survival and whether all plants share this feature. Finally, PDI loss of function mutations were shown to have a profound influence on glutelin maturation and sorting in rice (Table 1; Takemoto et al. 2002), which corresponds well to earlier investigations into the role of individual disulphide bonds in the structural maturation of a low molecular weight glutenin subunit (Orsi et al. 2001). However, the function of ERO proteins in plants has only recently been investigated, and has started with the cloning of the two ERO1p homologues present in *A. thaliana*, shown to be targeted to the endoplasmic reticulum and glycosylated (Dixon et al. 2003). Clearly, this topic has not received sufficient attention in the plant field and must be explored in more detail in the years to come.

4 Conclusions

Work on the activity of folding helpers of the plant ER has been dominated mainly by biochemical approaches that rely on a limited number of model secretory proteins. On the other hand, genetic approaches have indicated that proteins such as signal peptidase, calreticulin, PDI, endoplasmin and BiP are important housekeeping genes, and it is clear that a range of phenotypes can be generated by interfering with their expression. The results of such experiments underline the complex network of interdependence between a basic metabolic process such as protein maturation within the ER on the one hand, and organ differentiation, plant reproduction or stress response on the other. However, it is very difficult to distinguish between direct and indirect effects of loss of function mutants or gene knockouts.

Thus, whilst *A. thaliana* is obviously extremely valuable as a source of molecular tools and to demonstrate the overall importance of ER-located events for plant life, most of the detailed biochemical work on the plant ER originates from studies on the well-characterized seed storage proteins of crop plants and from protein expression in tobacco protoplasts. It is likely that further advances on the folding helpers of the plant ER will not only take

advantage of the possible interactions indicated by the above-mentioned genetic approaches, but will also need to rely on established or novel model systems and assays that are most suited for biochemical analysis of protein–protein interactions. In the spirit of such experimental strategies, genetic approaches themselves may have to be refined to permit precise gene replacement so that defined modifications can be tested within a relevant biological context. This would require efficient homologous recombination, and within the plant kingdom this is currently only possible in the moss *Physcomitrella patens*.

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