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The Plant Endoplasmic Reticulum

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Professor Dr. David G. Robinson
Ruprecht-Karls-University of Heidelberg
Heidelberger Institute for Plant Sciences (HIP)
Department Cell Biology
Im Neuenheimer Feld 230
D-69120 Heidelberg
Germany

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Editor

David G. Robinson studied Botany (B.Sc., 1969) and Biophysics (Ph.D., 1972) at the University of Leeds (UK). Postdoc and Asst. Prof. at Stanford University (1972–74). At the University of Göttingen 1974–99, as Professor for Plant Cytology. From 2000: Professor for Plant Cell Biology at the University of Heidelberg. First Director, Heidelberg Institute for Plant Sciences. Major research interests: vesicle-mediated protein trafficking, ER-Golgi dynamics, vacuole biogenesis and transport to the vacuole.

Preface

The endoplasmic reticulum (ER) is the mother of all membranes. Essentially all other membranes, except those of plastids and mitochondria, are derived from it through vesicle-mediated membrane trafficking. It serves, therefore, as the port of entry into the endomembrane system for proteins destined to reach the vacuole or the cell wall. Quality control machinery in the ER lumen presides over the export competence of these proteins, and coupled to selective sorting mechanisms for their entry into export vesicles, this elevates this organelle to the most important regulator of protein movement along the secretory pathway. Moreover, its functions can be upregulated during various stages of plant development, e.g., during storage protein deposition in seed maturation, or in answer to stress, e.g., the production of pathogen-related proteins, and the so-called unfolded protein response.

In addition to its function in the synthesis of luminal and integral membrane proteins, the ER is also the site of structural and storage lipids. Further important features of the ER are its role in the homeostasis of cellular calcium and – through the localisation of cytochrome P450 enzymes – in the synthesis of a number of plant hormones. These manifold activities are reflected in the great plasticity of the ER as a structure, with over a dozen specialized domains recognized for this organelle.

To my knowledge, a book devoted to the ER in plants has never been published; indeed, I am not aware of a similar book on this organelle existing for the animal field. There have been a couple of excellent reviews on the ER of plant cells (one by Vitale and Denecke in *The Plant Cell* and one by Staehelin in *The Plant Journal*), but these lie several years back and were, of necessity, somewhat limited in scope. Therefore, I think it is both timely and appropriate to put the spotlight onto the ER as a whole, especially since GFP technology has provided news ways of looking at the dynamics of this membrane, and its relationship to other organelles, especially the Golgi apparatus and peroxisomes.

February 2006 David. G. Robinson

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The Morphology and Dynamics of the ER

Hartmut Quader[1] (✉) · Michael Zachariadis[2]

[1]Biocentre Klein Flottbek, University of Hamburg, Germany
quader@botanik.uni-hamburg.de

[2]Department of Botany, University of Athens, Greece

Abstract The endoplasmic reticulum (ER) is a non-uniform compartment in plants as regards its morphology and function. It extends as a highly anastomosing membranous network throughout the cytoplasm, is the major compartment of membrane biogenesis, and has been verified to function as the starting site for the secretory pathway. Early electron microscopy studies revealed three morphological ER sub-domains: the smooth ER, the rough ER, and the nuclear envelope. In the last two decades vital staining procedures, immunological methods, and green fluorescent protein technology in connection with confocal laser scanning microscopy have extended and augmented our knowledge regarding the morphology of the different ER domains, especially the three-dimensional transition between the cortical tubular network, long tubular strands, and lamellar sheets during interphase and mitosis. The cytoskeleton in connection with the respective motor proteins and cations like Ca^{2+} and H^+ play a critical role in the regulation of ER organization in dividing, differentiating, and stressed cells. Although our understanding of ER morphology in plants has improved notably, our view still remains founded on a rather limited number of model cells.

1 Introduction

There is no standardized endoplasmic reticulum (ER) in plants. Descending from a single cell, in higher plants the zygote, plant cells may undergo rigorous morphological and metabolic changes before reaching their destination within an organism. Therefore, the course of development with periods of division and differentiation leads to various cell types, which will all have distinct ER organization according to their function. In the past few years our understanding of ER organization during the cell cycle, and in fully differentiated or differentiating cells during plant development, has improved considerably.

The ER extends as a highly anastomosing membranous network throughout the cytoplasm and represents in most plant cells the largest membrane system. It is the major compartment of membrane biogenesis and, ever since the membrane flow hypothesis was proposed (Mollenhauer and Morré 1980), also acts as the portal to the secretory pathway. It also contributes to several principal anabolic and catabolic cellular pathways, including the fine-tuning of the cytosolic Ca^{2+} concentration.

Morphologically the ER can be divided into three sub-domains: the smooth ER (sER), the rough ER (rER) (Fig. 1a), and the nuclear envelope (NE). In plants, desmotubule ER crossing plasmodesmata may be added as a fourth ER sub-domain linking the ER network of neighbouring cells. Plasmodesmata are structures which mediate intercellular communication (Staehelin 1996; see also Oparka and Wright, this volume). The amount of the NE remains almost constant during interphase, whereas the amount of the other ER sub-domains may vary according to metabolic demands.

Conventional ultrastructural studies showed longitudinally sectioned ER membrane fragments either without any associated ribosomes (the sER) or studded with ribosomes in a linear, spiral, or no clear arrangement (the rER). Ultrastructural studies using high-voltage electron microscopy have extended our view of ER organization in both animal (Walz 1982) and plant cells (Harris 1979). Such studies employed new post-fixation and post-staining techniques like osmium tetroxide/potassium ferricyanide (Hepler 1981) or zinc iodide/osmium tetroxide (Hawes et al. 1981), or high-pressure freezing (Craig and Staehelin 1988). These techniques not only provided evidence in favour of ER continuity, but also indicated that rER and sER occur in the form of flat, sheet-like sacs and tubular elements, referred to as cisternal ER (cER) and tubular ER (tER), respectively.

An even more complex view of ER morphology is obtained after visualizing the ER by means of light microscopy with ultraviolet microscopy, video-enhanced differential interference phase contrast microscopy (VeDIC) or fluorescence microscopy (Lichtscheidl and Hepler 1996). Actually, these procedures provided a reasonable insight into ER morphology some 40 years ago, but did not enjoy much recognition by the scientific community at the time (Drawert and Rüffer-Bock 1964; Url 1964). Light microscopy techniques, however, became the procedures of choice with the general availability of

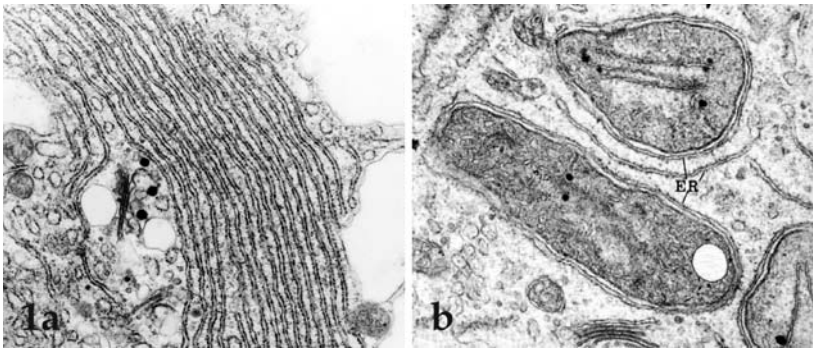

Fig. 1 Electron microscopic images of conventionally fixed ER. **a** Stack of rough ER in a pea root cell with a dictyosome at a close distance (courtesy of D.G. Robinson, Heidelberg). **b** ER in close association with chloroplasts (courtesy of P. Apostolakos, Athens)

VeDIC and confocal laser scanning microscopy (CLSM). ER visualization by CLSM profited enormously from the introduction of selective fluorescent stains about 20 years ago (Terasaki et al. 1984; Quader and Schnepf 1986; Hepler and Gunning 1998), and from indirect immunofluorescence techniques using antibodies that recognize the ER retention signal of ER luminal proteins (Napier et al. 1992) or ER resident proteins like the Ca^{2+}-binding protein calreticulin (Denecke et al. 1995). About a decade ago, green fluorescent protein (GFP) technology (Boevink et al. 1996; Haseloff et al. 1997; Hawes et al. 2001) provided an even greater input into ER morphology studies. A significant benefit of CLSM is the possibility of carrying out three-dimensional studies of ER organization in living cells (Hepler and Gunning 1998; Ridge et al. 1999; Cutler and Erhard 2002).

In general, ER morphology and its dynamics depend on the particular functions the cells of different tissues will have to perform for the sake of the organ, and thus of the whole organism. In the past, particular aspects of the organization and function of plant cell ER have been reviewed by several authors (Hepler et al. 1990; Lichtscheidl and Hepler 1996; Staehelin 1997; Hawes et al. 2001). In this chapter, we discuss the changes of ER morphology in meristem and differentiated cells, as related to cell cycle stages or physiological conditions.

2
ER Morphology and the Cytoskeleton

Many metabolic pathways in plant cells are under strict spatial control, as is apparent from distinct organelle distributions which are guaranteed by the two major cytoskeletal elements: microtubules (MTs) and actin filaments (AFs). MTs and AFs function in organelle patterning and motility as relatively stiff tracks along which the organelles are transported via specific motor proteins like dynein or kinesin (for MTs), and myosin (for AFs).

The controlled arrangement of the ER membrane meshwork throughout the cytoplasm is not conceivable without a guiding skeleton. In animal cells, MTs form tracks along which the ER network is constituted and altered in connection with the appropriate motor protein (Lee and Chen 1988; Terasaki 2000). In plant cells, ER tubules and small ER sheets have also been observed by electron microscopy in close nearness to MTs in the cell cortex of developing guard cells (Hodge and Palevitz 1984) and in pollen tubes (Lancelle et al. 1987). Most distinct are the MT–ER associations found during the mitotic stages (Hepler 1980) and during cytokinesis (Segui-Simarro et al. 2004). However, all the examples of spatial nearness between MTs and the ER during interphase resemble static situations, and bear the blemish that MTs and the ER have not been detected to co-align in plant cells over a longer distance. It thus seems very doubtful that these scarce contact sites are sufficient for the

observed controlled dynamic changes of the ER pattern. In vivo observations of ER organization and motility in onion epidermal cells show no perturbation in the presence of MT disassembling drugs, such as colchicine (Knebel et al. 1990), oryzalin, or trifluralin (Quader et al. 1989), when applied at concentrations specific for plant cells and for reasonable experimental periods. These results strongly support the view that MTs are not involved in ER organization in interphase and highly differentiated cells, although it has been claimed that MTs might be involved in the polar distribution of membranes including the ER (Mathur et al. 2003). A different situation may exist during mitosis and cytokinesis (see below).

Electron microscopy has revealed a spatial proximity between AF bundles and ER elements in fusiform cambium cells (Goosen de Roo et al. 1983), in developing guard cells (Palevitz and Hodge 1984), in differentiated root statocytes (Hensel 1987), in onion epidermis cells (Quader et al. 1987), in parenchyma cells of *Drosera* (Lichtscheidl et al. 1990), and in internodal cells of the giant alga *Chara* (Kachar and Reese 1988). Sliding of ER tubules along AFs has been convincingly demonstrated by in vitro video microscopy studies after gently extracting the cytoplasm of internodal cells of giant algae such as *Nitella* (Higashi-Fujime 1988) and *Chara* (Kachar and Reese 1988). We approached this question in onion bulb scale epidermal cells by following the redistribution of the ER after previously dislocating it by centrifugation (Quader et al. 1987). The ER starts to relocate in the form of bundles of long ER tubules and attains its former pattern after several hours depending on the centrifugal force applied. However, no recovery is observed in the presence of the AF disassembling drug cytochalasin D. This causes the transformation of parts of the tER into flat lamellar ER sheets (Quader et al. 1989). Lamellae form, in particular, at sites in the polygonal network where organelles accumulate (Quader et al. 1996). Depending on the physiological activity of the cells studied, the lamellar ER sheets may occupy large areas of the peripheral ER network. In mesophyll cells from *Vallisneria*, cytochalasin D also caused a change of the polygonal cortical ER tubules into lamellar sheets (Liebe and Menzel 1995).

AFs can only function as a framework for ER organization in correlation with a linking motor protein creating the force for ER motility. Using perfused internodal cells of *C. corallina*, Williamson (1979) showed that myosin-like filaments are associated in a stationary way with the ER at one end, while the other end is apparently in loose contact with the sub-cortical AF bundles. These filaments were later proved to be myosin (Grolig et al. 1988). The involvement of myosin in the dynamic distribution of cell organelles including the ER was confirmed in onion epidermal cells by localizing myosin through immunofluorescence, and by blocking its activity with the sulfhydryl reagent *N*-ethylmaleimide (NEM, Liebe and Quader 1994). Displacement of the ER by centrifugation leads to the dislocation of the ER and of myosin to the region of the centrifugal pole. After centrifugation, the previous ER and myosin pat-

tern was gradually restored. NEM, which leads to the complete inhibition of actomyosin-dependent organelle movement (Kohno and Shimmen 1988), not only arrested ER translocation, but also caused in onion epidermal cells the partial conversion of tER elements into lamellar sheets, whereas this agent apparently only froze the polygonal network in the alga *Vallisneria* (Liebe and Menzel 1995). Blocking the action of the myosin ATPase by 2,3-butanedione 2-monoxime caused the bulging and dilation of cortical ER tubules in the vicinity of plasmodesmata (Samaj et al. 2000).

In conclusion, while it is generally accepted for many algae and differentiated higher plant cells that ER organization depends on an intact actomyosin system, little is known regarding the situation in mosses and fern protonema cells.

3
ER Morphology and the Changing Demands of the Cell Cycle

While ultrastructural methods are suitable for studying the details of ER structure in all cell types, the use of fluorochromes such as the vital stain 3,3′-dihexyloxacarbocyanine iodide, $DiOC_6(3)$ (Terasaki et al. 1984) and GFP technology (Haseloff et al. 1997) are superior in visualizing dynamic changes in ER organization in three dimensions. Although $DiOC_6(3)$ and other fluorochromes appear to stain the great majority of different ER domains in plant cells, there is still no proof that all domains are equally labelled by the dyes or by GFP-tagged ER-targeted molecules.

3.1
ER Organization in Interphase Cells

Although the ER was first described by light microscopists, a more precise portrayal became possible only when Ledbetter and Porter (1963) introduced glutaraldehyde as a fixative for the electron microscopy of ultrathin sections. This technique, however, has a major disadvantage because it yields a rather limited three-dimensional view of cellular structures due to the minute thickness of the sections. Nevertheless, improvement in staining techniques gave a first glimpse into the 3-D pattern of the ER, and showed that it is an interconnected membrane system of tubules and flat sheets in remotely related differentiated interphase cells such as storage parenchyma cells of legume cotyledons, maize root cells, stamen hair cells, epidermal cells including guard cells, and moss caulonemata (Hepler et al. 1990). A distinct part of the ER was found in the cell cortex, largely present as a tubular network in close proximity to the plasma membrane (PM). In the vicinity of the nucleus it often exists as lamellar sheets covered with ribosomes. Lamellar sheets are not restricted to the nuclear region but may also occur embedded in the cortical

tER network, close to mitochondria, plastids (Fig. 1b), or other cell compartments (Hawes et al. 1981; Hepler 1981; Galatis and Apostolakos 1977).

Electron microscopic observations have been confirmed by tetracycline fluorescence studies on onion bulb scale epidermal cells (Drawert and Rüffer-Bock 1964), by $DiOC_6(3)$ staining (Quader and Schnepf 1986; Quader et al. 1987) (Fig. 2c), and by UV and VeDIC microscopy (Lichtscheidl and Url 1987; Allen and Brown 1988; Lichtscheidl and Weiss 1988). A polygonal meshwork of branching ER tubules with flat lamellar sheets fitted into that network interlace the cell cortex of higher plant cells and moss protonema cells (Hepler et al. 1990) (Fig. 2b,c). Since $DiOC_6(3)$ is a vital stain with little effect on the cellular activities at a low dose of exciting light, a spatial idea of ER patterning can be obtained by combining images from stacks of sections acquired in the *z* direction with the CLSM. This reveals the connection between the cortical ER network to ER elements like lamellar sheets located deeper in the cytoplasm in the vicinity of the nucleus or long tubular strands (Quader et al. 1987, 1989) (Fig. 2c). The long tubular strands seen in this way appear to be the form by which the ER membrane is displaced to distant cellular locations, like pulling on elastic. The velocity with which these ER tubules extend as long strands within the polygonal network range from about 0.5 to 8 μm/s (Lichtscheidl and Url 1990).

The continuum of the ER membrane system becomes particularly evident at sites where ER tubules flow into or leave flat ER sheets located deeper in the cytoplasm, giving the impression of a canvas fixed by several ropes. The results obtained with the vital stain $DiOC_6(3)$ have been confirmed in living cells by attempts employing GFP technology (Boevink et al. 1996; Brandizzi et al. 2003) (Fig. 2a). Splicing the ER retrieval peptide K/HDEL to GFP, or tagging GFP to domains of ER resident proteins such as BiP (Lee et al. 2002), calreticulin (Brandizzi et al. 2003), or proteins associated with (calmodulin-regulated ATPase, Hong et al. 1999) or residing in the ER membrane (calnexin, Irons et al. 2003), have resulted in a similar dynamic tER network with polygons and lamellar sheets of different shape and size. Astonishingly, the polygonal ER network is also displayed in *Arabidopsis* by a GFP-tagged MT-associated (plus end tracking) protein EB1 (Mathur et al. 2003).

The cortical polygonal network is subject to considerable dynamic modifications as a consequence of changing physiological conditions (Lee and Chen 1988; Knebel et al. 1990; Hepler et al. 1990; Ridge et al. 1999). ER tubules may expand two-dimensionally to lamellar sheets, which predominantly emanate at sites where three or more tubules merge (cross) or where closely located tubules fuse to a flat sheet. Conversely, lamellar sheets may also disintegrate into tubules (Quader 1990). The sliding movement of ER tubules in a polygon may lead to its transformation, resulting in a new polygon or in its elimination (Fig. 3).

The cortical ER network is tightly associated with the PM at particular sites, named immobile fixed sites as indicated by centrifugation experiments

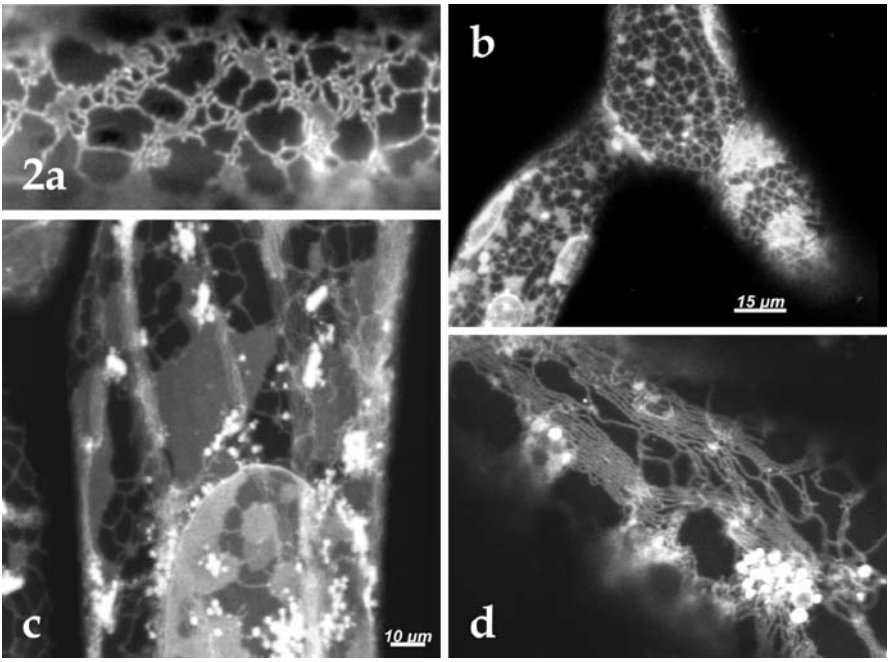

Fig. 2 ER visualized by GFP technology (**a**) or by confocal microscopy after vital staining with the fluorochrome $DiOC_6(3)$ (**b**–**d**). **a** Cortical polygonal network visualized in a tobacco BY-2 cell by GFP technology with an epifluorescence microscope. Bar as Fig. 2c (courtesy of Ch. Ritzenthaler, Strasbourg). **b** Polygonal ER network in the cortex of protonemata cells of *Funaria hygrometrica*. **c** Various morphological ER domains observable in the nuclear region of onion bulb epidermis cells. The image was processed from 15 single sections taken in the *z* direction. The polygonal ER network and large lamellar sheets are located in the cell periphery. Deeper in the cell the nucleus is recognizable due to its brightly fluorescent NE. Lamellar sheets are located in close nearness on both sides of the NE, verifiable by the fluorescence intensity of the two small lamellar sheets, the brighter one residing adjacent to the NE side directed toward the cell interior which is longitudinally traversed by long tubular strands. Bright fluorescent spots: oval form—amyloplasts; more rounded form—mitochondria. **d** High magnification of an area traversed by a bundle of long tubular ER strands displaying the close linkage to the polygonal network. Bar as Fig. 2c

(Quader et al. 1987; Liebe and Quader 1994), by combining stacks of images from the same plane of focus at short time intervals (Knebel et al. 1990), or by VeDIC observations (Lichtscheidl and Url 1990). The two membranes, however, never fuse. As regards the identity of these immobile fixed sites, the plasmodesmata of course come into question (see below and Oparka and Wright, this volume), but they cannot make up for all of them. Freeze fracture studies on actively secreting cells in pea root tips have revealed connections between the ER and strong PM indentation sites (Craig and Staehelin 1988).

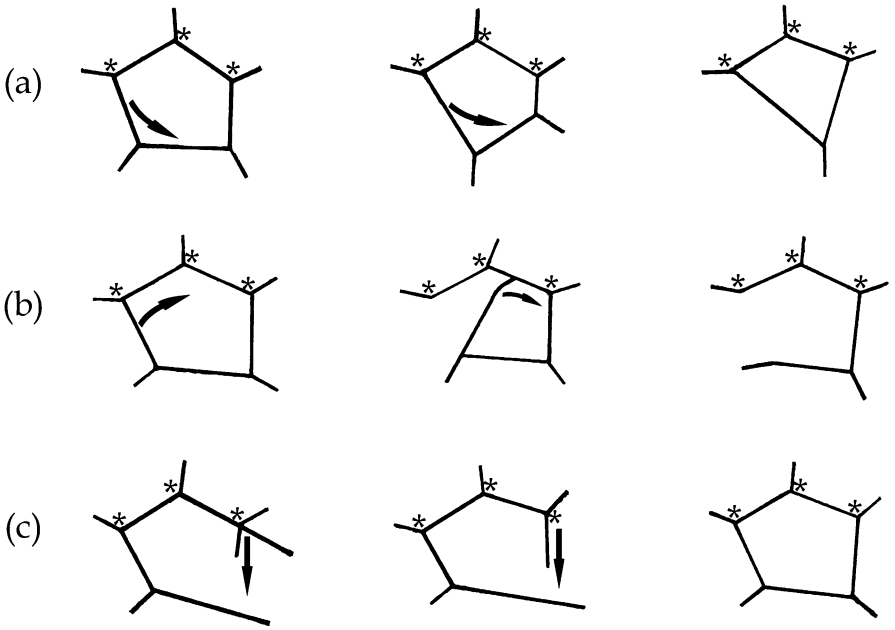

Fig. 3 ER tubule motility within the polygonal network. Diagrammatic representation of the three major types of ER tubule movement leading to altered or disintegrated polygons: **a** polygon transformation by tubular sliding; **b** polygon disintegration by tubular sliding, and **c** polygon formation by tubule branching. *Stars* mark immobile fixed sites, *arrows* indicate direction of tubular motion

Similar results were obtained in freeze-substituted *Drosera* epidermal cells (Lichtscheidl et al. 1990).

A particular situation occurs when neighbouring cells are interlinked by plasmodesmata. Ultrastructural studies have indicated that tER elements extend in a highly compressed or constricted form through this plasmodesmatal tunnel, thus forming an ER continuum between neighbouring cells (Hepler 1982). The sub-domain character of plasmodesmatal ER has been convincingly demonstrated by Grabski et al. (1993), who showed that a fluorescent diacylglycerol derivative locating predominantly to the ER and the NE can pass through plasmodesmata, but not fluorescent phospholipid analogues which locate predominantly to the PM.

ER tubules of primary plasmodesmata originate from portions of the ER extending through the area of the cell plate which become entrapped and compressed by phragmoplast vesicles during cell plate formation. (Hepler 1982; Hepler and Gunning 1998; see also Sect. 3.2). The participation of the ER in the formation of secondary plasmodesmata is also established (Lucas 1995), but little is known about the molecular processes involved in locating ER tubules to the site(s) of connection. Since the ER is closely associated with

the PM, it is imaginable that the ER tubule and the PM together are pushed or pulled through the forming cell wall channel.

The tubular ER is, however, not only in close spatial association to the PM, but it is also often seen to locate adjacent to other compartments such as the tonoplast and mitochondria in *Drosera* epidermal cells (Lichtscheidl et al. 1990) or moss plastids (Galatis and Apostolakos 1977) (Fig. 1b). These "sites of nearness" have been proposed to represent domains of lipid exchange among organelles between which vesicle trafficking does not occur (Staehelin 1997), but have also been suggested to be involved in bulk lipid or Ca^{2+} transfer (Levine 2004). A special case occurs in maturing pollen grains where the ER of the vegetative cell is found in close association with the PM of the generative cell (Hess 1993; Luegmayr 1993). Endosymbiotic bacteria as well as multicelluar parasitic intruders are often wrapped in layers of ER, forming a shield which separates the intruding organism from the host cytoplasm (see references in Lichtscheidl and Hepler 1996).

A special ER domain is the nuclear envelope (NE) which separates the nuclear matrix and the cytoplasm during interphase. The NE is not always of distinct spherical shape but is characterized by invaginations and grooves or may even assume a flattened disc-like shape (Collings et al. 2000). It is comprised of outer and inner NE membranes which join at the nuclear pore complex (NPC). The outer NE is morphologically continuous with the ER network and shows functional conformity since it has the ability to synthesize proteins (Mattaj 2004), whereas the inner NE is functionally directed to the nucleoplasma and anchors the nuclear lamina and the chromosomes. Most of our knowledge regarding proteins residing in NE membranes, such as the nuclear lamina receptor of the inner NE, comes from animal cells. Little is known about lipids and proteins specifically located to the plant NE (Meier 2000). The continuity of the ER and the NE is marked by a gate-like contraction of the ER tubule close to the confluence (Staehelin 1997), which does not prevent protein distribution from and into the NE (Napier et al. 1992; Denecke et al. 1995; Boevink et al. 1996; Zachariadis et al. 2001; Pay et al. 2002; Irons et al. 2003). GFP-tagged RAN-GTPase activating protein marks the NE rim but also distributes in the cytoplasm of interphase cells (Rose et al. 2004). The NE also becomes labelled in *Nicotiana benthamiana* by GFP-labelled tobacco mosaic virus movement protein (Reichel and Beachy 1998). Irons et al. (2003) succeeded in labelling the NE by expressing GFP-tagged human lamin B receptor in tobacco BY-2 suspension cells and followed the distribution of this inner NE protein during mitosis. Since higher plant cells lack centrioles, the MT organization and function of the perinuclear area/outer NE with respect to MT is noteworthy in view of the mitotic rearrangement of the NE (Schmit 2002).

3.2
ER Rearrangement During Mitosis and Cytokinesis

Plant cells, as most other eukaryotic cells, are distinguished by an open cell division, i.e. characterized by the breakdown of the NE while chromosomes pile up at the equatorial plane, and subsequent NE restoration after successful chromosome segregation. Distinct changes in the distribution of the ER have recently been indicated in dividing cells of different taxa (Tables 1 and 2). Early electron microscopy observations of ER reorganization, which mainly concerned the cell periphery, the region of the dividing nucleus, and the succeeding mitotic apparatus (MA) (Hepler 1980; Hawes et al. 1981; Hepler and Wolniak 1984) (Fig. 4a,b), have been confirmed and extended by studies involving ER visualization by immunofluorescence techniques (Napier et al. 1992; Denecke et al. 1995; Zachariadis et al. 2003) or GFP technology (Nebenführ et al. 2000; Irons et al. 2003).

The first striking insights into changes of the ER and NE during mitosis were provided by Hepler (1980), who investigated the fate of the NE–ER complex by osmium tetroxide/potassium ferricyanide staining in dividing barley cells and, in a subsequent study (Hepler 1982), the association of the ER with the developing cell plate and primary plasmodesmata during cytokinesis. He showed the aggregation of NE and ER elements in prometaphase after NE breakdown, partially as fenestrated lamellae at the spindle pole region. Moreover, parts of the stained membranes apparently encased the MA or even invaded the spindle. Using GFP-tagged lamin B receptor it was shown that the inner NE in dividing mammalian cells does not vesiculate during mitotic breakdown but equilibrates with the ER (Ellenberg et al. 1977), and that NE breakdown starts with a partial disassembly of the nuclear pore complex (Lenart et al. 2003). Using a similar construct, no other ER element but the NE is labelled in stably transformed tobacco BY-2 suspension cells during interphase (Irons et al. 2003). With the onset of metaphase, the fluorescently marked NE emerges at the pole region and to tubular structures around the MA, and becomes relocated to the NEs of the daughter cells during telophase/cytokinesis. The ER accumulating at the pole regions and at the MA rim obviously function as sites of NE retraction during mitosis.

But what is the situation during the stages preceding prometaphase and metaphase in which the mitotic players line up? The MT pattern during preprophase is characterized by the formation of a distinct narrow band in the cell cortex, the MT preprophase band (MT-PPB), at whose site the future cell plate will merge with the parental cell wall (Mineyuki 1999). The progressive breakdown of the MT-PPB at prophase is accompanied by the formation of the prophase spindle with MTs arranged along the nuclear rim progressing from the two pole regions towards the plane of division. In interphase cells the ER organization is similar in angiosperm and gymnosperm root cells,

Table 1 ER organization during mitosis and cell division: gymnosperms

Mitotic stage	ER arrangement (higher organization)
Interphase	All ER domains—peripheral polygonal network, lamellar flat sheets, NE—form a continuous meshwork
Preprophase	ER-PPB; peripheral polygonal network still exists but less compact
Prophase	Formation of a bipolar ER spindle constituting a sheath around the MA; NE breaks down
Pro-/ metaphase	No ER aggregation at the pole regions; progressing NE breakdown; transformation of major parts of the bipolar ER sheath into bundles of smooth K-NE-ER extending into metaphase spindle along kinetochore MTs and chromosome arms
Anaphase	Shortening of the K-NE-ER tubules in accord with kinetochore MTs, gathering finally at the pole regions; parallel interzonal ER tubules develop between the two daughter chromosome groups
Telophase/ cytokinesis	Increase of ER elements at the pole region and of the number of interzonal ER tubules; gradual formation of barrel-shaped ER phragmoplast; NE restoration

Table 2 ER organization during mitosis and cell division: angiosperms and pteridophytes

Mitotic stage	ER arrangement (loose organization)
Interphase	All ER domains—peripheral polygonal network, lamellar flat sheets, NE—form a continuous meshwork
Preprophase	No ER-PPB has hitherto been detected with certainty
Prophase	ER elements begin to accumulate at the pole regions; formation of a loose bipolar ER spindle which constitutes a sheath around the MA
Pro-/ metaphase	ER accumulation mainly at the pole regions; NE breaks down; bundles of smooth NE–ER tubules (K-NE-ER) extending from the pole region into the metaphase spindle along kinetochore MTs and chromosome arms
Anaphase	Strongest ER accumulation at the pole regions; shortening of the K-NE-ER tubules in accord with kinetochore MTs and progression of chromosome separation; ER tubules traverse the space between daughter chromosome groups
Telophase/ cytokinesis	Formation of an ER phragmoplast; ER elements are detected in the region of the forming cell plate

and in pteridophyte leaf cells with the tER network and flat sheets in the cell periphery and deeper in the cytoplasm, as revealed by immunofluorescence studies, although a distinct difference is observable in these plant systems during preprophase (Zachariadis et al. 2003).

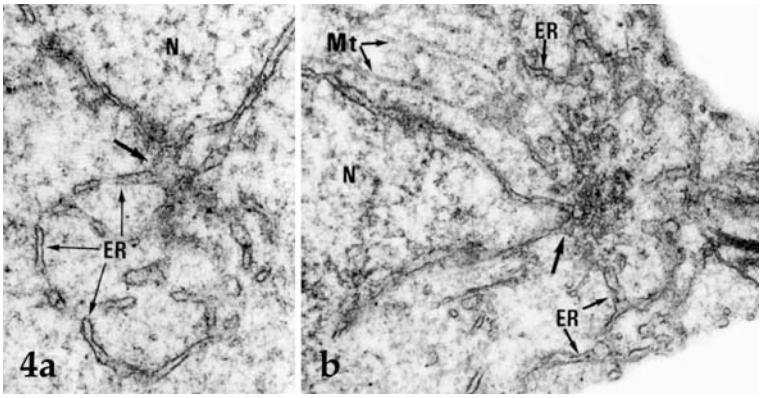

Fig. 4 ER organization during mitosis visualized by conventional electron microscopy. **a** ER gathering in the pole region of dividing cells of the moss *Marchantia* during early prometaphase (courtesy of P. Apostolakos, Athens); **b** as in (**a**), late prometaphase, indicating the association of prophase spindle microtubule with the ER (courtesy of P. Apostolakos, Athens)

In dividing root-tip cells of *Pinus*, the ER pattern at preprophase and prophase follows more or less that of microtubules forming a distinct band-like structure of parallel ER tubules succeeded by a tER prophase spindle. The three-dimensional reconstruction of a series of CLSM sections showed unequivocally that the observed band of ER tubules constitutes a ring underneath the PM, a tER-PPB, which in early preprophase is still loosely arranged and apparently becomes more compact during the transition to early prophase, following the maturation pattern of the MT-PPB (Fig. 5a) (Zachariadis et al. 2001, 2003). ER tubules have also been noticed among PPB MTs by electron microscopy (Mineyuki 1999). Of note is that the ER network traversing the cell cortex in interphase cells persists even after the formation of the tER-PPB, whereas all the MTs in the cell periphery except those of the MT-PPB disappear during PPB formation (Zachariadis et al. 2003). The function of the temporary tER-PPB is unknown, but it may play a role in Ca^{2+} sequestration/release which is important in regulating MT-PPB polymerization/depolymerization.

A tER-PPB could be visualized with certainty only in gymnosperms. It was not detectable in dividing leaf cells of the pteridophyte *A. nidulans*, and only sporadically in dividing angiosperm root-tip cells exposed to stress (Zachariadis 2003, unpublished observations).

In dividing *Pinus* root cells, from prophase through metaphase and anaphase, the ER distribution closely resembles that of the microtubules as it forms a tER prophase spindle (Fig. 5a). It becomes transformed into a bipolar metaphase spindle characterized by relatively imprecise pole regions and bundles of ER tubules that span the area between the poles and the

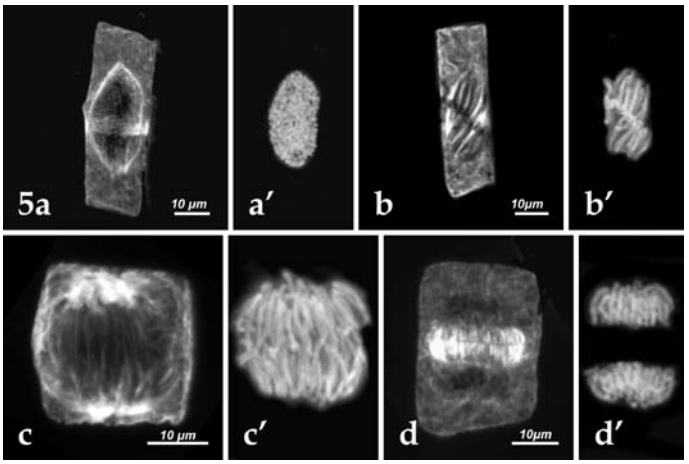

Fig. 5 ER organization during mitosis visualized by immunofluorescence techniques employing an antibody which recognizes the ER retention signal. Chromosomes were stained with propidium iodide. Note that the cortical ER network is present throughout mitosis/cytokinesis. **a** *Pinus brutia* root-tip cell, transition preprophase to prophase. The ER-PPB, the forming bipolar prophase ER spindle, and the arrangement of the chromosomes in the same cell. **b** Bundles of kinetochore ER tubules with broad pole regions during metaphase of a *P. brutia* root-tip cell and chromosome pattern. ER tubules traversing the interzonal area. Note the fir-tree character of the kinetochore ER bundles. **c** *Triticum turgidum* mid-anaphase root-tip cell with marked concentration of ER elements at the poles (mostly K-ERs) and ER tubules traversing the space between the two daughter chromosome groups. **d** ER phragmoplast of a dividing *Pinus* root-tip cell and chromosome arrangement

chromosomes' kinetochores (K-tER), probably following the kinetochore MT bundles (Fig. 5b). These well-defined K-tER bundles gradually shorten during anaphase, while a system of interzonal ER tubules is formed between the two separating chromosome groups. The ER concentrates at the spindle poles partly due to the retraction of the K-tER bundles (Zachariadis et al. 2003). An efficient ER reorganization occurs in the transition from late anaphase through telophase and cytokinesis. There are few ER elements recognizable at the phragmoplast-forming site (Segui-Simarro et al. 2004), although the interzonal ER tubules appear to multiply in number and traverse the space between the separated chromosome groups as loose bundles. Progressively, the ER bundles assume the form of a barrel-shaped young ER phragmoplast which converts, after shortening of the ER bundles, into a structure resembling the typical MT phragmoplast (Fig. 5d) (Hepler and Gunning 1998; Zachariadis et al. 2003).

The ER pattern in dividing *Triticum* root-tip cells differs from that of dividing *Pinus* root-tip cells, not only in the uncertainty regarding the formation of a tER-PPB, but also in the strong accumulation of tER elements at

the spindle poles (Hepler 1980; Hawes et al. 1981) and in the formation of a less distinct interzonal tER array (Fig. 5c). Similar differences apply to the redistribution of the ER in dividing *Asplenium* leaf cells (Zachariadis et al. 2003). The ER pattern observed in *Pinus* during cell division shows a higher degree of organization than in the other species. This is probably due to the molecular difference between the MTs of gymnosperm and angiosperm meristematic root cells, since the MTs in *Pinus* have acetylated tubulin and MTs formed by this tubulin derivative are known to have a relatively longer half-life (Gilmer et al. 1999). The extended turnover of the acetylated MTs might provide a more stable framework for the organization of the ER. In contrast, it may be difficult to detect a tER-PPB in dividing angiosperm cells because of the higher turnover of the PPB MTs.

Zachariadis and co-authors (2003) have suggested from inhibitor experiments that the control of ER organization may switch in dividing *Pinus* root cells from AF dependence during interphase to MTs during mitosis and cytokinesis, because in the presence of microtubule inhibitors like oryzalin the formation of the tER-PPB, tER metaphase spindle, and tER phragmoplast is prevented. The actomyosin system shown to guide the ER pattern in interphase cells is apparently not involved in the shaping of the tER-PPB in dividing *Pinus* root cells, although AFs also form a PPB-like structure in these cells (Zachariadis et al. 2001, 2003). MT-dependent ER reorganization during mitosis and cytokinesis correlates with the fact that vesicles are steered along phragmoplast MTs (Verma and Gu 1996). Mathur and co-workers (2003) reported a new EB1-like protein, AtEB1, in *Arabidopsis* that co-localizes to the growing MT plus ends and to motile endomembrane networks including the ER. Moreover, an integral membrane protein of the reticular sub-domain of the rough ER, p63, has been shown to bind MTs in vivo and in vitro in *Xenopus* (Klopfenstein et al. 1998). As yet no homologues of this protein have been found in plants.

4
ER Patterns During Cell Differentiation and Organ Development

Attempts to follow in vivo the dynamics of ER organization during development and differentiation of plant organs are still rather scarce. McCauley and Hepler (1990, 1992) studied the ER organization during bud formation in the moss *Funaria* by visualizing the ER with the fluorochrome $DiOC_6(3)$, and Ridge et al. (1999) followed the ER transition in developing *Arabidopsis* root and hypocotyl cells by employing GFP technology.

In tip-growing moss protonema cells, the dense ER meshwork in the cell cortex changes to a more open reticulate tubular network with occasional small lamellar sheets when growth slows down (see Fig. 2b) (McCauley and Hepler 1990, 1992). In bud initiative cells this wide tER network regains compactness after the asymmetric division and becomes even tighter during the

succeeding developmental phase of the bud initial. In caulonema cells and developing buds, ribosomes are found attached to the reticular tubular network as well as to the lamellar ER. The ER network of buds is covered more densely, whereas the lamallae apparently show no distinct difference in caulonema, branches, and buds (McCauley and Hepler 1992).

Root development involves the differentiation of meristematic cells into highly expanded parenchyma and epidermal cells, or in the root cap to elongated statocytes or secretory cells. Root but also hypocotyl cells of *Arabidospis* are distinguished in the cell cortex by highly perforated lamellar ER sheets during the early phase of expansion (Ridge et al. 1999). The overall size and the diameter of the holes of these perforated sheets are larger than those reported for the fenestrated lamellae observed in barley mitotic cells (Hepler 1981). The perforated ER sheets were only detectable in fast-growing cells and they began to diminish when growth came to an end. The perforation holes dilated, became segregated, and finally the cortical ER pattern resembled that of highly vacuolated non-growing cells. In root epidermal cells which had ceased expansion growth but entered polar root hair differentiation, the polygonal reticulum again condensed in the trichoblast starting at the site of root hair outgrowth (Ridge et al. 1999). The ER pattern in growing root hairs resembled that of tip-growing *Funaria* protonema cells (McCauley and Hepler 1992). The compact polygonal reticulum in the tip and in the non-growing basal region became gradually wider after growth ceased.

The observations by Ridge et al. (1999) are in agreement with previous ultrastructural studies of root cap differentiation (Stephenson and Hawes 1986) and with cotyledons during seedling growth (Harris and Chrispeels 1980), although the extent of the tubular and lamellar ER is not easily deduced by electron microscopy even using impregnation procedures that allow the study of thicker sections. During sieve cell maturation the transition from lamellar to tER is followed by tER relocation (Schulz 1988). The opposite, i.e. the formation of lamellar ER stacks, has been shown to occur during mustard seedling maturation (Bergfeld and Schopfer 1984). ER stack formation correlates with the disappearance of ER tubules and with seed dehydration. These ER aggregates probably represent a form of ER membrane storage during periods of dehydrated protoplasm, which is a situation also characteristic of mature pollen grains.

In general, a relatively wide polygonal network of tER in the cell cortex seems to be characteristic for very slow or non-growing plant cells, but in swiftly differentiating cells the amount, the distribution, and the proportion of ER tubules to lamellae undergo distinct changes.

5
The ER: A Stress-Responsive Compartment

Unfavourable radiation, water stress, severe temperature changes, wounding, and attacks by pathogens are possible environmental strain situations that plants have to cope with. The ER organization of affected cells may alter if the parameters involved in the signal transmitting cascade, like the proton and/or Ca^{2+} concentration, change. Morphological variations, in principle, concern a dynamic shift between the two major ER modifications, tubules, and lamellar sheets, frequently preceded or accompanied by motility changes (Table 3).

The influence of light on ER morphology has hitherto only been addressed in the alga *Acetabularia* (Liebe and Menzel 1994). Microscopy studies indicate that blue and UV light may affect ER organization in higher plants but detailed studies, e.g. on dose dependence, are missing. Regarding harsh water stress, plasmolysis studies showed that parts of the cortical ER adhere so strongly to the PM that they remain attached to the cell wall, forming a "Hechtian reticulum" linked to the protoplast by Hechtian strands (Oparka et al. 1994; Stickens and Verbelen 1996). The fate of the ER organization within the shrinking protoplast has not been addressed in these studies and

Table 3 Stress-induced changes in ER organization: formation of lamellar sheets from tubular elements or the disintegration of lamellar sheets to short tubules

Applied stress	Morphological changes		Refs.
	Breakdown of lamellar sheets	Formation of lamellar sheets	
Natural stress			
Low temperature	+++	—	a
High temperature (> 35 °C)	—	+++	b
Pathogen attack	—	+++	c
Water stress (dehydration)	—	+++	f
Artificial stress			
Lowering the intracellular pH (< 6.8)	+++	—	d
Increasing the intracellular pH (> 7.5)	—	+++	b
Perturbation of Ca^{2+} distribution	—	+++	e

a Quader et al. 1989
b Quader et al. 1996
c Leckie et al. 1995; Reichel and Beachy 1998; Takemoto et al. 2003
d Quader and Fast 1990
e Quader 1990; Bergfeld and Schopfer 1984

awaits thorough investigation. Stacks of lamellar ER are, however, induced by less severe water stress, which are reversed to ER tubules after stress release (Bergfeld and Schopfer 1984).

A low temperature, around 4 °C, causes in onion epidermal cells the disintegration of lamellar ER sheets to tubules and the disappearance of long tubular strands, leading to a distinct increase of short ER tubules (Quader et al. 1989). Although the peripheral polygonal network is hardly recognizable because of the vigorous meandering tubular elements it can, however, be assumed that it is retained. Contrary to chilling, elevated temperatures (35–40 °C) cause the formation of large lamellar ER sheets within the polygonal network and deeper in the cytoplasm, often leading to lamellar stacks (Quader et al. 1996). The disappearing long tubular strands most likely contribute to the genesis of the ER lamellae. The ER organization regains normal patterning after reverting the chilling or high temperature regime to about 20 °C within a suitable period (Quader et al. 1989; Quader et al. 1996).

Attacks of pathogenic viruses and fungi also cause the formation of large lamellar sheets. They temporarily form after infecting transgenic *N. benthamiana* with tobacco mosaic virus and turn back into tER elements in later stages of infection (Reichel and Beachy 1998). The authors suggest that the virus movement protein may play a role in the formation of the lamellar sheets. A similar reorganization of the ER has been observed in epidermal cells of pea after infection with the powdery mildew fungus *Erysiphe pisi* (Leckie et al. 1995) and of *A. thaliana* cotyledons infected with different oomycete pathogens (Takemoto et al. 2003). A dense ER seems to occur around the penetration site of the fungus, which has been discussed as a compact network of lamellar ER merging into a compressed reticulum of ER tubules resembling perforated ER sheets (see above; Hepler 1981; Ridge et al. 1999). Little is known about the function of the ER accumulating around haustorial necks, but a mere function in membrane lipid and protein synthesis in relation to the formation of the haustorium has been excluded because ER accumulation occurred also in cells without haustoria (Takemoto et al. 2003).

Wounding or treatment with the defence hormone methyl jasmonate induces in epidermal cells of rosette leaves of transgenic *Arabidopsis* plants the formation of so-called ER bodies, besides the known ER modifications (Gunning 1998; Hawes et al. 2001; Matsushima et al. 2003). Leaves which have not been wounded or treated with the hormone do not show these structures in which defence proteins seem to accumulate (Matsushima et al. 2003; see also Hara-Nishimura and Shimada, this volume).

Pathogenic attacks involve the partial degradation of the host cell wall. Enzymes derived from pathogens as pectinases, cellulases, hemicellulases, and proteinases selectively lyse cell wall components and thus may mimic a pathogen attack. Pectinase and protein K treatment caused the formation of large lamellar ER sheets similar to the infection by a pathogen, whereas cellu-

lases and hemicellulases did not significantly affect ER organization in onion bulb scale epidermal cells (Quader et al. 1996).

Systematic investigations to solve the molecular basis for the stress-induced changes of ER morphology are rather scarce. Low and high temperature, as well as the stress exerted through pathogen attack, not only affects ER organization but is also often accompanied by the inhibition of actomyosin-dependent intracellular movement. As already pointed out, an intact actomyosin system is required not only to pull ER tubules out of the polygonal network and to push the long ER strands deeper into the cytoplasm, but also to keep them in a stretched mode (see Sect. 2). Disassembly of the AF backbone, and also the inhibition of the motor protein myosin, results in the formation of lamellar ER sheets. Myosin carries out a dual function: besides being a motor for motility it also serves as a link between AFs and the ER tubules keeping the latter stretched. Without a functional actomyosin system, only the ER tubules of the polygonal network which are linked to fixed sites may maintain their stretched shape (Knebel et al. 1990). Ca^{2+} in connection with calmodulin (Shimmen et al. 2000) and protons (Nachmias et al. 1987) are known to control myosin activity. Artificially lowering the cytosolic pH by weak acid loading causes the breakdown of lamellar ER sheets into tubules (Quader and Fast 1990), whereas artificial cytosolic alkalinization by weak base loading leads to the opposite response: the formation of lamellar ER sheets (Quader et al. 1996). The long tER strands retract during both lowering and raising of the cytosolic pH.

The ionic situation, and here in particular the equilibrium of proton and calcium cations across the ER membrane, apparently plays an important role in ER organization. Interestingly, after lowering the cytosolic pH ER tubules can obviously not fuse to lamellar ER sheets, even when they are arranged in a highly compact manner.

The disintegration of the lamellar ER sheets to ER tubules by low temperature resembles the effect achieved by lowering the cytosolic pH. The latter has been shown to drop in suspension-cultured mung bean cells from 7.4 to 6.3 within an 18-h chilling period (Yoshida 1994). The induced disintegration of the lamellar ER sheets may therefore be due to the acidification of the cytoplasm by low temperature.

Individual or stacks of large lamellar ER sheets are formed in onion epidermal cells after interference with the cellular Ca^{2+} distribution, e.g. through application of the Ca^{2+} ionophore calcimycin, selective Ca^{2+}-chelating drugs, or calmodulin antagonists (Quader 1990). Motility is suppressed and organelles like mitochondria often accumulate in the area of the formed ER sheets. ER organization of perturbed cells recovers after washing out the drugs (Quader 1990). At the beginning, tubular ER loops develop at the rim of the ER sheets, which successively become pulled away as long tER strands to other areas of the cell (see Fig. 2d). Recovery is inhibited in the presence of actomyosin blocking substances (Quader 1990). The actomyosin system

is, however, only indispensable for the movement of ER tubules, because calcimycin-induced lamellar ER sheets disintegrate to tubules after artificially lowering the cytosolic pH by weak acid loading with calcimycin and actomyosin-inhibiting drugs still present (Quader et al. 1996).

6 Prospects for the Future

There has been a considerable gain in knowledge regarding the dynamics of ER morphology in fully differentiated plant cells through the use of vital fluorochromes and GFP technology in connection with the CLSM. GFP technology is best suited to studying ER biogenesis, ER morphology and its functional relationships, molecular aspects involved in ER organization changes during cell differentiation and plant organ development, or as a response to stress. GFP can be tagged to proteins located either in the lumen, the membrane, or closely associated with ER domains. To distinguish in living cells between ER domains involved in protein synthesis and others by fluorescence microscopy would be most desirable. Other questions will concern the possible association of the ER with other organelles such as mitochondria, vacuoles, and the PM, the role of AFs and MTs in ER organization in accordance with the cell cycle, and how Ca^{2+} or protons are involved in the regulation of ER morphology dynamics. Mutants such as the *Arabidopsis* "pilz" group genes (Mayer et al. 1999), which are indispensable for microtubule organization, might be useful for studies of the role of MTs in ER organization during mitosis and cytokinesis.

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Targeting of RNAs to ER Subdomains and its Relationship to Protein Localization

Haruhiko Washida[1] · Andrew J. Crofts[1] · Shigeki Hamada[1,2] · Thomas W. Okita[1] (✉)

[1]Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, USA
okita@wsu.edu

[2]*Present address:*
Department of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, 060-8589 Sapporo, Japan

Abstract The targeting of proteins to specific subcellular regions is directed by a variety of signal elements. Many of these signals consist of amino acid residues (peptide sorting signals) arranged contiguously or in a three-dimensional array. In addition to posttranslational processes, proteins can also be localized to specific regions of the cell by the targeting of their cognate RNA. Ongoing studies in developing rice endosperm have shown that the RNAs that code for the major storage proteins are localized to specific subdomains of the cortical endoplasmic reticulum (ER), and that there is a tight correlation between the initial site of RNA localization and the final destination of the encoded protein in the endomembrane system. The segregation of RNA onto distinct ER subdomains may be a necessary and sufficient step for the localization of the coded protein in the cell.

1 Introduction

The endoplasmic reticulum (ER) serves as the entry site for proteins that are to be secreted or located at one or more locations in the endomembrane system in eukaryotic cells. Proteins are targeted to the secretory system with the emergence of the signal peptide during initial protein synthesis, which is recognized by the signal recognition particle. The resulting translationally arrested complex (mRNA–ribosomes–translation factors) is then mobilized to the ER, which enables the translocation of the growing polypeptide chain to continue through the ER membrane to the lumen. Within this organelle the polypeptide is folded and assembled to a correct conformational maturation state aided by a plethora of resident molecular chaperones. The presence of additional peptide sorting signals or interacting domains may enable the protein to be either retained within the ER or transported to other destinations within the endomembrane system (Vitale and Denecke 1999; Vitale and Ceriotti 2004). In the latter instance, proteins can be exported from the ER by two distinct pathways, one involving the Golgi apparatus

and a second that is suggested to be Golgi-independent (Hara-Nishimura et al. 1998).

An ideal system for studying the biochemical and cellular events of ER-dependent translation is developing seeds. During seed development, one or more organs synthesize vast quantities of reserve protein, which assemble within the ER lumen itself or within protein storage vacuoles to form discrete organelles termed protein bodies. One question that immediately arises is why some plants utilize the ER lumen whereas others develop protein storage vacuoles. Although the exact cellular basis for this is not known, it is clear that the nature of the storage protein does not dictate its ultimate storage site. For example, members of the prolamine superfamily that share common structural domains are not stored in the same compartment. The 2S albumins of several dicotyledonous plants and prolamins of wheat and barley are transported to the protein storage vacuole, whereas the maize prolamins (zeins) assemble within the ER lumen to form intracisternal inclusions (Shewry et al. 1995). Although peptide sorting signals as well as peptide interacting domains are ultimately responsible for the final destination of proteins, evidence is beginning to emerge that the intracellular location of a protein may also be influenced by where it is being translated on the ER (Crofts et al. 2004). In this chapter we elaborate on this hypothesis by discussing recent advances in the analysis of the relationship between RNA targeting to specific ER subdomains and protein localization.

2
RNA Localization in Animals and the Role of the ER

RNA localization is recognized as an important process in controlling the synthesis of proteins at specific sites within the cell. More than 100 messenger RNAs are now known to be targeted in a wide variety of eukaryotic cells. This process is essential for cell fate determination in yeast (Chartrand et al. 2001), during early vertebrate development (Bashurullar et al. 1998; Palacios and Johnston 2001), in polar cell growth of somatic cells (Ainger et al. 1997; Carson et al. 1998; Shestakova et al. 2001), and in mediating cell motility (Kloc et al. 2002). The use of this mechanism for several different developmental and cellular processes suggests that the process of RNA localization is common to all eukaryotes.

Several studies have implicated a role for the ER in the localization of RNAs and, in turn, for localized protein secretion during oogenesis. In *Xenopus laevis*, many maternal RNAs are found concentrated in the animal or vegetal pole of the oocyte and early embryo. One well-characterized protein is *Vg1*, which codes for a transforming growth factor-β and is required for mesoderm induction and right-left symmetry of the embryo (Kloc et al. 2001). In early stage oocytes, *Vg1* is initially distributed throughout the cytoplasm but, as

the oocyte matures, it then becomes tightly associated with a crescent-shaped region of ER and then later with a wedge-shaped ER subdomain (Kloc and Etkin 1998). In late stage oocytes, *Vg1* RNA and closely aligned ER vesicles are redistributed to the vegetal cortex. The localization pattern of *Vg1* is dependent on intact microtubules and kinesin II (Betley 2004). The involvement of this molecular motor, previously established as functioning in membrane trafficking, provides additional support for a role of the ER in *Vg1* transport and localization.

Further evidence for the involvement of the ER in *Vg1* RNA localization is suggested by the putative role of *trans*-acting factors that recognize the *cis*-sequence determinants required for RNA localization. The localization of *Vg1* RNA to the vegetal pole requires a 366-nucleotide element located in the 3′ untranslated region (3′UTR) which is recognized by several proteins (Mowry and Melton 1992). One identified protein is Vera/*Vg1* RBP which is found enriched in ER fractions and is able to accommodate the anchoring of *Vg1* RNA to the ER (Mowry 1996). Overall, the available evidence indicates that *Vg1* RNA piggybacks on ER which is transported on microtubules to the vegetal cortex.

In addition to providing the basis for RNA movement, the ER may provide specialized domains for protein synthesis. The transcript for the yeast plasma membrane protein Ist2p is localized to the bud tip and the protein is deposited locally (Juschke et al. 2004). In *Drosophila*, the anterioposterior and dorsoventral axes of the future embryo are specified by the secreted *Gurken*, a transforming growth factor-α-like protein. *Gurken* RNA is transported and localized to ER at the dorsal/anterior corner of the oocyte resulting in localized exocytosis at this site (Herpers and Rabouille 2004). Hence, RNA localization to specific ER subdomains at discrete cellular locations may be an alternative process for targeted secretion.

3 RNA Localization in Plants

Other than RNA sorting in developing seeds, which will be the subject of the remaining sections of this article, there are relatively few reports on the localization of RNAs in plants. The interested reader is referred to two recent reviews (Okita and Choi 2002; Crofts et al. 2004) which describe published examples of this phenomenon in higher plants and algae. One study that deserves mention is the localization of expansin RNAs. Expansins are extracellular proteins that are involved in cell enlargement, pollen tube growth, and abscission as a result of their ability to induce cell wall relaxation and extension (Cosgrove 2000). Im et al. (2000) showed that the different expansin mRNAs were evident as distinct patches located at either the apical or basipetal end of xylem precursor cells. More specifically, *ZeEXP1* and *ZeExp3*

mRNAs were found at the apical end just below the apical meristem, whereas *ZeExp2* mRNA was found at the basal end. The nature of the RNA patch was not identified, but given the secretory nature of expansins, the "patch" is likely to be a complex of ER membranes. Hence, the targeting of expansin RNAs to a specific subdomain of ER located at either the apical or basal end of the protoxylem cells may function to direct polarized trafficking of the secreted expansin to specific cell wall regions to allow intrusive cell growth.

4
Prolamine and Glutelin RNA Localization in Rice

Plant seeds accumulate storage proteins which serve as a source of nitrogen and carbon for the developing seedling. The storage proteins are coded by two major gene superfamilies, prolamin and globulin (Shewry 1995; Shewry et al. 1995). Unlike other plants, which utilize only one of these storage protein types as their dominant reserve, rice is unique in that it accumulates major quantities of both storage protein types. In addition to accumulating the alcohol-soluble prolamine, the typical storage reserve of cereals, rice contains vast quantities of glutelin, a protein structurally homologous to the 11S globulins. Rice also accumulates significant quantities of a 26-kDa α-globulin. Although the solubility properties of this protein have led it to be defined as globulin-like, it also contains peptide domains of the prolamine superfamily and is related to the rice prolamines. These rice storage proteins are deposited as separate entities within different compartments of the endomembrane system, either the vacuole or ER lumen (Fig. 1). Interestingly, oat also utilizes both storage protein types but they are stored together in a single compartment, the protein storage vacuole (Muench and Okita 1997).

Information on how these proteins are deposited within the ER and protein storage vacuole is rather limited. In view of their close structural identity to the 11S globulins, it is likely that many of the cellular processes involved in the synthesis, transport, and deposition of glutelins are identical to those observed for the 11S globulins (Robinson and Hinz 1999). The newly synthesized protein is exported to the Golgi where it forms dense vesicles which then fuse to the storage vacuole (Krishnan et al. 1986). Protein export and/or dense vesicle formation is likely to depend on the presence of vacuolar peptide sorting signals, as demonstrated for phaseolin (Frigerio et al. 1998). One or more peptide sorting signals are recognized by a receptor located at the transitional ER or an early Golgi compartment, which concentrates glutelin into a dense vesicle that has been detected in developing rice endosperm (Krishnan et al. 1986). The 26-kDa globulin has also been observed to form Golgi-associated dense vesicles (Krishnan et al. 1992), suggesting that they are transported to the storage vacuole by a similar if not identical process.

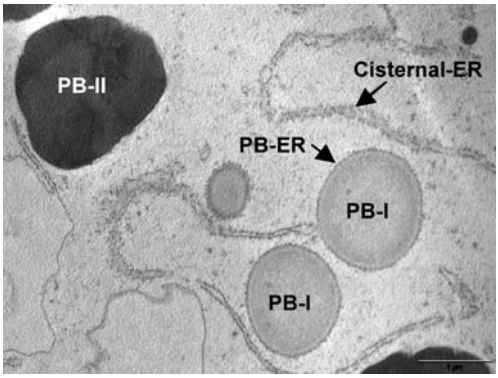

Fig. 1 Morphology of protein bodies in rice. Electron micrograph of a rice endosperm section. Depicted are spherical prolamine-containing protein bodies (PB-I) bounded by rough ER (PB-ER), irregularly shaped glutelin-containing protein bodies (PB-II), and cisternal ER. *Scale bar* = 1 μm

The accumulation of prolamine within the ER lumen could also be dependent on a receptor-based mechanism involving a lumenal peptide sorting signal, although none has ever been identified in storage proteins that utilize this site for storage. Assembly and accumulation within the ER lumen is more likely a product of protein–protein interactions, both homotypic and heterotypic (Kim et al. 2002). For example, the maize β- and γ-zeins are capable of self-assembling into stable lumenal granules in transgenic plants, whereas the α- and δ-zeins are unable to do so and are dependent on interaction with the former pair of zeins for ER retention (Herman and Larkins 1999). This assembly of prolamins into an intracisternal inclusion granule must occur at a rate much faster than its potential export from the ER. Moreover, the inclusion granules must be highly ordered structurally to prevent an unfolded protein response leading to their degradation.

Receptor-dependent and -independent protein interactions are likely responsible for the distinct protein bodies formed in rice. One question that is immediately raised here is how are heterotypic interactions between newly synthesized prolamine and glutelin polypeptides prevented from occurring? One possibility is that these proteins are rapidly sorted from each other; for example, glutelins may be rapidly exported at the transitional ER sites. Alternatively, they are synthesized and initially deposited at spatially distinct subdomains of the ER membrane complex. Indeed, rice endosperm cells contain two morphologically distinct ER subdomains; cisternal ER consisting of single lamellar membranes distributed throughout the cell and protein body ER (PB-ER) which delimits the prolamine protein body (PB-I) (Krishnan et al. 1986; Li et al. 1993). Both membrane types are continuous and constitute the cortical ER in developing rice endosperm (Fig. 1) (Muench et al. 2000).

These distinct ER subdomains may serve to spatially separate the synthesis and, in turn, the entry of these proteins into the secretory pathway. Such a suggestion was supported by biochemical analysis of subcellular fractions enriched for these membranes. Prolamine PBs, purified by repeated sucrose density gradient centrifugation, contained polyA-RNA which directed the synthesis of only prolamines, suggesting that the PB-ERs were enriched for prolamine mRNAs (Yamagata et al. 1986). On the other hand, Kim et al. (1993) demonstrated an approximately two-fold greater abundance of glutelin transcripts over prolamine transcripts in membrane-bound polysome fractions, indicating that this fraction, composed mainly of cisternal ER, is enriched for glutelin mRNAs.

The segregation of these storage protein RNAs to distinct ER subdomains was unequivocally demonstrated by Li et al. (1993). Estimation of the storage protein content in purified PB-I and cisternal ER fractions and in thin tissue sections using high-resolution in situ hybridization techniques showed that the cisternal ER contained more than a twofold molar excess of glutelin mRNA over prolamine transcripts, while prolamine mRNAs were present at seven- to ten-fold molar excess over glutelin transcripts in the PB-ER (Li et al. 1993). The results of this study demonstrated that the storage protein RNAs were not randomly distributed on the ER but, instead, localized to specific subdomains of this membrane complex. Although not directly demonstrated, these results indicate that the storage proteins gain entry into the secretory pathway at spatially separated sites within the ER.

The segregation of these storage protein RNAs to distinct ER subdomains could occur directly by the directed targeting of the RNAs. Alternatively, the localization of the rice storage protein RNAs to distinct ER subdomains could be accomplished by a peptide-based mechanism. Under this scenario, emergence of the signal peptide during translation would be recognized by a signal recognition particle (SRP), which would arrest translation and mediate interaction with the SRP receptor and Sec61p complex on the ER. Distinct SRP–receptor combinations or additional factors would dictate the specificity towards the PB-ER or cisternal-ER membranes. Interestingly, despite the fact that the signal peptides of rice prolamines share considerable homology with those from the maize zeins, which are also deposited as intracisternal inclusion granules, the mature primary sequences of these proteins are unrelated (Masumura et al. 1990; Mitsukawa and Tanaka 1991). The glutelin signal peptide has no sequence similarity to these prolamine sequences and, like other 11S globulin signal peptides, contains two cysteine residues interdigitated by five leucines. Interestingly, the rice genome contains two SRP 54 polypeptides, the subunit of the SRP complex which recognizes the signal peptides (*Oryza sativa* genome database), suggesting the possibility of unique SRPs that recognize distinct signal peptide sequences. Despite these features, the available evidence indicates that prolamine RNAs are localized to the PB-ER by an RNA-based mechanism.

5
Rice and Maize Prolamine RNAs are Localized by an RNA-Dependent Mechanism

The high-resolution in situ hybridization approach, although sensitive, was extremely laborious and alternative more facile approaches were clearly required to study how these RNAs were sorted to the PB-ER and cisternal ER. Choi et al. (2000) developed a light microscopy-based approach using an in situ reverse transcriptase-mediated polymerase chain reaction (RT-PCR) with fluorescently labeled nucleotides to study RNA localization in developing rice endosperm cells. Figure 2 shows that fluorescence patterns obtained by in situ RT-PCR and their spatial relationships to the PB-ER are markedly distinct for prolamine and glutelin RNAs. Prolamine RNAs are distributed as small, spherical, and sometimes ringlike structures of 1–2 μm diameter which are identical to the structures labeled by BiP antibodies and visualized by indirect immunofluorescence (Choi et al. 2000). Consistent with this view is that these structures were also stained by $DiOC_6$ and rhodamine B hexyl ester, two ER vital stains which specifically label PB-I because they also react with the hydrophobic prolamine polypeptides as well as the ER membrane (Choi et al. 2000) (Fig. 2). In contrast, glutelin RNAs are seen as much larger irregularly shaped patches located adjacent to PB-I, a pattern indicative of their localization on cisternal membranes (Fig. 2).

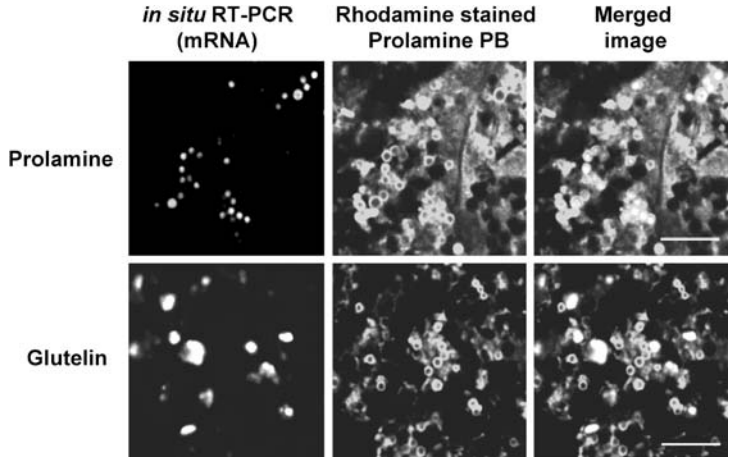

Fig. 2 Prolamine and glutelin mRNA localization as viewed by in situ RT-PCR. Prolamine and glutelin RNAs were detected by in situ RT-PCR using gene-specific primers and Oregon Green 488 d-UTP. The spherical PB-ERs were visualized by staining the sections with Rhodamine B hexyl ester (*middle images*). Confocal microscopic analysis showed that prolamine RNAs were localized to spherical PB-ERs. In contrast, glutelin RNAs were distributed as large patches indicative of their location on cisternal ER located near PB-ER. *Scale bar* = 10 μm

The development of facile methods to assess RNA localization enabled a systematic study to determine whether this phenomenon was dependent on either an RNA or peptide-based elements. A series of transgenic plants expressing synthetic prolamine RNAs were generated and analyzed for RNA localization. They included a wild-type prolamine gene and three prolamine variants containing a substituted glutelin 3′UTR in place of the normal prolamine 3′UTR, a signal peptide deleted version, and an AUG translational knockout. These various prolamine transgenes were also tagged with unique DNA sequences to distinguish the transcripts made from the transgene from those derived from endogenous genes. The signal peptide deleted prolamine RNA was faithfully localized to the PB-ER, indicating that the signal peptide and the corresponding coding sequences were not required. In contrast, the prolamine glutelin 3′UTR RNA was mistargeted to the cisternal ER. As the epitope-tagged prolamine coded by this prolamine RNA variant was readily detected by immunoblot analysis, these results indicate that prolamine RNA localization is not dependent on the prolamine primary sequence. Interestingly, although the prolamine primary amino acid sequence is not essential for RNA targeting to the PB-ER, an intact AUG translation initiation codon is required for correct prolamine RNA localization to the PB-ER (Choi et al. 2000; Hamada et al. 2003b). This result indicates that the regulated prolamine RNA pathway requires the participation of the translational machinery. On the surface, this latter conclusion would appear to conflict with our earlier interpretation of RNA-directed localization. However, the requirement for translation and RNA targeting are not strictly coupled and can be contributed by different sequences, as demonstrated by the analysis of RNA reporters containing translatable green fluorescent protein (GFP) or β-glucuronidase (GUS) sequences. Placement of prolamine sequences in the 3′UTR was able to direct the reporter RNAs to the PB-ER. Consequently, the requirement for translation is provided by the GUS or GFP reporter, with the RNA *cis* elements conferring PB-ER targeting located in the 3′UTR (Choi et al. 2000).

The dependence on RNA localization for protein localization appeared to be a unique feature of the rice system, even though other cereals, e.g., maize and sorghum, accumulate storage proteins in similar compartments to those used by rice. Indeed, based on in situ hybridization analysis using endosperm thin sections, RNAs for the maize zeins were reported to be essentially randomly distributed on the PB-ER and cisternal-ER membranes in developing maize endosperm (Kim et al. 2002). Protein body formation in maize was suggested to occur by diffusion of the zeins to the intracisternal inclusion granule followed by self- and interassembly of the various zein polypeptide classes. Curiously, heterologous expression of the 10-kDa δ-zein RNA in developing rice endosperm showed a striking localization pattern. Unlike the random distribution of the 10-kDa δ-zein RNA reported in maize endosperm, the localization of this RNA was clearly restricted to the PB-ER (Hamada et al. 2003b).

To resolve this apparent discrepancy, Washida et al. (2004) assessed the distribution of the storage protein RNAs in developing maize endosperm using the in situ RT-PCR technique mentioned earlier. In developing maize endosperm cells, the PB-ER was visualized and the localization was confirmed by both double immunofluorescence using 10-kDa δ-zein antibody and rhodamine B hexyl ester staining. Analysis of the mRNA localization using in situ RT-PCR revealed that maize zein RNAs coding for 22-kDa α-zein, 15-kDa β-zein, 27-kDa γ-zein, and 10-kDa δ-zein were localized to ER-bounded zein protein bodies (Washida et al. 2004). In contrast, RNAs coding for 51-kDa legumin-1, a storage protein sharing homology to the 11S globulins and rice glutelin, were distributed on adjacent cisternal ER. These results indicate that the same RNA targeting mechanism is at work in rice and maize, and that this mechanism has been conserved since the divergence of these species 50 million years ago.

The apparent differences in RNA distribution patterns depending on the technique used deserve comment. The in situ RT-PCR technique is significantly more sensitive as the tissue sample is much thicker, resulting in a larger target size as compared to the ultrathin sections of tissue analyzed by in situ hybridization at the electron microscopy level. The basis for the apparent random localization of zein RNAs when viewed by electron microscopy (Kim et al. 2002) is not known. However, the measured gold particle densities in this study were about an order of magnitude lower than those measured in rice (Li et al. 1993). One possibility that may account for the observed random distribution of RNAs was a preferential loss of RNAs associated with the PB-ER during the in situ hybridization procedure, as suggested by Kim et al. (2002).

6
Prolamine RNA Transport from the Nucleus to the PB-ER

RNA transport is well characterized in several systems (Bor and Davis 2004). In polarized somatic cells and during early vertebrate development, endogenous RNAs are visualized as large granules (Barbarse et al. 1995; Ainger et al. 1997; Carson et al. 1998; Roock et al. 2000; Krichevsky and Kosik 2001) or particles (Sundell and Singer 1990; Ferrandon et al. 1994; Forristall et al. 1995; Kloc and Etkin 1995) that move at rates of up to 4 to 6 μm/min via cytoskeleton-associated motors (Bassell and Singer 1997; Chartrand et al. 2001; Saxton 2001; Kloc et al. 2002; Tekotte and Davis 2002). RNA movement can be monitored in real time in living cells using a method based on a modification of the yeast three-hybrid system developed to detect RNA binding proteins (Bertrand et al. 1998; Takizawa and Vale 2000). In this system, two genes are expressed to monitor RNA transport. One gene encodes GFP fused to the MS2 coat protein, a high-affinity RNA binding protein from the single-

stranded MS2 RNA phage which recognizes a specific 19-nucleotide RNA stem loop. This reporter protein also contains a nuclear localization signal (NLS) to restrict it to the nucleus in the absence of any RNA target. A second gene transcribes a hybrid RNA containing prolamine RNA sequence and tandem repeated (6×) MS2 RNA binding sites fused to a GUS reporter gene. When these two genes are expressed simultaneously, the MS2-GFP binds to one or more of the MS2 binding sites, which enables one to follow the movement of this RNA in real time by following the GFP fluorescence.

Hamada et al. (2003a) adapted this GFP two-gene monitoring system to follow prolamine RNA movement in developing rice endosperm and heterologously in tobacco BY-2 cells. When the MS2-GFP fusion protein was expressed by itself in BY-2 cells, native fluorescence was observed predominantly within the nucleus. When coexpressed with GUS-MS2-prolamine RNA, GFP was localized to the periphery of the cell, the presence of the large vacuole in these cells apparently restricting GFP fluorescence to the thin cytoplasm adjacent to the cell wall (Hamada et al. 2003a). A similar pattern was also evident in developing rice endosperm cells when MS2-GFP fusion protein was expressed alone. However, an entirely different distribution pattern was observed when both genes were expressed. Numerous small particles ranging in size from 0.3 to 2 μm in diameter were readily evident. Although many of the particles were stationary, several moved within the focal plane across the cell demonstrating RNA movement. These prolamine transport particles generally moved unidirectionally in a stop-and-go manner, with an estimated average velocity of 0.3–0.4 μm/s and instantaneous velocities of up to 10 μm/s (Hamada et al. 2003a). These characteristics are indicative of movement driven by a cytoskeleton-associated motor protein (Bassell et al. 1999; Jansen 1999, 2001; Tekotte and Davis 2002).

The dependence on the cytoskeleton for RNA movement was supported by the use of drugs that disrupt this cellular structure. Under optimal conditions, RNA movement particles were easily observed for up to 30 min. When treated with cytochalasin D and latrunculin B, which disrupt actin filaments, particle movement was rapidly suppressed with only Brownian movement noted. Movement was also arrested by 2,3-butanedione monoxime, an inhibitor of the ATPase activity of skeletal myosin (Ostap 2002). In contrast, the microtubule drug nocodazole had no significant effect on RNA movement (Hamada et al. 2003a). The results indicate that RNA particle movement is dependent on intact microfilaments.

7
The Dependence of RNA Transport on Zip Codes

The *cis* elements or zip codes (Singer 1993) responsible for prolamine RNA targeting to the PB-ER were defined by studying the localization of RNAs

coded by a series of GFP-prolamine cDNA genes in transgenic rice endosperm cells (Hamada et al. 2003b). GFP RNAs, which normally are localized to the cisternal ER, are redirected to the PB-ER when prolamine RNA sequences are attached to the 3′UTR. Deletion analysis of the prolamine sequences showed that two zip codes are required for PB-ER targeting. One zip code is located downstream adjacent to the sequence encoding the signal peptides, while a second *cis* element is located in the proximal half of the 3′UTR (Hamada et al. 2003b). Both *cis* elements share considerable homology, suggesting that recognition by *trans*-acting factors is sequence dependent.

The majority of *cis* elements responsible for RNA targeting do not share a common sequence, but are dependent on the formation of an exact secondary and/or tertiary structure. This is supported by ongoing studies to identify the *cis* elements responsible for the targeting of the 10-kDa δ-zein RNA to the PB-ER in developing rice endosperm. Despite the relatively small size of this transcript, the RNA appears to have at least four *cis* elements. Three are located within the proximal two-thirds of the coding sequence including the signal peptide and a fourth is located in the 3′UTR (Washida et al. unpublished). A direct alignment of the RNA sequences of these four regions indicates very little sequence homology and no common motifs are evident. Moreover, there is no sequence similarity between the *cis* element of rice prolamine (Hamada et al. 2003b) and those of the 10-kDa δ-zein. This lack of sequence conservation between these RNAs suggests that the *cis* element responsible for PB-ER targeting is structural in nature and that its sequence is less important.

Both the rice prolamine and maize 10-kDa δ-zein require at least two *cis* elements for restricted localization to the PB-ER. These multiple, functionally redundant *cis* elements may act synergistically to ensure efficient transport. Other examples of mRNAs with multiple redundant *cis* elements are found in *Xenopus* oocytes (*Vg1*: Desher et al. 1998; Mowry and Cote 1999; Yaniv and Yisraeli 2001; *Fatvg*: Chan et al. 1999), *Drosophila* oocytes (*Nanos*: Bergsten et al. 2001), and budding yeast (*Ash1*: Gonzalez et al. 1999; Chartrand et al. 1999). In many cases, the location of these multiple *cis* elements is restricted to the 3′UTR. The presence of one or more *cis* elements in the coding regions of the rice prolamine and maize 10-kDa δ-zein may confer another function in addition to their role in RNA localization. The yeast *Ash1* RNA has four *cis* elements, three of which are located within the coding region. Chartrand et al. (2002) obtained evidence that these three *cis* elements are required for transport and localization of *Ash1* RNA to the bud tip, and for preventing premature synthesis of the protein during its transport to this location. If the rice and maize *cis* elements also play a similar dual function, this would be consistent with the requirement for translation initiation where rice prolamine RNAs are transported in translationally arrested complexes to the PB-ER.

8
Multiple RNA Transport Pathways to the Cortical ER

The presence of a single rice prolamine or maize 10-kDa δ – zein *cis* element results in localization of the RNA not only to the PB-ER but also to the cisternal ER. In the absence of any *cis* element, RNAs are localized to the cisternal ER. These results support the existence of a default RNA transport to the cisternal ER in addition to a regulated transport pathway to the PB-ER. RNA transport to the ER occurs even for RNAs that code for cytoplasmic-localized proteins (Hamada et al. 2003a), indicating that the bulk, if not all, of protein synthesis occurs near or on the ER.

Although glutelin RNAs are localized to the cisternal ER, their transport and targeting to this ER subdomain also occurs by a regulated pathway. Replacement of the prolamine or 10-kDa δ-zein 3′UTR with the glutelin 3′UTR results in their redirection from the PB-ER to the cisternal ER. These results suggest the presence of one or more *cis* elements in the glutelin 3′UTR, and the existence of a regulated glutelin pathway to the cisternal ER which is dominant over the regulated prolamine pathway to the PB-ER (Choi et al. 2000; Hamada et al. 2003b).

9
Mistargeting of RNA to ER Subdomains Affects Protein Localization

The sorting of rice storage protein RNAs to distinct ER subdomains, and the packaging of these proteins into separate endomembrane compartments, suggests that RNA localization and protein localization are interrelated. Such a relationship is obvious for prolamine RNAs and the immediate assembly of the coded protein product upon translocation into the lumen. Additionally, proteins synthesized on the cisternal ER would presumably be in close proximity to the transitional ER, a requirement for their export to the Golgi complex or storage vacuole. Because storage protein RNA localization requires specific *cis* elements, this hypothesis relating RNA and protein localization can be directly tested. A hybrid 10-kDa δ-zein RNA containing a 3′UTR from the nopaline synthase (NOS) gene is normally localized to the PB-ER in developing rice endosperm as mentioned before. Immunocytochemical studies of ultrathin sections and transmission electron microscopic analysis showed that 10-kDa δ-zein polypeptides are localized to PB-I, the site of their synthesis (Fig. 3). Replacement of the 3′NOS sequence with the glutelin 3′UTR results in the displacement of the RNA from the PB-ER to cisternal ER (Hamada et al. 2003b). This change in RNA localization is mediated by the *cis* element(s) contained within the glutelin 3′UTR, and takes place even though the 10-kDa δ-zein coding sequence contains three *cis* elements that direct PB-ER localization of the RNA. Further analysis has shown that this altered RNA

localization pattern also changes the localization pattern of the 10-kDa δ-zein protein. The 10-kDa δ-zein was not found in PB-I but in the storage vacuole PB-II (Fig. 3). The final destination of the 10-kDa δ-zein protein therefore depends on which specific ER subdomain is used as the initial site of protein synthesis.

Several questions that arise from this study center on the mechanism by which the 10-kDa δ-zein protein, which is normally deposited as part of an intracisternal inclusion granule, is transported from the cisternal ER to PB-II. One property of the 10-kDa δ-zein is that it appears to be incapable of forming a protein body-like structure by itself in transgenic plants. Lending and Larkins (1989) have demonstrated that the synthesis of the various zein classes shows a strict temporal and spatial pattern during endosperm development, with the β- and γ-zeins laid down first followed by the α- and δ-zeins, which displace the former zein classes to the peripheral regions of the protein bodies. Coexpression of γ- or β-zein is needed for stable α- and δ-zein accumulation and aggregation to form protein bodies in transgenic tobacco (Bagga et al. 1995; Coleman et al. 1996; Bagga et al. 1997; Kim et al. 2002; Coleman et al. 2004), suggesting that the 10-kDa δ-zein (as well as α-zeins) is incapable of self-assembly in a larger macromolecular structure and requires

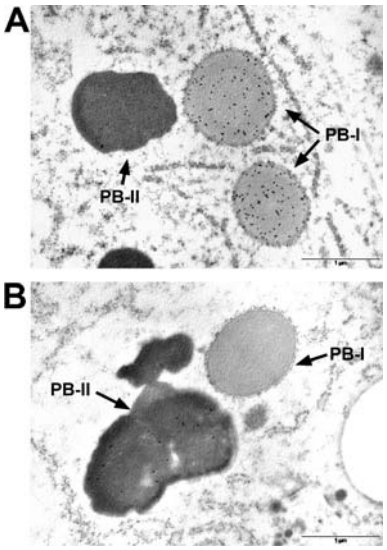

Fig. 3 Protein-A gold immunocytochemistry showing the localization of 10-kDa δ-zein in distinct protein bodies. The 10-kDa δ-zein and hybrid zein–glutelin constructs were introduced into rice by *Agrobacterium*-mediated transformation. In situ RT-PCR analysis revealed that 10-kDa δ-zein RNAs were localized only to the PB-ER, whereas the hybrid zein–glutelin RNAs were targeted to cisternal ER (Hamada et al. 2003b). δ-Zein polypeptides coded by RNAs containing the NOS 3′UTR are observed in PB-I (**a**) , while those coded by RNAs containing the glutelin 3′UTR are localized to PB-II (**b**). *Scale bar* = 1 μm

heterotypic interactions with other proteins. In the absence of interacting partners, the 10-kDa δ-zein is likely to be competent for ER export and transport to the storage vacuole.

A second question relates to how 10-kDa δ-zein is targeted to the storage vacuole PB-II. In general, the mechanism by which storage proteins are sorted to the storage vacuole is poorly understood. Both 7S and 11S globulins are exported from the ER where they are concentrated into dense vesicles at the *cis*-Golgi (Hillmer et al. 2001). Only phaseolin, a 7S globulin, has been shown to possess a vacuolar targeting signal at its C-terminus (Frigerio et al. 1998), although such targeting signals have also been suggested for the 11S globulins (Saalbach et al. 1991). The presence of a vacuolar sorting signal indicates the existence of a receptor that is responsible for ER export and dense vesicle formation. The 10-kDa δ-zein polypeptides may have a cryptic peptide signal that targets them to the storage vacuoles or they may be escorted to this organelle by their heterotypic interaction with glutelins. Immunocytochemical studies showed that the 10-kDa δ-zeins are embedded in the crystalline (glutelin-containing) parts of PB-II, suggesting the possible interaction of these proteins. Conversely, transport to the storage vacuole in rice may be simply a default process. Further studies on protein transport to the storage vacuole in rice should provide information on the existence of any of these pathways.

The preceding description clearly demonstrates that at least for one protein, the 10-kDa δ-zein, the localization of its RNA has a profound impact on where the protein is deposited in the cell. The converse experiment to support the relationship between RNA localization and protein localization is to mistarget an RNA from the cisternal ER to the PB-ER. The sunflower seed 2S albumin (SSA) is stored in the protein storage vacuole in both sunflower and transgenic rice. Closer examination showed that SSA protein is localized on the periphery of PB-II (Washida et al. unpublished) much like the 26-kDa globulin (Krishnan et al. 1986). Consistent with this location in the storage vacuole, its RNA is observed on the cisternal ER much like that seen for glutelin RNAs. Targeting of SSA RNA to the cisternal ER suggests the presence of *cis* elements functionally equivalent to those present in glutelin RNA (Hamada et al. 2003b)

The SSA RNA was modified to contain the 5′ coding sequences of the 10-kDa δ-zein RNA, which contains one or two PB-ER *cis* elements. In both instances, a portion of the hybrid 10-kDa δ-zein–SSA RNA was found to be partially mislocalized to the PB-ER in addition to the remainder which was targeted to its normal location on the cisternal ER. Cytologic examination using SSA antibodies showed that the SSA protein was found in both PB-I and PB-II (Washida et al. unpublished). Although SSA RNA could not be totally displaced to the PB-ER, the results do support the hypothesis that the PB-ER delimits a unique ER subdomain for the confined localization of proteins.

In PB-I, SSA was distributed to the periphery of the inclusion granule. Such a spatial arrangement is similar to that seen for the zein protein bodies, where

the β- and γ-zeins are displaced to the periphery around the α- and δ-zeins. The peripheral distribution pattern also indicates that SSA does not interact with prolamines and self-assembles around the more hydrophobic prolamine inclusion granule. The physical processes that account for the self-assembly of SSA within PB-I are not known, but it is likely that they are identical to those used for the concentration of SSA in Golgi-derived dense vesicles.

10 Conclusion

Recent studies have demonstrated that the localized secretion of proteins is accomplished by targeting their RNAs to ER located near the site of secretion. Ongoing studies in developing rice endosperm have demonstrated that RNA localization to specific ER subdomains is also responsible for the final destination of the encoded protein in the endomembrane system. Three RNA transport pathways have been identified: two regulated pathways requiring targeting *cis* elements and a third default pathway. Current efforts are focused on the characterization of the *trans*-acting RNA binding proteins and their interacting partners, which allow multiple RNA localization pathways from the nucleus to the distinct subdomains of the cortical ER. These molecular cellular studies are being complemented by parallel studies on genetic mutants that contain abnormal amounts of the 57-kDa glutelin precursor, which is normally processed into basic and acidic subunits in the protein vacuole (Kumamaru et al. 1988). Several of these mutants have been identified as RNA sorting mutants, in addition to mutants defective in proglutelin transport to the storage vacuole and proteolytic processing. This combined multifaceted approach will hopefully identify the major players responsible for formation of the RNA particle, and its transport and localization to specific subdomains of the cortical ER.

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

Alessandro Vitale[1] (✉) · Jürgen Denecke[2]

[1]Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, via Bassini 15, 20129 Milano, Italy
vitale@ibba.cnr.it

[2]Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

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.

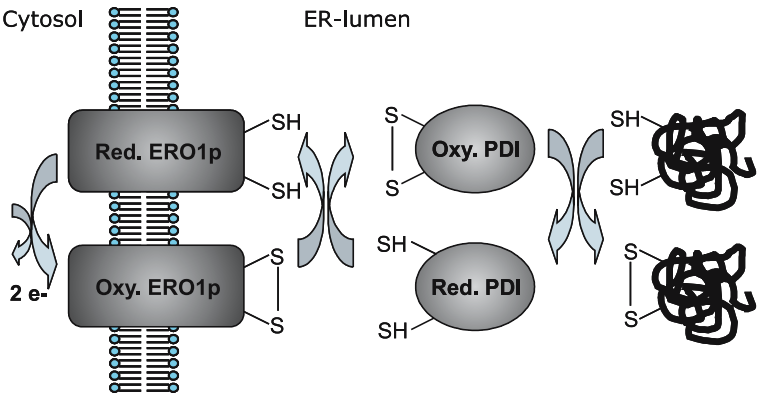

Fig. 1 Illustration of the role of the ER membrane protein ERO1 in the transfer of oxidative equivalents. The ER lumen is separated from the reducing cytosol via a membrane. Oxidized ERO1p can transfer oxidizing equivalents to the soluble ER luminal protein PDI, which can then be handed over to a folding 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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Endoplasmic Reticulum-associated Protein Degradation in Plant Cells

Aldo Ceriotti[1] (✉) · Lynne M. Roberts[2]

[1]Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, Via Bassini 15, 20133 Milan, Italy
ceriotti@ibba.cnr.it

[2]Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Abstract The endoplasmic reticulum (ER) is equipped with a quality control function that retains misfolded and unassembled proteins and allows only structurally mature polypeptides to be transported to their final destination. The retained proteins are eventually retro-translocated to the cytosol and destroyed by a process called endoplasmic reticulum-associated degradation (ERAD). Besides being involved in the degradation of aberrant polypeptides, the ERAD pathway is also used to regulate cellular functions and is exploited by some plant and bacterial toxins to reach the cytosol after internalization by target cells. After summarizing the general characteristics of the ERAD pathway, we describe the features of known plant ERAD substrates and of the plant degradative machinery, highlighting the role of protein disposal in the response to ER stress.

1 Introduction

Protein folding is an error-prone process, and while certain polypeptides can fold quite efficiently, a relevant fraction of other proteins fails to reach a native conformation even under normal conditions. These misfolded polypeptides may be endowed with new and deleterious activities, act as dominant negative mutants, or may more generally get involved in spurious interactions that interfere with normal cellular functions. As such, they must be rapidly eliminated. Indeed, when the overall efficiency of protein biogenesis in a mammalian cell population was measured, it was found that up to two-thirds of newly synthesized polypeptides were rapidly destroyed (Schubert et al. 2000). In addition, cellular proteins are subjected to post-synthetic damage due to the action of reactive small molecules, proteases, and to changes in primary sequence (protein ageing). These post-synthetic changes, as well as exposure to high temperatures, can lead to a degree of protein unfolding that can be deleterious to the cell. It is therefore clear that cells must have a way to deal with this continually generated mass of aberrant and potentially damaging protein. The way the endoplasmic reticulum (ER) copes with the problem of disposing of misfolded proteins is the subject of this chapter.

2
Retention in the ER as a Prelude to Degradation

Many of the proteins that are inserted into the ER lumen or into the ER membrane are not residents of this organelle and so must be transported to other intracellular sites, or be secreted. About 20 years ago, it became apparent that proteins entering the ER are subjected to a quality-control mechanism that allows only correctly folded and (when applicable) assembled polypeptides to be transported to their final destination (reviewed in Ellgaard and Helenius 2003). It was later realized that rather than being degraded within the lumen of the ER, soluble and membrane bound secretory proteins that fail to fold are destroyed after being dislocated to the cytosol, where they become substrates of proteasomes. Thus, although the vacuole can also contribute to the disposal of ER and secretory proteins (Hong et al. 1996; Tamura et al. 2004), retention and proteasomal degradation appears to be a common mechanism that cells use to avoid the potentially dangerous expression of aberrant polypeptides. This disposal pathway is often referred to as ER-associated protein degradation (ERAD) and considerable progress has been made during the last decade towards understanding its individual steps at the molecular level.

Different mechanisms can contribute to the retention of misfolded proteins in the ER. One of them could be the absence of an exposed export signal. According to current models, export of proteins from the ER can occur by bulk-flow or can be mediated by receptors that recognize specific signals on the exported proteins (see chapt. by Aniento et al., this volume). If these signals are not exposed on the surface of the molecule because of misfolding, the protein will be retained in the folding compartment. The retention of unfolded proteins in the ER can also be due to their association with molecular chaperones, which in turn are maintained in the ER at steady state because they bear signals for retrieval from downstream compartments or because they form an insoluble matrix and are therefore excluded from budding vesicles.

A role for protein trafficking in the ERAD pathway is suggested by the finding that a generalized block in the transport of proteins to the Golgi complex can inhibit the degradation of certain substrates (reviewed in Ahner and Brodsky 2004). In addition, it has been shown that certain misfolded proteins can leave the ER in transport vesicles only to be returned prior to degradation (Vashist et al. 2001; Yamamoto et al. 2001; Sato et al. 2004). However, since a block in protein transport can also have a direct impact on ER structure and homeostasis, it remains to be established whether certain proteins must recycle from the Golgi to be degraded, or whether the observed effects are due to a general perturbation of ER functions.

In certain cases, aggregation can contribute to the retention of unfolded protein in the ER. Misfolded proteins are often aggregation prone, and their inclusion in large complexes is likely to limit their diffusion through the ER and their insertion into transport vesicles. While in some cases aggre-

gate formation does not hamper the subsequent degradation of the misfolded polypeptide (Molinari et al. 2002), in other cases the ER seems to be unable to efficiently disrupt such complexes, leading to the prolonged accumulation of the misfolded proteins (Sparvoli et al. 2001).

3 An Overview of the ERAD Pathway

In general, the ERAD process can be divided in at least four stages: (i) recognition of the aberrant polypeptide, (ii) dislocation, (iii) release from the ER surface, and iv) degradation. However, it is becoming clear that rather than constituting a single defined pathway, ERAD can be considered as a collection of different pathways that may involve different players depending on the characteristics of the protein substrate in question.

Different mechanisms can contribute to the recognition of misfolded proteins in the ER. The same molecular chaperones that assist the folding of newly synthesized polypeptides have been often implicated in the process that prepares aberrant proteins for degradation. Indeed, chaperone interactions, aside from their value in facilitating protein folding may be important in maintaining proteins in soluble form and/or in targeting any terminally misfolded proteins to the next step of the ERAD pathway (dislocation).

The plant homologue of the immunoglobulin heavy chain binding protein (BiP) is a major ER chaperone of the Hsp70 protein family. Certain ERAD substrates have been found to bind extensively to BiP in the ER, their degradation being tightly associated with their release from the chaperone (Knittler et al. 1995; Skowronek et al. 1998; Chillarón et al. 2000; Molinari et al. 2002). Degradation of some, but not all, ERAD substrates has been found to be impaired in the presence of different BiP mutants (Plemper et al. 1997; Brodsky et al. 1999; Nishikawa et al. 2001), and aggregation of some misfolded proteins has been found to be exacerbated in yeast strains carrying mutated BiP (Nishikawa et al. 2001; Kabani et al. 2003). So, although BiP may play different roles in ERAD, one of them appears to be to maintain particular protein substrates in a soluble, retrotranslocation-competent state.

Different ER oxidoreductases including yeast Eps1p (Wang and Chang 2003), ERp57 (Antoniou et al. 2002), and protein disulfide isomerase (PDI) (Molinari et al. 2002; Tsai et al. 2001) have also been implicated in the disposal of ERAD substrates. Although extensive unfolding may not be strictly required for dislocation (Tirosh et al. 2002; Fiebiger et al. 2002), reduction of disulfide bonds has been shown to precede dislocation of IgM heavy chains (Fagioli et al. 2001) and ER oxidoreductases are obvious candidates as the catalyzers of reducing reactions required to prepare disulphide-bonded substrates for the membrane translocation step (Molinari et al. 2002). However, it should be noted that yeast PDI has been implicated in the disposal of

a cysteine-free polypeptide, indicating that substrate reduction is not the only activity of PDI involved in quality control (Gillece et al. 1999). A physiological redox state is important not only to allow substrate reduction, but also for the general functioning of the ERAD machinery, since a cysteine-free protein is stabilized in the ER by treatments that affect intracellular redox potential or free thiol status (Tortorella et al. 1998). Finally, it is interesting to note that the degradation of certain membrane proteins has been found to require the action of cytosolic chaperones such as Hsp70 and Hsp104p, the latter being a yeast protein belonging to the AAA-ATPase superfamily (Hill and Cooper 2000; Zhang et al. 2001; Taxis et al. 2003). The activity of cytosolic chaperones appears to be specifically required for the disposal of large or tightly folded cytosolic domains of certain membrane proteins (Taxis et al. 2003).

Although interaction with chaperones is clearly important to prepare ERAD substrates for degradation, it is also becoming evident that other factors contribute to divert unfolded proteins from the biosynthetic to the degradative pathway. Within this context, the best characterized recognition mechanism within the ER is the one based on specific modifications of N-linked glycan chains. Many of the proteins that are inserted into the ER are modified by the addition of a core glycan of 14 saccharides (Fig. 1), which is transferred from a lipid carrier to asparagine residues within the sequon Asn-X-Ser/Thr, where X can be any amino acid but proline. The structure of the glycan initially transferred to the protein is the same in virtually all eukaryotes, and this high level of conservation likely reflects a conserved functional role. The large polar structures of core glycans can have a direct effect on protein stability and solubility, but also contribute to protein folding by mediating the interaction of nascent and newly synthesized glycoproteins with two ER chaperones, calnexin and calreticulin (see chapt. by Vitale and Denecke, this volume). While calnexin is a type I membrane protein, calreticulin is a soluble protein. Briefly, these chaperones are related to the legume lectin family and both of them interact with monoglucosylated oligosaccharides produced by the action of ER glucosidase I and glucosidase II (Fig. 1). Most importantly, monoglucosylated oligosaccharides are also produced by the action of UDP-glucose:glycoprotein glucosyltransferase (GT), a folding sensor that can selectively and iteratively add a glucose residue to misfolded (but not to folded) proteins, giving them further opportunities to interact with calnexin and calreticulin, and thus to complete structural maturation.

In several cases, it has been observed that inhibition of glucose trimming, and hence of calnexin/calreticulin binding, accelerates the degradation of misfolded proteins (de Virgilio et al. 1999; Wilson et al. 2000; Chung et al. 2000; Molinari et al. 2002; Mancini et al. 2003). Although this indicates that entry into the calnexin/calreticulin cycle can protect certain substrates from degradation, stabilizing ERAD substrates in their monoglucosylated form not always resulted in a prolonged half-life. While degradation of certain substrates was retarded (Molinari et al. 2002; Cabral et al. 2002; Oda et al. 2003),

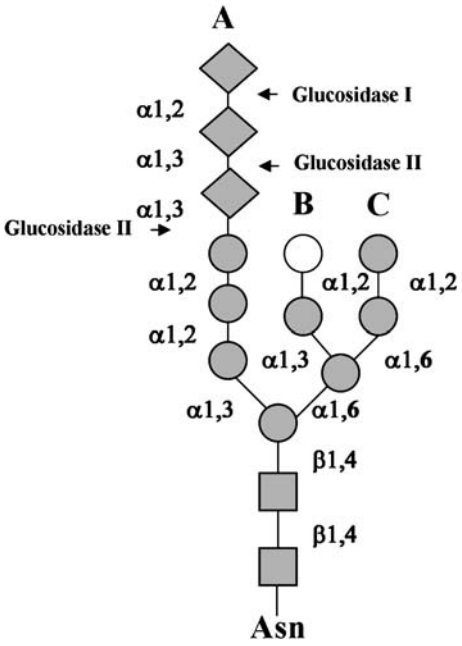

Fig. 1 Structure of the N-linked core oligosaccharide. A branched oligosaccharide composed of 14 units is presynthesized and transferred en bloc to specific asparagine (Asn) residues. The three branches are indicated with the letters A, B and C. The oligosaccharide contains two N-acetylglucosamines (*squares*), nine mannoses (*circles*) and three glucoses (*diamonds*). The site of action of glucosidase I and II are indicated. Yeast and mammalian ER α1,2-mannosidase I preferentially removes the mannose residue present at end of the B branch (*empty circle*)

the degradation of others was left unaffected (Chung et al. 2000; Fagioli and Sitia 2001; Mancini et al. 2003) or even accelerated (Liu et al. 1999). These data would suggest that in many cases glycoprotein degradation can occur both via a pathway involving an interaction with calnexin/calreticulin and via a pathway that does not involve these chaperones. In addition, it appears that some substrates can escape the attention of lectin chaperones and proceed along the ERAD pathway even when glucosidase action is blocked, while others are more sensitive to treatments that favor the accumulation of monoglucosylated glycans. These observations indicate that glucose trimming plays an important but complex role in the ERAD pathway.

The trimming of mannose residues is a further modification of glycan structure that has been implicated in the regulation of ERAD. The ER of yeast and mammals contains a Class I α1,2-mannosidase that removes a mannose from the B-branch of the oligosaccharide to yield the $Man_8GlcNAc_2$ B isomer (Fig. 1). If this process is inhibited using kifunensine or 1-deoxymannojirymicin, or the mannosidase gene is disrupted, degra-

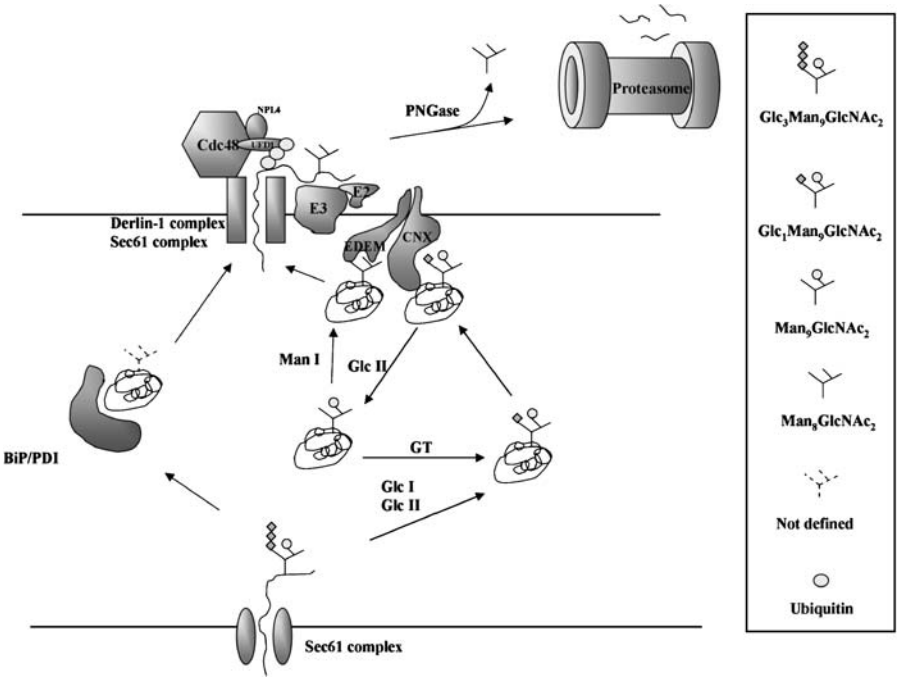

Fig. 2 Schematic model of the ERAD pathway. After being inserted in the ER through the Sec61 channel, proteins fold with the assistance of molecular chaperones and folding enzymes such as BiP and PDI. These folding assistants help maintaining unfolded proteins in solution before they manage to fold or are delivered to the retro-translocation channel. In the case of glycoproteins, folding can be assisted by the lectin chaperones calnexin (CNX) and calreticulin. Although not depicted in the figure, folding substrates can sequentially bind different chaperones. Binding to calnexin and calreticulin requires trimming of glucose residues by glucosidase I (Glc I) and II (Glc II). Further trimming by glucosidase II releases the substrate protein from calnexin. If the released protein fails to fold, it can be reglucosylated by UDP-glucose:glycoprotein glucosyltransferase (GT). Prolonged residence in the ER allows trimming of one or more mannose residues by ER mannosidase I (Man I), and the mannose trimmed glycan mediates binding to EDEM. Since calnexin and EDEM interact, it is possible that substrates are directly transferred from CNX to EDEM. The Sec61 complex and a protein complex containing Derlin-1 have been implicated in the retro-translocation step. Retrotranslocated proteins are ubiquitinylated by the concerted action of ubiquitin-conjugating enzymes (E2) and of ubiquitin-protein ligases (E3). The p97/Cdc48-Ufd1-Npl4 complex is recruited to the ER membrane by the Derlin-1 complex and contributes to the dislocation of the ERAD substrate, which is then escorted to the proteasome for destruction. During these late stages of ERAD, glycoprotein substrates can be deglycosylated by the action of cytosolc peptide:*N*-glycanase (PNGase)

dation of many glycoprotein substrates is impaired (Su et al. 1993; Knop et al. 1996b; Liu et al. 1997; Jakob et al. 1998; de Virgilio et al. 1999; Chung et al. 2000; Wilson et al. 2000; Fagioli and Sitia 2001). Conversely, overex-

pression of ER mannosidase I enhanced the degradation of a glycoprotein substrate (Hosokawa et al. 2003). Even in the case of a substrate that is stabilized by an interaction with calnexin, mannosidase inhibition blocked degradation when glucosidase action (and thus calnexin binding) was also hampered (Wilson et al. 2000). This indicates that the stabilization afforded by inhibition of mannose trimming was not due to enhanced binding to calnexin. So, how do mannosidase inhibitors impede the degradation of glycoprotein substrates? In yeast, deletion of a gene encoding a non essential protein known as homologous to mannosidase I (Htm1p/Mnl1p), selectively causes a reduction in the rate of degradation of mutant glycoproteins, but not of unglycosylated substrates (Jakob et al. 2001; Nakatsukasa et al. 2001). The Htm1p/Mnl1p protein lacks two cysteine residues that are essential for α-mannosidase activity, so it is not involved in the processing of N-linked oligosaccharides. Rather, it is thought to act as a mannose-specific lectin, able to recognize de-mannosylated oligosaccharides. Similarly, an ER degradation enhancing α-mannosidase-like protein (EDEM) that lacks mannosidase activity has been characterized in mammals and its overexpression shown to accelerate degradation of a misfolded protein (Hosokawa et al. 2001). The mammalian protein can complement the yeast orthologue, highlighting the conservation of the quality control system in evolutionarily distant organisms (Gnann et al. 2004). EDEM was found to interact with calnexin, and some substrates appear to be transferred from calnexin to EDEM for degradation (Oda et al. 2003; Molinari et al. 2003). A model has been therefore proposed in which, after release from calnexin, misfolded proteins either reenter the calnexin cycle by the action of GT or are sequestered by EDEM for degradation (Fig. 2). The specificity of the putative lectins remains to be determined but, although $Man_8GlcNAc_2$ was initially thought to represent the main signal for degradation, several lines of evidence indicate that further trimming intermediates may be recognized by the quality control system (Ermonval et al. 2001; Kitzmüller et al. 2003; Frenkel et al. 2003; Herscovics 2001; Herscovics et al. 2002; Hosokawa et al. 2003). It should also be noted that demannosylation per se is unlikely to constitute a signal for degradation since for instance, most glycoproteins are trimmed to $Man_8GlcNAc_2$ in *Saccharomyces cerevisiae*, and mannose-trimmed glycans can be found in long-lived residents of the ER (Matsuoka et al. 1994; Navazio et al. 2002). A cooperation between mannose-binding lectins and ER chaperones may therefore be crucial to confer to the recognition machinery the required specificity toward misfolded glycoproteins. The recent identification of other lectin-like proteins involved in ERAD highlights the complexity of the mechanisms used to target proteins to degradation (Buschhorn et al. 2004; Olivari et al. 2005).

The transient nature of the dislocating polypeptide has so far hampered a detailed characterization of the process by which ERAD substrates cross the ER membrane. Biochemical and genetic evidence indicate that retrotranslocation may occur through the Sec61 complex, the same protein con-

ducting channel that mediates import into the ER. Different ERAD substrates have been found to interact with the Sec61 complex (Wiertz et al. 1996; de Virgilio et al. 1998; Wesche et al. 1999), and the degradation of several substrates is affected in different yeast *sec61* mutants (Plemper et al. 1997; Pilon et al. 1997; Zhou and Schekman 1999; Simpson et al. 1999). However, recent data indicate that the ER membrane contains another protein complex that may play a role in dislocation (Lilley and Ploegh 2004; Ye et al. 2004). This complex contains the membrane proteins Derlin-1 and VIMP (VCP-interacting membrane protein, where VCP is another name for p97/Cdc48, see below), Derlin-1 being the mammalian homologue of the yeast Der1p, a protein that is required for the degradation of a subset of ERAD substrates in this organism (Knop et al. 1996a; Taxis et al. 2003).

In general, dislocation to the cytosol is accompanied by substrate ubiquitinylation, a modification that constitutes a crucial signal for proteasomal degradation. Accordingly, different ubiquitin conjugating enzymes and ubiquitin protein ligases have been shown to be required for the degradation of ERAD substrates (Biederer et al. 1996; Bays et al. 2001; Swanson et al. 2001; Fang et al. 2001; Yoshida et al. 2002; Yoshida et al. 2003b). When polyubiquitination is impaired, many substrates are not dislocated indicating that recognition of the ubiquitin moieties is a pre-requisite for the extraction of misfolded proteins from the membrane (Biederer et al. 1997; Bordallo et al. 1998; de Virgilio et al. 1998; Kikkert et al. 2001; Jarosch et al. 2002). Although ubiquitin chains were initially thought to provide the ratchet for extraction of the protein by proteasomes, accumulating evidence indicates that they may also facilitate recognition by other cytosolic factors that are able to complete the extraction of the dislocating protein. One identified factor is the cytosolic complex containing the AAA-ATPase p97 (also called valosin-containing protein, or Cdc48p in yeast) and its partners Ufd1 and Npl4 (Ye et al. 2001). p97/Cdc48p forms homohexamers that are thought to undergo ATP-dependent movements and can potentially work as an unfoldase, disassembling protein complexes in a fashion that is reminiscent of the disassembly of SNARE complexes by the AAA-ATPase NSF. An interaction with the Derlin-1 complex is likely to place p97 in the right position to take care of ERAD substrates soon after they appear on the cytosolic side of the ER membrane, possibly facilitating their release from interacting proteins (Rabinovich et al. 2002; Ye et al. 2003; Ye et al. 2004) (Fig. 2). p97 can bind substrates independent from ubiquitination, but acts synergistically with the cofactor Ufd1 in the recognition of polyubiquitin chains (Ye et al. 2003). In addition, its yeast homologue Cdc48p has been proposed to cooperate with a set of ubiquitin-binding factors to escort dislocated substrates to the proteasome for degradation (Medicherla et al. 2004; Richly et al. 2005).

As mentioned above, many of the proteins that are inserted into the ER are modified by the addition of N-linked glycans. These glycan chains not only play a role in the recognition within the ER but also contribute

to the identification of the dislocating substrate by ubiquitin ligases able to recognize high-mannose glycan chains (Yoshida et al. 2002, 2003b). Removal of these glycoprotein substrates also involves the action of a cytosolic peptide:*N*-glycanase (PNGase), a deglycosylating enzyme (reviewed in Suzuki and Lennarz 2003). Both the yeast and the mammalian enzymes selectively act on high-mannose glycans and not on complex-type oligosaccharides. In addition, these enzymes act on unfolded glycoproteins but are unable to remove N-linked chains from folded ones (Hirsch et al. 2003, 2004). This enzyme therefore has the requisite activities to act on unfolded polypeptides dislocated from the ER. PNGases from vertebrates and insects also contain a PUB/PUG domain that has been implicated in the association with a variety of ubiquitin/proteasome pathway-related proteins and proposed to recruit a glycoprotein-degradation complex (Suzuki et al. 2001; Suzuki and Lennarz 2003). This domain is not present in the yeast and *Arabidopsis thaliana* homologues (Suzuki et al. 2001), suggesting that different protein complexes may take care of dislocated glycoproteins in different organisms. Although alternative proteolytic systems have been implicated in the degradation of certain substrates (Fayadat et al. 2000; Cabral et al. 2000; Mancini et al. 2003; Brandizzi et al. 2003; Schmitz et al. 2004) the proteasome-mediated destruction of the dislocated protein commonly constitutes the final phase of the ERAD pathway. Since misfolded proteins presented by the dislocation apparatus to the cytosol are likely to rapidly aggregate if left unattended, the dislocation and degradation steps of ERAD are normally tightly coupled, and cytosolic degradation intermediates cannot normally be detected unless proteasomal activity is blocked. This tight link between dislocation and degradation may also explain why certain ERAD substrates accumulate in the ER rather than in the cytosol when proteasomal activity is inhibited (Chillarón and Haas 2000; Mancini et al. 2000).

4 The Emerging Similarities and Differences of the Plant ERAD Pathway

Evidence in favor of the existence of a mechanism of protein retention and disposal in the plant ER was provided by studies on the bean storage protein phaseolin. Phaseolin is a homotrimeric protein of the 7S class that accumulates in the storage vacuoles of bean (*Phaseolus vulgaris*) cotyledons during seed development. Trimer formation is required for the intracellular transport of this protein, and is under a complex regulation involving the interaction with BiP and the trimming of glucose residues from the N-linked glycans (Lupattelli et al. 1997; Foresti et al. 2003). Trimer assembly is mediated by two sets of α-helices present in the monomer, and deletion of either of these domains leads to the synthesis of an assembly-defective protein (Ceriotti et al. 1991; Foresti et al. 2003). When expressed in tobacco protoplasts or transgenic

tobacco plants, assembly-defective phaseolin shows a prolonged association with BiP (Pedrazzini et al. 1994; Foresti et al. 2003), and is degraded by a pathway that is not affected by brefeldin A (BFA), a fungal metabolite that blocks Golgi-mediated traffic to the vacuole (Pedrazzini et al. 1997; Nebenführ et al. 2002). Although it still remains to be established whether cytosolic proteasomes are involved in the eventual degradation of defective phaseolin, it can be concluded that some aspects of the fate of this protein are shared with other ERAD substrates. These aspects include extensive binding to molecular chaperones, retention in the ER and BFA-insensitive degradation.

A second substrate found to be degraded in a BFA-insensitive fashion is the A chain of the plant toxin ricin. Ricin is a ribosome-inactivating protein synthesized by castor bean (*Ricinus communis*) endosperm cells. The mature protein consists of a catalytic chain (RTA) linked by a single disulfide bond and non-covalent interactions to a cell binding B chain (RTB). RTA inactivates ribosomes by specifically depurinating a site in 23S/26S/28S rRNA that is critical for the binding of elongation factors, a modification that leads to an irreversible block in protein synthesis (reviewed in Hartley and Lord 2004). To kill mammalian cells, ricin must therefore cross a cellular membrane to reach the cytosol where its target ribosomes are located. Mammalian cell intoxication begins with the endocytic uptake of ricin holotoxin and its retrograde transport to the ER where the holotoxin is reduced with the help of oxidoreductases present in this compartment (Lord et al. 2003; Spooner et al. 2004). RTA then appears to enter the ERAD pathway and is dislocated to the cytosol (Wesche et al. 1999). Similarly to ricin, other protein toxins have been shown to hijack the ERAD pathway to reach their cytosolic targets (Lord et al. 2003). Clearly, in order to act as cellular poison, a proportion of RTA must uncouple from this pathway to avoid complete destruction by proteasomes, a feature that distinguishes this and other toxins that retro-translocate from the ER from standard ERAD substrates.

In castor bean endosperm cells, ricin is synthesized as a precursor in which the RTA and RTB are connected by a linker peptide of 12 amino acids that has been shown to be required for correct targeting to the vacuole (Frigerio et al. 1998, 2001; Jolliffe et al. 2003). In this context, RTA is not recognized as an ERAD substrate. The ricin precursor is then transported out of the ER and delivered to the vacuole, where a propeptide preceding RTA and the linker peptide are removed, generating mature ricin. When plant cells are forced to express RTA by itself however, the glycosylated RTA initially segregated within the ER lumen becomes cytosolic and is degraded in a BFA-insensitive manner (Frigerio et al. 1998; Di Cola et al. 2001). Treatment with proteasome inhibitors stabilizes RTA expressed in tobacco protoplasts, demonstrating the involvement of this multicatalytic complex in RTA degradation (Di Cola et al. 2001). Although generally following the ERAD pathway, RTA behaves differently to other substrates so far analyzed in yeast and mammalian cells. As mentioned above, ERAD is generally characterized by a tight coupling

between the dislocation and degradation steps, that avoids the transient accumulation of potentially dangerous proteins in the cytosol. In contrast, fractionation experiments could clearly show the accumulation of dislocated RTA in the cytosol even in the absence of proteasome inhibitors, indicating that some specific feature allows this protein to escape prompt recognition by the cytosolic degradative machinery (Di Cola et al. 2001).

Although the ubiquitinylation of the N-terminal amino acid is involved in the degradation of certain substrates in mammalian cells, ubiquitin is normally linked to the substrate through an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the target protein. Like other toxins that are thought to dislocate from the ER, RTA is characterized by a low lysine content, a characteristic that likely reduces the chances of this protein being ubiquitinylated when appearing on the cytosolic face of the ER. The idea that the transient accumulation of dislocated RTA in the protoplast cytosol was indeed due to the low lysine content of this protein was confirmed by the finding that increasing the number of lysine residues in RTA from two to six was sufficient to convert RTA into a more standard substrate that was unable to accumulate in the cytosol unless proteasomes were inhibited (Di Cola et al. 2005). Consistent with this view was the finding that mutation of the two endogenous lysyl residues present in native RTA caused a marked stabilization of the protein in the cytosol of tobacco protoplasts. Although ubiquitinylation of RTA could not be directly demonstrated, it is likely that this dependence of the rate of degradation on lysine content reflects differential ubiquitinylation of the three RTA forms.

Since an active ubiquitinylation machinery is normally required for the extraction of proteins from the ER, it is unclear how the lysine-free RTA could dislocate as efficiently as the lysine-containing versions of the protein. One possibility is that N-terminal ubiquitinylation is sufficient for RTA extraction, but not for rapid targeting to the proteasome. Alternatively, ubiquitinylation may be required only to release the dislocated protein from the ER surface and not for the actual transfer through the membrane. If this is the case, RTA may have evolved a mechanism to leave the dislocation apparatus without the help of ubiquitin-dependent chaperones. Since ribosomes have been shown to favor RTA refolding in vitro (Argent et al. 2000), it is possible that an interaction between unfolded RTA emerging from the ER membrane and the ribosome triggers toxin refolding and release into the cytosol.

The accumulation of deglycosylated substrates in the cytosol is normally observed only when proteasomal activity is inhibited. This has been taken to mean that deglycosylation and degradation are normally tightly linked, in accord with the observation that PNGase can be present in a complex with the proteasome itself (Park et al. 2001). However, this observation would also be compatible with a model in which glycopeptides are first generated by the proteolytic activities of the proteasomes, and then deglycosylated by a cytosolic PNGase. Since the transfer to PNGase is not strictly required for pro-

tein disposal in yeast and mammals (Suzuki et al. 2000; Blom et al. 2004), it is conceivable that, depending on the level of coupling between dislocation and degradation, PNGase may work either before or after the proteasome along the glycoprotein disposal pathway. The finding that deglycosylated lysine-free RTA accumulated in the cytosol under physiological conditions when proteasomes were active established that deglycosylation can precede proteasomal degradation and suggested that ubiquitinylation plays an important role in the final stages of plant ERAD (Di Cola et al. 2005).

The effects of changing lysine content on RTA degradation also highlighted a difference in the way plant and mammalian cells "see" specific lysine residues and use them to target a protein for degradation. While the lysine-free form of RTA was stabilized and supertoxic in the cytosol of tobacco protoplasts, it retained native potency (when re-associated in vitro with RTB) towards mammalian cells (Deeks et al. 2002). These data suggest that the endogenous lysine residues in RTA do not act as targets for degradation of this protein in mammalian cells, in striking contrast with the observations made from the expression of RTA in plant cells. It is likely that the two lysines have been maintained through evolution precisely because they are not used by mammalian cells as targets for ubiquitinylation. At the same time, we may speculate that their presence in the preproricin sequence, and recognition by the ubiquitinylation machinery, may be advantageous in castor bean endosperm in situations where the preproricin-producing cells may need to deal with truncated forms arising from premature termination of translation or from the translation of aberrant mRNAs (Schubert et al. 2000).

Another protein shown to dislocate to the cytosol in plant cells without being immediately degraded is sGFP-P, a fusion between the green fluorescent protein (GFP) and the P-region of maize calreticulin. In tobacco protoplasts, accumulation of this protein was not enhanced by treatment with BFA or over-expression of Sec12 (which is known to cause a block in the ER-to-Golgi transport), and the protein was slowly degraded via a non-proteasomal pathway (Brandizzi et al. 2003). When this GFP fusion protein was expressed in the epidermal cells of *Nicotiana benthamiana* leaves it was found to label not only the nuclear envelope and the ER (as expected for an ER-targeted protein), but also the cytosol, and was found to be transported into the nucleoplasm in a microtubule-dependent fashion. In the case of this protein, the mechanisms allowing uncoupling of dislocation and degradation remain unclear and analysis of further substrates will be required to determine whether distinct phases of dislocation and degradation are more common in plants that could be expected based on the results obtained in yeast and mammalian cells. Clearly, the finding that the fluorescence emitted by this fusion was evident in the cytosol and nucleoplasm indicated that either dislocation occurred without unfolding of the GFP domain, or that sGFP-P could refold in plant cytosol. While GFP itself can efficiently refold after denaturation, another dislocated substrate containing a GFP moiety was found not to refold

in vitro suggesting that dislocation of folded proteins remains a possibility (Fiebiger et al. 2002).

Studies on the fate of the barley mildew resistance o (MLO) protein have recently helped shed light on the plant ERAD machinery. MLO is an integral membrane protein with seven transmembrane helices, an extracellular N-terminus and a cytosolic C-terminus and normally accumulates in the plasma membrane (Devoto et al. 1999). The MLO protein inhibits a resistance reaction to the powdery mildew pathogen, and a series of *mlo* mutant alleles that confer resistance to this pathogen have been described. Analysis of several mutants revealed that some did not accumulate detectable levels of the MLO protein, whilst having normal levels of transcripts. Analysis of the stability of fusions containing either wild-type or mutated MLO proteins revealed that while the fusions containing the wild-type protein were stable, the ones containing mutants were destabilized, and suggested that the lack of MLO expression was through a quality control mechanism. Accordingly, the degradation of an ustable (MLO-1) allele in Arabidopsis protoplasts was found to be BFA-insensitive and could be slowed down by proteasome inhibitors. Thus, the lack of expression of certain *mlo* alleles is due to a quality control mechanism that impedes expression of the mutant proteins at the cell surface by diverting them for proteasomal degradation (Müller et al. 2005). Alleles that were found to be unstable in Arabidopsis were generally found to be similarly unstable in yeast and human cells, suggesting that the recognition mechanisms are evolutionarily conserved. This work has provided a direct characterization of a component of the plant ERAD machinery, showing that degradation of MLO mutants requires the action of a plant p97/Cdc48 homologue. The Arabidopsis genome contains three homologues of the AAA-ATPase Cdc48/p97 (Lord et al. 2002; Müller et al. 2005). When mutations in the ATPase domains were introduced in one of these genes, and the mutated protein was coexpressed with the unstable MLO-1 fusion protein in Arabidopsis protoplast, degradation of the ERAD substrate was greatly impaired (Müller et al. 2005). This, together with the observation that the MLO-1 protein is ubiquitinylated in vivo, indicates that also in plant cells the Cdc48/p97 protein and ubiquitinylation play an important role in the degradation of certain aberrant proteins.

Besides clearly demonstrating the existence in plants of a pathway analogous to the one described in other systems, the work performed to date has begun to reveal some of the mechanisms that govern substrate recognition, dislocation and degradation in plants. Both phaseolin and sGFP-P are substrates for BiP binding, and this association may be required to maintain these particular proteins in a degradation-competent state. Although only a fraction of assembly-defective phaseolin was ever found in association with BiP, the disappearance of total and BiP-bound defective phaseolin followed similar kinetics, indicating that dissociation from BiP and degradation are kinetically linked (Pedrazzini et al. 1997). A BiP binding site has been mapped

to a region of phaseolin that is also involved in trimer assembly (Foresti et al. 2003). When this region was appended to the C-terminus of GFP it did not disturb protein folding (as judged by the GFP fluorescence) but did stimulate BiP binding. Whether addition of this BiP binding site causes a destabilization of the GFP-phaseolin fusion has not yet been determined.

Whether mannose trimming regulates the degradation of plant glycoproteins is currently not known, and so far the issue has been examined only in the case of RTA. When expressed in tobacco protoplasts, this protein is modified by the removal of one or more mannose residues (Di Cola et al. 2001). Since a mannosidase inhibitor did not affect degradation and since different RTA glycoforms were observed in the cytosol when trimming was undisturbed, it appears that the recognition of RTA as an unfolded protein and its dislocation do not rely on the recognition of a single, specific glycan structure (Di Cola et al. 2005). RTA has been shown to interact with liposomes containing negatively charged phospholipids in vitro, and this interaction is known to induce a conformational change in the protein that may facilitate the recognition of RTA as an ERAD substrate (Day et al. 2002). It is thus possible that RTA may have evolved a specific and glycan-independent mechanism to allow its recognition as an unfolded protein in the ER of intoxicated cells. Clearly, analysis of other glycoprotein substrates will be required to determine the role played by mannose trimming in the plant ERAD pathway.

5
The ERAD Pathway and the Unfolded Protein Response

Besides being equipped with a pathway dedicated to the disposal of misfolded proteins, the ER also contains molecules able to sense the presence of aberrant polypeptides and to trigger a cellular response—the unfolded protein response, UPR. This response invariably includes the increased expression of ER chaperones and folding enzymes and can be triggered by any condition able to compromise folding in the ER. In fact, it can be activated not only by exogenous stresses such as the one imposed by pathogen infection, but also by endogenous ones, such as genetic mutations or the differentiation of professional secretory cells. Experimentally, inhibition of N-glycosylation or treatment with reducing agents are commonly used to induce UPR in different systems, including plants. In developing bean cotyledons, tunicamycin treatment leads to an increase in the synthesis of BiP (D'Amico et al. 1992) and the levels of BiP, PDI, calnexin and calreticulin are increased in different plant tissues exposed to treatments that cause accumulation of misfolded protein in the ER (Denecke et al. 1991, 1995; Koizumi 1996; Koizumi et al. 2001). In addition, genetic mutations that lead to the synthesis of aberrant zein polypeptides cause UPR in developing maize endosperm (Marocco et al. 1991; Coleman et al. 1995; Kim et al. 2004).

While some basic features of UPR have been conserved throughout evolution, plants, yeast and mammals may have developed somewhat different strategies to respond to ER stress. In yeast, ER stress is sensed by the Ire1 protein, a type I transmembrane protein containing a luminal domain that senses ER stress and a cytosolic kinase and endoribonuclease domain. BiP associates to the luminal domain of Ire1p, but when unfolded proteins accumulate the concentration of free BiP decreases and this leads to BiP dissociation, followed by Ire1p oligomerization and autophosporylation. In turn, the cytosolic domain of Ire1p catalyzes the splicing of the *HAC1* mRNA that is then translated into a transcription factor that activates a large set of genes (Okamura et al. 2000; Travers et al. 2000). Genome-wide expression analysis in yeast revealed an intimate link between UPR and ERAD. In addition to the genes encoding molecular chaperones and folding enzymes, many other genes encoding proteins involved in secretion or in the biogenesis of the secretory pathway were found to be up-regulated during UPR (Travers et al. 2000). Among these were several genes known to be involved in ERAD. In addition, the rapid degradation of an ERAD substrate was found to require UPR activation, and UPR was found to be constitutively activated in strains carrying mutations in ERAD components.

In mammals, UPR is under a more complex control. An initial phase during which chaperone genes are induced mainly by the action of the ER-membrane bound transcription factor ATF6, is followed by a second phase during which ERAD gene transcription is activated in a mechanism that involves mammalian homologues of yeast Ire1p and the transcription factor XBP1 (Yoshida et al. 2003a). In addition to Ire1p homologues, the ER of mammalian cells contains an additional transmembrane kinase (PERK), which is similarly activated when BiP dissociates from the luminal sensor domain (Bertolotti et al. 2000). The cytosolic kinase domain of this protein phosphorylates the β-subunit of the translation initiation factor eIF2, causing an inhibition of protein synthesis and the activation of a specific set of genes (Harding et al. 1999; Okada et al. 2002). The first of these effects is thought to reduce the demands made on the ER, and to collaborate with the induction of chaperone synthesis in the recovery from the stress condition.

In plants, BiP transcription is regulated by a feedback mechanism that involves the monitoring of BiP levels (Leborgne-Castel et al. 1999). Homologues of Ire1p are present in Arabidopsis and rice (Koizumi et al. 2001) and a bZIP transcription factor having characteristics similar in part to those of ATF6 has been identified in Arabidopsis (Iwata and Koizumi, 2005). The expression analysis of a large number of Arabidopsis genes led to the identification of UPR induced and repressed genes (Martinez and Chrispeels 2003; Noh et al. 2003). Among the genes induced during UPR were those encoding putative α, β and γ-subunits of the Sec61 translocon, and a putative ubiquitin gene (Martinez and Chrispeels 2003). Since the Sec61 translocon and ubiquitin have implicated in ERAD, it is possible that the induction of these

genes contributes to maintaining an efficient disposal system in the plant ER during stress.

6 Conclusions

While we are still far from a thorough characterization of the ERAD pathway in plants, available information indicates that mechanisms able to extract misfolded polypeptides from the ER lumen and ER membrane are active in plant cells and that the proteasome is in some cases responsible for the degradation of these dislocated polypeptides. The role of ERAD may not be limited to the destruction of structurally defective proteins, and plant cells may exploit this potentially versatile pathway for the fine tuning of certain metabolic processes. It is well established that different cellular pathways including hormonal response, photomorphogenesis and pathogen defense are regulated by the controlled degradation of specific proteins. In addition, the vast array of ubiquitin-protein ligases identified in the Arabidopsis genome suggests the presence of many ubiquitination cascades able to recognize unique degradation signals (Vierstra 2003). By contrast, since ERAD is based on the identification of ill-defined features presented by structurally defective proteins, selective degradation could be achieved by a regulated alteration of the folding state, as in the case of yeast 3-hydroxy 3-methylglutaryl coenzyme A reductase (Hampton 2002; Shearer and Hampton 2005). It is thus possible, if not likely, that ERAD will be found to play a much more pervasive role in plant biology than initially thought.

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ER-to-Golgi Transport: The COPII-Pathway

Fernando Aniento[1] · Ken Matsuoka[2] · David G. Robinson[3] (✉)

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

[2]RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Japan

[3]Dept. Cell Biology, Heidelberg Institute of Plant Sciences, University of Heidelberg, Heidelberg, Germany
david.robinson@urz.uni-heidelberg.de

Abstract The endoplasmic reticulum (ER) is the starting site of the journey of newly synthesized proteins to the apoplast, plasma membrane and to the vacuolar compartments. Transport between these membrane compartments of the secretory pathway in eukaryotic cells is mediated by vesicles, which are produced by a budding mechanism involving coat proteins that capture specific cargo molecules and help package them into coated vesicles. These vesicles are known as COPII-coated vesicles, and are usually isolated after their induction in vitro using microsomal membranes, cytosol and a non-hydrolyzable GTP-analogue. COPII-coated vesicles are formed at specific sites in the ER known as ER-exit sites (ERES). ERES are well-characterized in mammalian cells, and can be recognized in some algae, but controversy surrounds their identification in higher plant cells.

1 COPII—Coat Proteins

In yeast and mammalian cells, a protein-covered transport vesicle 50–90 nm in diameter known as a COPII- (**co**at **p**rotein II) vesicle, is the transport intermediate that mediates the export of proteins from the ER. The COPII coat consists of three components: two dimeric protein complexes (the Sec23/24p complex and the Sec13/31p complex) and Sar1p, a small GTP-binding protein (Lee et al. 2004).

Genetic analysis in yeast and biochemical analysis of these proteins using an in vitro reconstitution system have revealed that COPII vesicles are created by the concerted action of subunits of the coat that interact with each other. Of these coat constituents, Sar1p is the key regulator of vesicle formation through the action of its GTPase activity. The Sec23/24p complex predominantly act as the cargo capturing component, and has a bow-tie like structure with each half of the tie being either the Sec23 or the Sec24 protein. This nearly symmetrical structure conforms with the fact that Sec24p has an approximately 20 kDa *N*-terminal domain with the remaining 80 kDa domain having significant homology with Sec23p. Sec23p also functions as the activating factor for Sar1p. The Sec13/31p complex on the other hand, which

locates to the most external layer of the COPII coat, acts as the assembler of the COPII vesicle structure. This protein complex also assumes a nearly symmetrical structure with two terminal globular domains at each end of the central cylindrical domain (Matsuoka et al. 2001). This symmetry is dependent upon the tetrameric nature of this protein complex with two 30 kDa Sec13p and two 130 kDa Sec31p subunits (Lederkremer et al. 2001). Because the globular Sec13p has a WD40 repeat (Saxena et al. 1996) that associates with the *N*-terminal region of Sec31p where a WD40 repeat is also present, it is likely that the globular structures at both ends of the Sec13/31 complex consist of the WD40 repeat regions of Sec13p and Sec31p.

The assembled coat is constituted from small hexagonal and pentagonal units (Matsuoka et al. 2001). Such a polygon-based structure not only allows for the assembly of small vesicles, but for other structures as well, such as tubules and sheets (Aldersey-Williams 1995). Indeed, the COPII coat assembled on the surface of liposomes not only makes vesicles (Matsuoka et al. 1998), but can also form coated tubules in some cases (Antonny et al. 2001). This property of the COPII coat enables not only the capture of small secretory proteins, but might also accommodate large and long structures, such as assembled collagen in mammalian cells (see below).

Membrane-associated proteins are also involved in COPII vesicle formation. Here, the most important membrane component for COPII vesicle formation is Sec12p, which is the GDP-GTP exchange factor (GEF) for Sar1p. A number of other membrane proteins, such as SNAREs, p24 proteins and other cargo receptors and/or transmembrane cargo proteins also contribute to the formation of the COPII vesicle, possibly supplying high affinity sites for the attachment of the Sec23/24 complex onto the ER membrane.

1.1 Evidence for COPII Proteins in Plants

In plants, the analysis of COPII-mediated transport from the ER is a little behind that of yeast and mammals. Earlier complementation work of yeast sec12ts mutants with *Arabidopsis* cDNA was the starting point of the characterization of plant genes for COPII proteins (d'Enfert et al. 1992). Both *Arabidopsis* Sec12 and Sar1 cDNAs were identified in this study. Further identification and characterization of these proteins was carried out *in planta* (Bar-Peled and Raikhel 1997) or with yeast mutants (Kim et al. 1997; Takeuchi et al. 1998). Subsequently, using the EST data base of Arabidopsis, an antibody against an Arabidopsis Sec23 homologue was generated and used to analyze the localization of this protein in the cell (Movafeghi et al. 1999; Yang et al. 2005). As a part of the ER was recognized by this antiserum, the presence of the COPII machinery was first confirmed to exist in plants. With the completion of the *Arabidopsis* genome, we can now find all the orthologues to the yeast gene products for COPII vesicle formation described above. Inter-

estingly, there is no homologue in either the *Arabidopsis* nor human genome for the yeast Sec16 protein, although this peripheral membrane protein is essential for the generation of COPII vesicles from the ER in *Saccharomyces cerevisiae* (Supek et al. 2002).

Several Sec23 and Sec24 homologues are present in the genomes of higher eukaryotes. *Arabidopsis* for example has 10 members belonging to this family (Fig. 1A). Seven of them are relatively close to yeast Sec23p, and the others are related to yeast Sec24p and its homologues Lst1p and Iss1p. In yeast, Iss1p forms a complex with Sec23p and this complex can substitute for the Sec23/24 complex both in vivo and in vitro (Kurihara et al. 2000). In contrast, the Lst1p-Sec23p complex cannot substitute for the Sec23/24 complex. The Lst1p-containing complex is, however, required for the proper export of a plasma membrane ATPase Pmp1p from the ER in yeast (Simoni et al. 2000). Therefore, it is possible that distinct complexes of plant Sec23 and Sec24 ho-

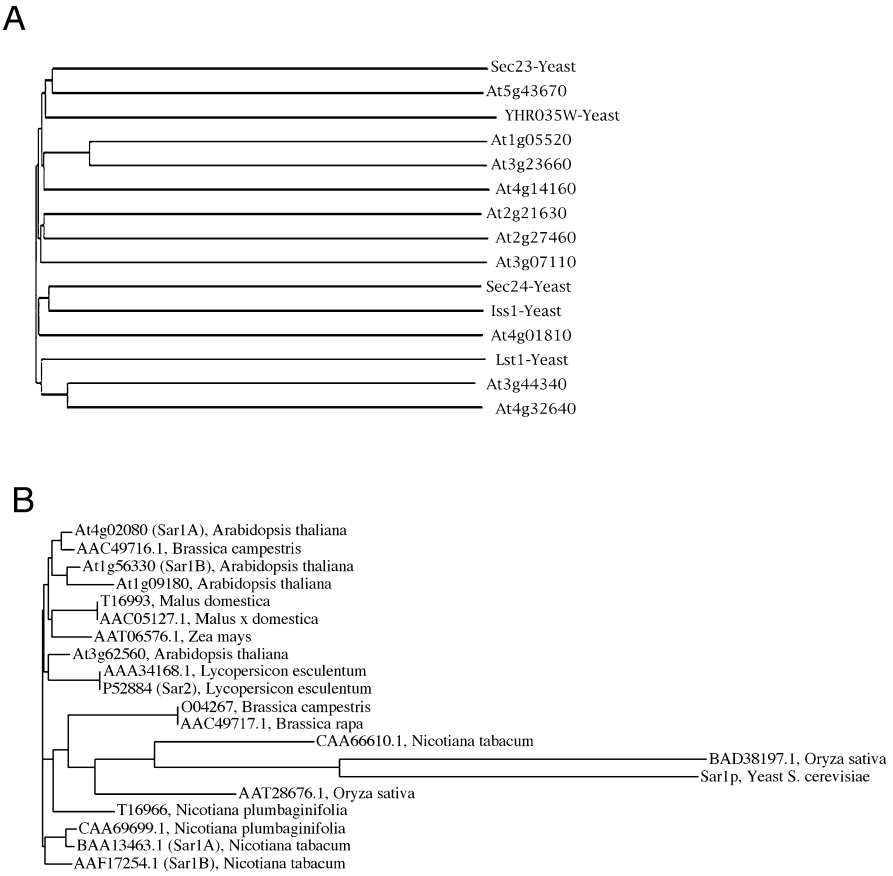

Fig. 1 Phylogenetic relationships of COPII proteins. **A** Sec23, Sec24. **B** Sar1

mologues may contribute to the differential export of distinct proteins from the ER, indeed, a recent analysis of the yeast Sec24 proteins supports this possibility (Milleer et al. 2002, 2003). However, single knock out *Arabidopsis* plants for two Sec23 homologues and a Sec24 homologue did not show any detectable phenotype (Matsuoka and Narisawa, 2006, personal communication). This observation raises the possibility that some of the homologous genes in *Arabidopsis* are simply redundant genes, as is the case with yeast Iss1 and Sec24. In any case, a recent in vitro analysis of the *Arabidopsis* Sec23/24 complex indicates that plant homologues of these proteins play a role in the export of membrane cargo molecules in the ER (Contreras et al. 2004). Details of this analysis are described below.

In contrast to the large number of Sec23/24 family proteins, only two Sec13 genes (At2g3005, At3g01340) and two Sec31 genes (At3g63460, At1g18830) are present in the *Arabidopsis* genome. Recent observations on ER export sites (ERES) in tobacco cells have indicated that plant Sec13 plays a similar role in intracellular protein transport as in other organisms (see below and Yang et al. 2005). The sizes of *Arabidopsis* Sec31 proteins are about 10–30 kDa smaller than yeast Sec31p, although the overall primary structures are similar: an *N*-terminal WD40 domain, central acidic domain, subsequent proline/serine rich domains and a conserved *C*-terminal domain of about 10 kDa. Although direct evidence for the involvement of plant Sec31 proteins in the ER-to-Golgi transport is still lacking, evidence for a role for these proteins in plant development is now accumulating. A double knock out of Sec31 genes causes synthetic lethality in *Arabidopsis* (Matsuoka and Narisawa, 2006, personal communication). Moreover, a point mutation in one of the Sec31 genes causes an abnormal morphology of guard cells (Hino et al. 2005). A yeast two hybrid assay has shown that the rice Sec31 gene product can interact with the alpha subunit of rice heterotrimeric G protein (Kato et al. 2000). Although the major location of rice heterotrimeric G protein is the plasma membrane (Kato et al. 2004), integration of the alpha subunit of heterotrimeric G protein into membrane occurs in the ER. Thus, it will be interesting in the future to test for the participation of plant Sec31 proteins in cargo capturing during export from the ER.

1.2 Role of Sar1 in Secretion

The small GTP-binding protein Sar1p is a crucial player in the formation of COPII-coated vesicles and in the uncoating of the vesicle. It is a small protein of about 20 kDa without any lipid modification. On the basis of the analysis of the yeast protein, the following scheme for the Sar1 GTPase cycle has been established (Lee et al. 2004; Watanabe and Riezman 2004). Firstly, the GTP-form of Sar1p is generated by the action of Sec12p, which is an integral membrane protein and functions as a GDP-GTP exchange factor for Sar1p. The GTP-

form of Sar1p has higher affinity to the ER as well as to lipid bilayers, and the GTP form of Sar1p generated by the action of Sec12p binds to the ER membrane. The GTP-form of Sar1 has a higher affinity for the Sec23/24 complex and recruits this soluble protein complex to the ER membrane from the cytosol. Sec23p's GTPase activation of Sar1p facilitates the hydrolysis of GTP by Sar1p and decreases the affinity of this protein for the membrane. However, binding to the membrane of Sec23/24 with the GTP-form of Sar1 is stabilized when Sec23/24p also binds to the membrane cargo to make a tertiary complex. The rate of GTP hydrolysis in this tertiary complex is slower than that of Sar1p and Sec23/24 alone and this slow release allows the assembly of COPII coat with the Sec13/31 complex on the ER membrane to occur (Sato 2004).

In contrast to the limited evidence for the participation of Sec13, 23, 24, and 31 proteins in ER-export in plants, much evidence has accumulated for the role of Sar1p in the export of cargo proteins from the ER. Like other COPII components, plants have several different isoforms of Sar1 proteins. Phylogenetic analysis (Fig. 1B) has suggested that the divergence of Sar1p in plants is independent of the functional differentiation during evolution. Instead, it would seem that the mutation of Sar1 proteins has simply followed the evolution of species. Thus, the general action of plant Sar1p might not be so divergent as is the case for other small GTP binding proteins, such as the Rabs (Vernoud et al. 2003).

Characterization of the role of Sar1 in plant cells has been tested using information obtained on other related small GTPases, as the mutation that fixes the protein as a GTP- or GDP-bound form in these proteins has been established. In the case of *Arabidopsis* and tobacco Sar1A proteins, substitution of the 74th histidine residue (H74L) with leucine makes a GTP-fixed mutant, and with asparagine for the 39th threonine residue a GDP-fixed mutant is formed. It has been reported that the overexpression of these proteins with GFP-tagged AtRer1 or AtErd2, both of which are membrane proteins rapidly recycling between the ER and Golgi, caused the accumulation of both GFP-tagged proteins in the ER in transiently expressed *Arabidopsis* and tobacco cells (Takeuchi et al. 2000). This observation indicates that *Arabidopsis* and tobacco Sar1 proteins play a crucial role for ER-to-Golgi transport in higher plants. A similar result was reported using soluble cargo proteins and a tobacco protoplast expression system with both intact and YFP-tagged Sar1 proteins (Phillipson et al. 2001; daSilva et al. 2004). However, these observations are somewhat inconsistent with similar analyses obtained with mammalian cells.

In mammalian cells, microinjection of such GTP- or GDP-restricted mutants allowed for the labelling of ERES (Storrie et al. 1998; Ward et al. 2001). When expressed transiently in tobacco leaf epidermis YFP-tagged Sar1 gave rise to large (roughly Golgi-sized) punctate fluorescent signals which were also deemed to be ERES (daSilva et al. 2004). In contrast, over-expression of mutant Sar1 proteins in tobacco or Arabidopsis cells led to the uniform

distribution of reporter proteins in the ER (Takeuchi et al. 2000). This suggests that the distribution of YFP-tagged Sar1 may not represent true ERES since such fusion proteins may have the ability to form aggregates. Purified tobacco Sar1 protein can assume a homo-dimeric form at high concentrations (K. Matsuoka, 2006, personal communication), as is the case with yeast Sar1p (Matsuoka et al. 2001). If Sar1 binds with low affinity this could increase the likelihood of aggregate formation. GFP and its derivatives can also dimerize at high concentrations, although mutation can prevent the formation of such dimers (Zacharias et al. 2002). In their 2004 paper daSilva et al. did not use such mutants as controls. Since the immunofluorescent detection of endogenous Sar1 in tobacco BY-2 cells revealed numerous smaller punctae (Yang et al. 2005 and discussed below), it is possible that the overexpression of these GDP- or GTP-forms of Sar1 or wild-type YFP-tagged Sar not only disrupts ER-to-Golgi traffic but may also lead to structural aberrations in the early secretory pathway of plant cells.

In addition to dominant-negative Sar1, the *Arabidopsis* Sec12 homologue also prevents the efficient secretion of soluble cargo proteins when this protein is overexpressed (Phillipson et al. 2001). This phenomenon was explained by the fact that the overexpressed Sec12 titrated out the functional Sar1 from the cell. However, it is not clear if this overexpression predominantly prevented the COPII-cycle or whether overexpression resulted in a malfunctioning of the whole ER membrane system thus indirectly affecting ER-to-Golgi transport, since the product of the overexpression of the fluorescently protein-tagged Sec12 distributed throughout the whole ER membrane system. In the yeast *Pichia pastoris*, the localization of Sec12 is restricted to the ERES whereas in baker's yeast *Sacchoromyces cerevisiae* Sec12 is localized throughout the whole of the ER, correlating with the absence of a specific domain for ER exit sites (Rossanese et al. 1999). Future analysis on the intracellular localization of the endogenous Sec12 protein in plant cells in comparison with the localization of ERES will reveal if overexpression of this protein directly affects the GTPase cycle of Sar1 protein.

2
ER Export Signals and Cargo Recognition

In order to be included into COPII-coated vesicles, transmembrane cargo proteins must interact with one or more components of the coat. Therefore, domains of Sar1 and the Sec23–Sec24 dimer should be available for binding to integral membrane cargo proteins (Bi et al. 2002). Indeed, different types of signals in the cytosolic tails of these proteins have been shown to bind to Sar1–Sec23–Sec24 pre-budding complexes (Aridor et al. 1998; Kuehn et al. 1998), and therefore are held to act as ER export signals. These include di-acidic, di-hydrophobic and di-basic motifs.

2.1 Di-Acidic Sequence (DXE/EXE) Motifs

Export motifs based on acidic residues were initially characterized in studies of the transport of the vesicular stomatitis virus glycoprotein (VSV-G), which has served as a model secretory protein in studies on folding and export from the ER. VSV-G, a type I transmembrane protein that traffics to the cell surface, is abundantly expressed in VSV-infected cells and concentrated into ER-derived transport vesicles (Nishimura and Balch 1997; Doms et al. 1998). VSV-G possesses a cytoplasmically exposed *C*-terminal tail of 29 residues that is required for export out of the ER. A conserved YTDIEM motif is present in this tail sequence and was found to be necessary for efficient export of VSV-G from the ER (Nishimura and Balch 1997; Sevier et al. 2000). Mutation of any one of the underlined consensus residues results in a VSV-G protein that assembles into a normal homotrimer that is only slowly exported out of the ER. On the other hand, transfer of the YXDXE sequence to a transport-neutral membrane protein accelerates its export rate, although not to the levels of wild-type VSV-G transport.

The so called di-acidic sequence (**DXE**) motif contained within the VSV-G tail sequence is found in many other secretory proteins (see Table 1) that are efficiently exported from the ER, including the Kir2.1 potassium channel protein (Ma et al. 2001), and the yeast membrane proteins Sys1p and Gap1p (Kappeler et al. 1997; Malkus et al. 2002). Moreover, Sys1p is dependent on its di-acidic residues for direct binding to Sec23/24p (Vostmeier and Gallwitz 2001) and Gap1p requires its di-acidic motif to form pre-budding complexes with Sar1 and Sec23–Sec24 (Malkus et al. 2002). Recently, the first evidence for a di-acidic sequence in the export of proteins from the ER of plant cells has become available. Hanton et al. (2005) have examined the ER-export competence of two Golgi-localized proteins: the sugar nucleotide transporter GONSTI (Handford et al. 2004), and the golgin CASP (Renna et al. 2005, see below), respectively type I and type II membrane-spanning proteins. Not only is the export of these two proteins dependent upon a di-acidic signal, but the transplantation of a cytoplasmic domain containing this signal to an ER-resident membrane protein instigated the exit of the latter. Similar results have been obtained in studies on the guard cell plasma membrane—located in potassium channel KAT1, the cytosolic tail of this protein possesses two putative di-acidic sequences: DAE (aa 392–394) and DTE (aa 555–557). When the sequence DTE was mutated to AAA no change in the localization of KAT1 was observed. In contrast, when the DAE sequence was mutated to AAA, KAT1 was retained in the ER and nuclear envelope (Homann, 2006, personal communication).

2.2
Di-Aromatic or Di-Hydrophobic Motifs

Another type of transport ER exit signal for membrane cargo consists of a pair of bulky hydrophobic residues and have therefore been described as di-aromatic or di-hydrophobic motifs. An example of a protein bearing this type of signal is the membrane protein ERGIC53, a putative cargo receptor for some glycoproteins which cycle between the ER and Golgi compartments (Appenzeller et al. 1999). This type I transmembrane protein possesses a cytoplasmic tail sequence of 16 residues that is required for proper localization. More specifically, a conserved pair of aromatic residues at the extreme *C*-terminus of ERGIC53 is necessary for transport out of the ER (Kappeler et al. 1997, and see Table 1). There is some flexibility in this signal as other bulky hydrophobic amino acids can substitute for this *C*-terminal signal (Nufer et al. 2002). There is also evidence for a role for these terminal residues in binding to COPII subunits (Kappeler et al. 1997; Nufer et al. 2002), although only the phenylalanine in position – 2 (F509) is required for COPII binding (Nufer et al. 2003). ER export of ERGIC53 also requires a glutamine residue in its cytosolic tail (Q501), which itself does not increases COPII binding, but probably assists in the optimal presentation of F509 to the COPII machinery (Nufer et al. 2003). ER export of ERGIC53 is also dependent on disulfide bond-stabilized oligomerization, which in turn depends on the presence of a polar and two aromatic residues in the transmembrane domain (Nufer et al. 2003). ERGIC53 homologs in yeast also possess bulky hydrophobic residues at their *C*-termini (LL) that are required for export out of the ER and proper localization. Furthermore, when bulky hydrophobic residues are placed at the *C*-terminus of a transmembrane reporter protein, transport to the Golgi is accelerated (Nakamura et al. 1998; Nufer et al. 2002), although not to the rates observed for endogenous ERGIC53.

An additional conserved ER export signal has been identified in the tail sequence of the ERGIC53 family of proteins (Sato and Nakano 2002). This tyrosine-containing motif is $\sim$ 12 amino acid from the *C*-terminal signal (see Emp46p in Table 1). Both motifs are required for assembly into COPII prebudding complexes and for ER export. Other di-aromatic motifs (FF, YY or FY) are found in a similar position in membrane proteins that exit the ER such as the p24 family of proteins (Fiedler et al. 1996; Dominguez et al. 1998) (see below) and the Erv41–Erv46 complex (Otte and Barlowe 2002). Interestingly, many of the proteins described, including ERGIC53, VSV-G and p24 family proteins form oligomeric complexes, such that a given exported protein would presumably display multiple signals to the COPII budding machinery. Indeed, other reports suggest that multiple signals are needed for efficient export of the Can1p arginine permease (Malkus et al. 2002), the Erv41–Erv46 complex (Otte and Barlowe 2002) and an ATP-binding cassette transporter protein, Yor1p (Epping and Moye-Rowley 2002). A requirement

Table 1 Characterized ER export signals

Export signal	Protein	Interacting coat subunits	Refs.
Di-acidic motifs (EXD/EXE)			
I**Y**T**D**I**E**MNRLGK (– 1)	VSV-G	Not tested	Nishimura & Balch 1997; Sevier et al. 2000
ANS**F**C**YENE**VAL (– 45)	Kir2.1	Not tested	Ma et al. 2001
QSPIQLK**D**L**E**SQI (– 1)	Sys1p	Sec23/24p	Vostmeier & Gallwitz 2001
AEKM**D**I**D**TGR (– 34)	Gap1p	Sar1p; Sec23/24p	Malkus et al. 2002
Di-hydrophobic motifs			
YIMYRSQQEAAAKK**FF** (– 1)	ERGIC-53	Sec23/24p	Kappeler et al. 1997
YYMFRINQDIKKVK**LL** (– 1)	Emp46p	Sec23/24p	Sato & Nakano 2002
RR**FF**EVTSLV (– 1)	Emp24p	Sec13/31p>Sar1p>Sec23/24p	Belden & Barlowe 2001
KN**YF**KTKHII (– 1)	Erv25p	Sec13/31p>Sec23/24p	Belden & Barlowe 2001
YLRR**FF**KAKKLIE (– 1)	p23	Sec23/24p	Dominguez et al. 1998
YLKR**FF**EVRRVV (– 1)	p24	Sec23/24p	Dominguez et al. 1998
YLKR**YF**HKKKLI (– 1)	Atp24	Sec23/24p	Contreras et al. 2004b
YQPDDKTKG**IL**DR (– 1)	Erv41p	Sar1p; Sec23/24p	Otte & Barlowe 2002
KL**FY**KAQRSIWGKKSQ (– 1)	Erv46p	Sar1p; Sec23/24p	Otte & Barlowe 2002
Di-basic motifs ([RK](X)[RK])			
(1) MLQW**RRRH**CCFAKM	β1,3GalT2	Sar1p	Giraudo & Maccioni 2003
(1) M**KS**R**GR**FGWWSVR	Tobacco prolyl hydroxylase	Not tested	Yuasa et al. 2005

Underlined residues are required for export from the endoplasmic reticulum (ER). Numbering is from the *C*-terminal end, where the terminus corresponds to (– 1), or from the *N*-terminal end, where the terminus corresponds to (1)

for multiple signals in secretory proteins might be an important element in ER quality control. One might envisage a scenario whereby a single export motif contained on unfolded or unassembled subunits of protein oligomers would possess weak binding affinities for coat subunits, whereas folded and assembled protein complexes would present a combinatorial signal with a higher binding affinity for coat subunits. A requirement for combinatorial signals could operate concomitantly with the ER-retention machinery that retains unfolded proteins (Ellgard and Helenius 2003) to efficiently exclude unassembled oligomers from ER-derived vesicles.

Of particular interest is the case of the p24 family of putative cargo receptors, which are localized to the early secretory pathway, i.e. the intermediate compartment and the Golgi complex in mammalian cells (Stamnes et al. 1995; Sohn et al. 1996; Rojo et al. 1997; Dominguez et al. 1998; Füllerkrug et al. 1999; Gommel et al. 1999; Emery et al. 2000, 2003). All p24 proteins have one absolutely conserved phenylalanine in their cytoplasmic tail, which in many cases corresponds to the – 7 position (with respect to the *C*-terminus), while in the – 8 position there is often a bulky hydrophobic residue, in most cases another phenylalanine (see Table 2). These pair of aromatic residues have been shown to bind COPII subunits, in particular the Sec23/24p dimer (Dominguez et al. 1998). Some p24 proteins also have a classical dilysine motif in the – 3,– 4 position, which binds COPI and mediates Golgi to ER retrograde transport (Cosson and Letourneur 1994; Letourneur et al. 1994), but in other cases the lysines are replaced by arginines or histidines, which do not bind to coatomer (Dominguez et al. 1998; Marzioch et al. 1999).

Actually, p24 proteins can be sub-divided into four sub-families, based in the sequence of their cytosolic carboxi-terminus (Emery et al. 2000, and see Table 2). While all of them have been shown to bind COPII subunits via their dihydrophobic motifs, although with varying efficiencies, only members of the p23 and p25 (with higher efficiency) subfamilies can bind COPI via their dilysine motif (Dominguez et al. 1998). As mentioned before, p24 proteins also have the ability to form hetero-oligomeric complexes (Belden and Barlowe 1996; Füllerkrug et al. 1999; Gommel et al. 1999; Marzioch et al. 1999; Jenne et al. 2002). Such oligomerization links p24 proteins bearing retrograde and anterograde sorting signals, and thereby confers them with the ability to cycle constitutively through the early secretory pathway (Nickel et al. 1997; Füllerkrug et al. 1999; Gommel et al. 1999; Rojo et al. 2000).

Yeast cells also contain p24 proteins with the ability to bind only COPII (like Emp24p) or both COPI and COPII (Erv25p) (Belden and Barlowe 2001, and see Table 2). In plant cells, there are several members of the p24 family (up to 10 different proteins in *Arabidopsis thaliana*; Contreras et al. 2004a). In clear contrast to the situation in mammals and yeast, all the members of the plant p24 family are of the p25 type, and thus have the ability to bind both COPI and COPII subunits (see Table 2). While the dilysine motif in the – 3,– 4 position is required for ARF1 and COPI binding, the dihydrophobic (FF or

Table 2 Sorting signals in the carboxi-terminal cytosolic tail of proteins of the p24 family involved in the interaction with COPI and/or COPII coat proteins

Mammalian p24 proteins	
p23	VFYLRR**FF**KA**KK**LIE
p25	MRHLKS**FF**EA**KK**LV
p24	IYYLKR**FF**EVRRVV
p26	VLLLKS**FF**TEKRPISRAVHS
Yeast p24 proteins	
Erv25p	VNYLKN**YF**KT**KH**II
Emp24p	IYYLRR**FF**EVTSLV
Erp1p	MKHLGK**FF**VKQ**K**IL
Erp6p	MKSLRS**FF**VKQ**K**VL
Erp3p	ILEFIFRESR**KH**NV
Plant p24 proteins	
NP176075	ILYLKQ**YF**EK**KK**LI
NP172429	VLYLKQ**YF**EK**KK**LI
At1g21900	ILYLKR**YF**HK**KK**LI
NP187689	VLYLKR**YF**LK**KK**LI
NP564256	FVHLKT**FF**EK**KK**VI
AAM47917	FLHLKT**FF**EK**KK**VI
NP178428	FWHLKT**FF**EK**KK**LI
AAM15035	FWHLKT**FF**EK**KK**LI
NP_172854	FWHLKT**FF**QK**KK**LI
NP189550	LRHLKS**FL**ER**KK**LL

Proteins of the p24 family are characterized by the presence of a pair of hydrophobic residues (often in the – 7,– 8 position with respect to the cytosolic *C*-terminus) which allow them to bind COPII subunits and therefore to be included in COPII vesicles mediating ER export. Some of them also have a dilysine motif in the – 3,– 4 position, which is involved in COPI binding and retrograde transport from the Golgi to the ER. This is the case of p23 or p25 in mammalian cells or Erv25p in yeast cells. All the members of the p24 family in plant cells have both a dilysine motif, involved in COPI binding, and a dihydrophobic motif, involved in COPII binding but also in COPI binding in cooperation with the dilysine motif. As a consequence, plant p24 proteins have a stronger affinity for COPI than for COPII

YF) motif in the – 7,– 8 position is necessary and sufficient for COPII binding, in particular the Sec23/24p subunits, but also cooperates strongly with the dilysine motif in ARF1 and COPI binding (Contreras et al. 2004 a,b). As a consequence, COPI from plant sources has a stronger affinity for p24 cytosolic tails than COPII, a situation which is not observed when using COPI and COPII subunits from animal sources (i.e. rat liver). Only in the absence of the dilysine motif in the – 3,– 4 position, or after COPI depletion can COPII sub-

units bind to the p24 cytosolic tail (Contreras et al. 2004b). It is tempting to postulate that the molecular characteristics of these two components in plants may reflect the morphological differences in the early secretory pathway between plants (which do not have an ERGIC) and other eukaryotes. While the presence of both sorting motifs may allow p24 proteins to be selectively incorporated in both COPI- and COPII-vesicles, plant cells may need a more efficient mechanism for retrieval of ER resident proteins from the cis-Golgi than required in mammalian cells.

2.3 Di-Basic Motifs

Glycosyltransferases are type II membrane proteins, with an *N*-terminal domain comprising a short cytoplasmic tail, a transmembrane region and a luminally oriented *C*-terminal domain bearing the catalytic site. They are resident proteins of the Golgi complex and have been shown to bind Sec23p through their cytoplasmic tail, suggesting that they leave the ER in COPII vesicles (Dominguez et al. 1998). A third class of ER export motif, based on di-basic motifs, [RK](X)[RK], is present at the cytoplasmic tail of Golgi resident glycosyltransferases, and is required for these proteins to exit the ER (see Table 1). This motif is located proximal to the transmembrane border and it directly interacts with the COPII component Sar1p, suggesting a role for Sar1p in sorting, in addition to Sec23/24p (Giraudo and Maccioni 2003). Analysis of the amino acid sequence of plant glycosyltransferases also reveals the presence of basic residues which could act as ER export signals, but their role in ER export has not yet been demonstrated.

However, a role for basic residues in ER export has been recently shown for a tobacco membrane-anchored prolyl hydroxylase (PH) (Yuasa et al. 2005). This protein is an integral type II membrane protein. Its membrane-anchored nature is specific for plants, because no integral PH has been found in animals. Membrane fractionation and immunocytochemical studies indicate that this protein localizes both to the endoplasmic reticulum and the Golgi apparatus. Using GFP constructs, it has been shown that basic amino acids in the cytoplasmic, *N*-terminal region of PH play a role in its export from the ER: when the basic amino acids in this sequence (KSRGR) were changed to non-charged hydrophilic amino acids, the protein was distributed exclusively in the ER (Yuasa et al. 2005). It remains to be established whether these motifs interact with Sar1p or other COPII subunits.

2.4 The Mechanism(s) of Cargo Recognition

Several lines of evidence indicate that a family of Sec24 proteins functions in cargo recognition. Furthermore, the presence of multiple Sec24 homologues

appears to expand the variety of cargo that must be efficiently exported from the ER. Yeast cells express two additional Sec24-like proteins: Lst1 and Iss1, and higher eukaryotes are endowed with at least four Sec24 isoforms (Pagano et al. 1999, and see above). In yeast, the Lst1 subunit is not essential for COPII-dependent export but is required for efficient export of specific transmembrane cargos from the ER (Roberg et al. 1999; Shimoni et al. 2000). Both Sec23–Sec24 and Sec23–Lst1 proteins can be incorporated into a continuous COPII structure, suggesting that heterogeneity in the coat could increase the variety of cargo accommodated by a COPII-coated vesicle (Shimoni et al. 2000). In a functional sorting assay it was shown that both Sec23–Sec24 and Sec23–Lst1 can function independently in assembly of COPII coats; however, the spectrum of cargo packaged into vesicles synthesized with Sec23–Sec24 was quite distinct from those generated with Sec23–Lst1 (Miller et al. 2002). These observations, coupled with the fact that Sec23–Sec24 displays binding affinities for both di-acidic (Vostmeier and Gallwitz 2001) and di-hydrophobic motifs (Kappeler et al. 1997; Dominguez et al. 1998; Belden and Barlowe 2001), support a direct role for Sec24 in cargo recognition. Sec24p has indeed been shown to contain multiple cargo binding sites to ensure capture of diverse membrane proteins into transport vesicles (Miller et al. 2003).

Although plant cells also contain several Sec24 isoforms, there is no information on the binding specificity of Sec24 towards different ER export signals. Alternatively, Sar1 may also contribute to cargo recognition through direct association with export signals to form stable prebudding complexes. In this respect, Sar1p has also been shown to interact with export cargo, including VSV-G (Aridor et al. 1998), the v-SNAREs Bos1p and Bet1p (Springer and Schekman 1998), p24 proteins (Belden and Barlowe 2001) and the Erv41p/Erv46p complex (Otte and Barlowe 2002). In the case of glycosyltransferases, binding to Sar1p specifically involves di-basic motifs (Giraudo and Maccioni 2003).

3
Visualization of ER-Export

3.1
Organisms with Transitional ER

ERES are either randomly distributed throughout the ER or are clustered in discrete domains termed “transitional ER” (tER). Such domains are characterized by a high density of budding profiles which do not require cryofixation for their preservation (Sasso et al. 1994; Bannykh et al. 1996; Ladinsky et al. 1999), which react positively toward COPII antibodies in immunogold labelling (Orci et al. 1991; Tang et al. 2000, 2001; Horstmann et al. 2002). Characteristically, ribosomes are absent from the budding area, but nevertheless

are present on the other side of the ER. tER is often seen in glandular cells engaged in secretion, but is also frequently encountered in unicellular organisms where the nuclear envelope is occasionally seen to assume the role of tER (e.g. Tanaka and Noguchi 2000). A particularly good example of tER is seen in the model green alga *Chlamydomonas* (Zhang and Robinson 1986). In this organism, several Golgi stacks sit in a kind of amplexus formed by ER running out of the nuclear envelope. This orients the Golgi stacks with their *trans* cisternae facing towards the nuclear envelope. Different stages in the budding process can easily be recognized, ranging from dome-shaped to long-necked protuberances (see Fig. 2). The tips of these structures usually have a fuzzy COPII-like coat.

The fission yeast *Pichia pastoris*, unlike *Saccharomyces cerevisiae*, has Golgi stacks which lie juxtaposed to tER (Rossanese et al. 1999). The cause of the different morphology of the ER-Golgi continuum in the two yeasts was formerly held to be the greater length of the lumenal domain of Sec12 in *Pichia*, thus allowing for the oligomerization of adjacent GEFs (Bevis et al. 2002). However, more recent research has implicated "scaffolding" proteins such as Sec16p in maintaining the close proximity of tER and Golgi stacks in Pichia (Soderholm et al. 2004). Perhaps a significant feature of all cells having a tER is that cytoplasmic streaming is minimal, thus ensuring that tER and Golgi lie and remain close to one another.

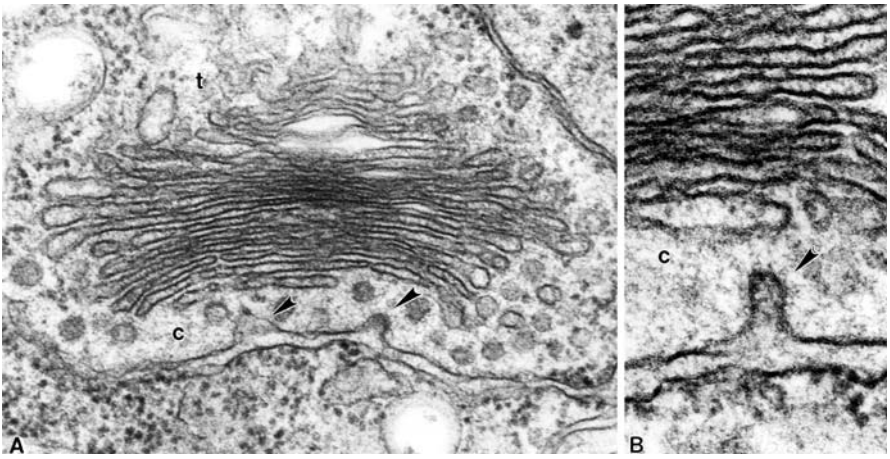

Fig. 2 The Golgi-ER interface in *Chlamydomonas noctigama*. **A** Overview of transitional Er with typical vesicle budding profiles (*arrowheads*). c – *cis*, t – *trans* Faces of the Golgi stack. **B** High magnification of a budding profile on the ER. A fine coating is visible on the surface of the bud. Conventional fixation with glutaraldehyde and osmium tetroxide

3.2 ERES in Mammalian Cells

In mammalian cells, especially cultured cells whose secretion modus is primarily of the constitutive type ERES are distributed uniformly over the surface of the ER (Hammond and Glick 2000; Stephens et al. 2000). In contrast to the Golgi apparatus which is typically perinuclear, the ER in these cells is mainly located in the cell cortex with the consequence that ER to Golgi trafficking in these cells has a long-distance component. A typical feature of mammalian cells is therefore the ERGIC (ER to Golgi intermediate compartment), which morphologically resembles a cluster of tubules and vesicles ("VTCs"; Martinez-Menarguez et al. 1999; Murshid and Presley 2004). ERGIC/VTC arises by the homotypic fusion of COPII vesicles (Stephens and Pepperkok 2001; Duden 2003). However, *en route* to the Golgi apparatus ERGIC is coated with COPI rather than COPII proteins (Shima et al. 1999; Stephens et al. 2000). This reflects its primary role as a site of capture and recycling of ER-resident proteins (Klumpermann 2000).

ERES in cultured cells have been visualized by fluorescence microscopy using either antibodies generated against COPII-coat proteins (Sar1: Aridor et al. 2004; Sec13: Shugrue et al. 1999; Hammond and Glick 2000; Stephens et al. 2000; Sec23: Stephens et al. 2000; Sec31: Rust et al. 2002), or through the expression of (X)FP-fusion constructs with Sec13 (e.g. Hammond and Glick 2000; Ward et al. 2001), and Sec24 (e.g. Stephens et al. 2000; Stephens 2003). Although sizes are difficult to determine from fluorescent images, individual ERES appear to be around 300–400 nm in diameter (Stephens 2003; Watson et al. 2004). ERES are formed *de novo* during interphase but can fuse and divide. They are relatively immobile (displacement time of 5–15 $\mu m\, h^{-1}$), and remain visible for several minutes (Stephens 2003), during which time COPII proteins continually cycle on and off. FRAP measurements indicate that the individual proteins have different turnover kinetics, but the $t_{1/2}$ for each is less than 5 seconds (Watson et al. 2004). Concomitant with the breakdown of the Golgi apparatus (Shorter and Warren 2002), ERES also appear to be dismantled during mitosis in mammalian cells (Stephens 2003).

Microtubules are responsible for both the maintenance of the Golgi apparatus in the nuclear region, and for guiding cargo vehicles between the ER and the Golgi in mammalian cells (Murshid and Presley 2004; Polishchuk and Mironov 2004; Watson et al. 2004). Depolymerization of microtubules through agents such as nocodazole leads to the formation of "mini-Golgis", which are somewhat reminiscent of plant Golgi stacks; washing out the drug leads to clustering of the stacks and formation of the perinuclear Golgi complex (Ho et al. 1989). Not only do ERGIC/VTCs move along microtubules towards the Golgi complex with the help of a dynein/dynactin molecular motor (Murshid and Presley 2004), but it now seems that microtubules also interact with ERES. Recent research has shown that ERES line up along re-

polymerizing microtubules, and that there is a direct interaction between Sec23 and the $p150^{Glued}$ component of the dynactin complex (Watson et al. 2004). This suggests that ER export is functionally coupled to the cytoskeleton.

3.3 ERES in Higher Plants

Vesiculation profiles at the ER in thin sections have only rarely been recorded in the literature dealing with higher plant ultrastructure (e.g. Craig and Staehelin 1988; Staehelin 1997; Ritzenthaler et al. 2002; see also Fig. 4), suggesting that ERES in this cell type are less frequent than in mammalian cells. It could be that this is related to the fact that plants generally secrete less protein than animal cells. On the other hand, in those situations where large amounts of secreted or vacuolar proteins are synthesized (e.g. aleurone cells in germinating cereal grains, or cotyledon parenchyma cells in developing legume seeds) ERES have proved to be just as difficult to visualize in thin sections.

Recently, two papers have addressed this problem by employing GFP-technology (daSilva et al. 2004) and immunofluorescence combined with GFP-technology (Yang et al. 2005). There are several observations in which these two studies show agreement. Firstly, the overexpression of COPII proteins (Sar1, Sec13) is, in the short term, without affect on secretion. This indicates that they are already in excess in the cytosol and are not limiting factors for the formation of ERES. Secondly, the expression of Sar1-mutants, locked into either the GDP- or GTP-forms effectively blocks secretion, and has deleterious affects on ERES morphology. Thirdly, Sec12, the GEF which is required for Sar1 recruitment, is distributed throughout the ER, and is not specifically collected into areas around ERES. In other respects, there are marked differences in the results obtained in these two investigations. Perhaps the most significant of these is the relative densities of ERES and Golgi stacks. Whereas the data of daSilva et al. (2004) suggest a 1 : 1 ratio between similarly sized structures, according to Yang et al. (2005) ERES are smaller and greatly outnumber the Golgi stacks. As a consequence, Yang et al. (2005) proposed that not every visualized ER-bound COPII site was an active ERES, i.e. only those sites in close association with a Golgi stack would actually be involved in cargo export. The necessity for this suggestion came from the realization that otherwise there would be no control over ER export or Golgi capture.

daSilva et al. (2004) have hypothesized that ERES and Golgi stacks are tightly coupled and function as mobile "secretory units". However, the measurements made in support of this claim (performed on tobacco leaf epidermal cells) were obtained on Golgi stacks which were relatively slow moving ($0.1–0.3\ \mu m\ sec^{-1}$). In contrast, in tobacco BY-2 cells Golgi stacks move in a stop-go manner with speeds of around $3\ \mu m\ sec^{-1}$ (Nebenführ et al. 1999).

In the latter cells, COPII proteins can be seen to transiently associate with the rims of Golgi stacks (Yang et al. 2005; see also Fig. 3C–H). Although the degree of COPII-Golgi association became higher the slower the Golgi stacks moved, Yang et al. (2005) reported cases where stationary Golgi stacks had no

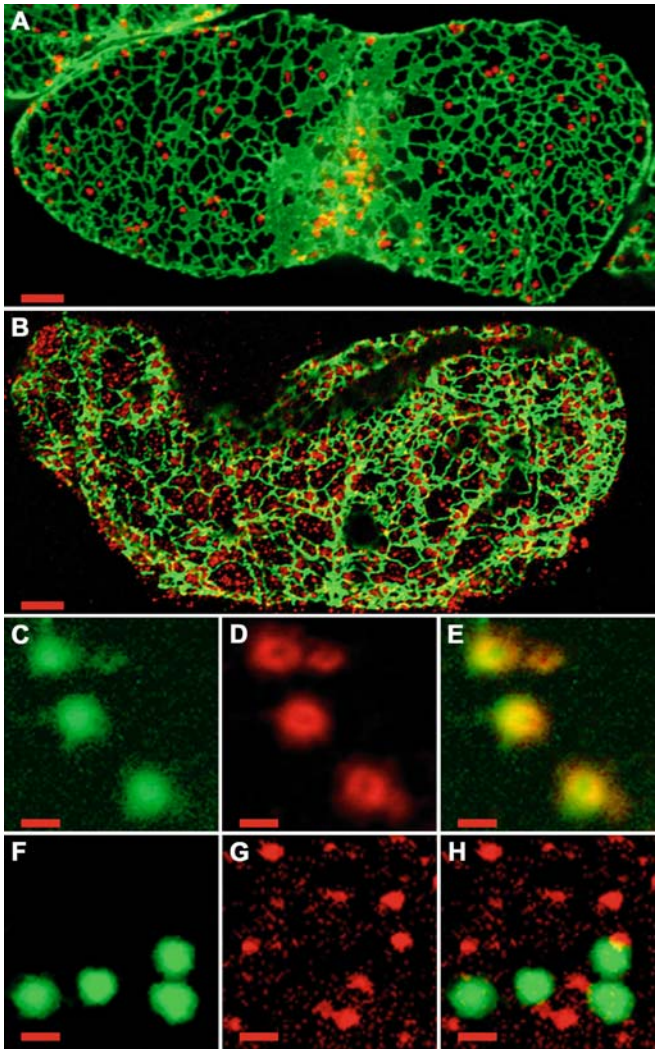

Fig. 3 COPI and COPII labelling in relation to the ER and Golgi apparatus in tobacco BY-2 cells. **A** Immunostaining with AtArf1 against a background of HDEL-GFP (ER-marker). **B** The same, but immunostaining with AtSar1. Note the density of punctae is much higher than in **A**. **C–E** Immunostaining with AtArf1 against a background of Man-I-GFP (Golgi-marker). **F–H** The same, but immunostaining with AtSar1. *Magnification bars* = 5 μm (**A**,**B**), 1 μm (**C**,**D**)

attached COPII. On the other hand, earlier FRAP experiments have indicated that Golgi proteins can be transported into both mobile and immobilized Golgi upon recovery from photobleaching (Brandizzi et al. 2002; Brandizzi and Hawes 2004). Thus, movement (and stoppage!) per se do not appear to be crucial to the loading of Golgi stacks with ER export cargo. Nevertheless, movement is a characteristic feature of the plant Golgi apparatus, and, in contrast to mammalian cells, it is microfilament based (Hawes et al. 2003; Hawes 2004). The motor connecting the stacks to the actin filaments is likely to be a plant-specific myosin, and indeed preliminary data is available showing the localization of a transiently expressed YFP-myosin to Golgi stacks in BY-2 cells (Nebenführ, 2006, personal communication).

Although higher plant ERES appear to be randomly distributed like the mammalian counterparts (see Fig. 3A,B), according to Yang et al. (2005) they differ in three respects. Firstly, plant ERES do not disappear during mitosis, a feature obviously related to the persistence of the Golgi apparatus during this event. Secondly, they appear to be much more dynamic in the sense that COPII proteins cycle on and off the ER membrane at a much faster rate. Thirdly, the fact that COPII proteins can be detected at the periphery of moving Golgi stacks suggests that either ERES detach from the surface of the ER or are dragged along in the plane of the membrane. In the first case scenario the COPII-labelled structures would constitute a kind of pre-fusion complex. Temporary contact with a moving stack would trigger the release and tethering of such complexes. For the second case scenario to work, Golgi stacks and ERES must be directly connected via tubules (see below) or held together by scaffolding proteins.

A number of scaffolding/matrix proteins have been characterized in mammalian cells, some of which fulfill the function of tethering factors (see Barr and Short 2003 for a review). The first plant homologue to such a protein has recently been cloned and characterized (Renna et al. 2005). AtCASP can be classified as a "golgin", but whether it functions purely as a tethering factor, or in stabilizing Golgi stacks, and/or in addition to maintaining an ER-Golgi "secretory unit" is pure speculation at the moment.

3.4
Tubules vs. Vesicles: a Never-Ending Argument

COPI and COPII vesicles were discovered on the basis of vesicle budding assays performed in vitro with subcellular fractions enriched in Golgi or ER membranes respectively (Schekman and Orci 1996; Balch 2004). Although the essentiality of the COPI and COPII coat protein recruiting machineries for successful protein transport through the early secretory pathway is generally accepted (see Bonifacino and Glick 2004; Lee et al. 2004 for reviews), the actual existence of these vesicles in vivo is still questioned by some researchers (e.g. Hawes 2004; Polischuk and Mironov 2004). The notion that

tubules rather than free vesicles may be responsible for COPII-mediated ER exit has been fueled by observations on mammalian cells secreting procollagen, which when assembled in the ER lumen are considered to be too large a cargo to fit into 60–80 nm diameter COPII vesicles (Bonfanti et al. 1998; Lamandé and Bateman 1999). Nevertheless, procollagen export out of the ER is definitely COPII-dependent (Stephens and Pepperkok 2002). A possible solution to this apparent paradox has recently been provided by Mironov et al. (2003) who, on the basis of correlative light and electron microscopy with tomography have provided evidence for the en bloc protrusion of the ER membrane in the immediate vicinity of ERESs. These saccules are then supposed to separate from the ER and mature into ERGIC-like transport carriers.

Since higher plant cells do not export procollagen-like fibres out of the ER and do not possess a motile ERGIC it is difficult to evaluate the above results

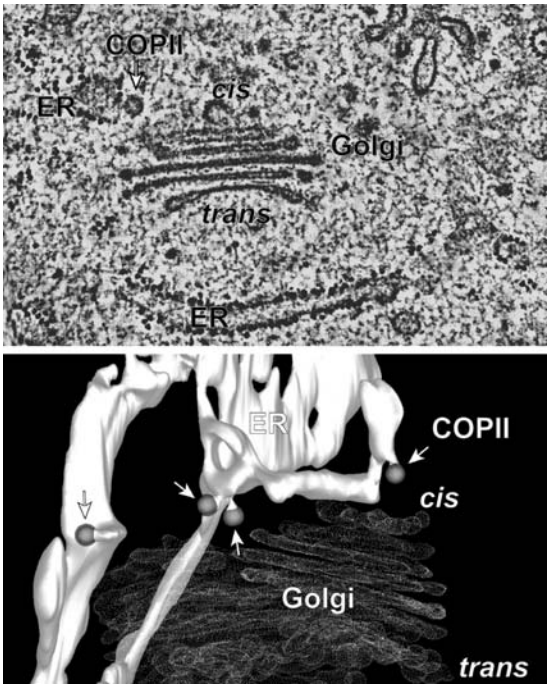

Fig. 4 *Top*: Tomographic slice of a high-pressure frozen/freeze-substituted venus flytrap (*Dionaea muscipula*) glandular cell. The ER export site adjacent to a Golgi stack shows a typical COPII vesicle budding profile (*arrowhead*). Courtesy of Z. Gergely and L.A. Staehelin. *Bottom*: 3D electron tomography-based model of a transitional ER site with budding COPII vesicles and an adjacent Golgi stack in a high-pressure frozen/freeze-substituted alfalfa (*Medicago sativa*) root tip meristem cell. Note the dispersed organization of the four COPII vesicle budding sites and that the stack is oriented with its *cis*-side facing the forming COPII vesicles. Courtesy of B.-H. Kang and L.A. Staehelin

in the context of the published data on ERESs in higher plant cells. On the other hand, direct tubular contacts between the ER and the Golgi apparatus in plant cells have been reported on several occasions in the plant literature (e.g. Juniper et al. 1982; Harris and Oparka 1983). However, their low frequency would seem to make them occasional rather than regular transfer connections between the ER and the Golgi apparatus. Moreover, neither the close proximity of Golgi stacks and ER in higher plant cells, nor the acto-myosin driven movement of these stacks over the surface of the ER constitutes a priori a reason for thinking that bidirectional vesicle trafficking between these compartments does not occur.

Although the interface between the tER and the Golgi stacks is very narrow in the yeast *Pichia pastoris*, tomographic analysis of serial sections prepared from high pressure frozen/freeze-substituted samples clearly reveals vesicles of a size comparable to COPII vesicles (Mogelsvang et al. 2003). A similar analysis has recently been performed on meristem cells of *Arabidopsis* (Kang and Staehelin, 2006, personal communication). This reveals the presence of putative COPII vesicles budding from the surface of the ER in the immediate vicinity of Golgi stacks (Fig. 4A,B). As might be expected for a cell type which is geared up for the secretion of polysaccharides rather than protein, the density of the COPII buds is not high and this is most probably the reason why they are seen so infrequently in individual sections passing through a Golgi stack.

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Transport of Proteases to the Vacuole: ER Export Bypassing Golgi?

Takashi Okamoto

Department of Biological Sciences, Tokyo Metropolitan University, Minami-osawa 1-1, Hachioji, 192-0397 Tokyo, Japan
okamoto-takashi@c.metro-u.ac.jp

Abstract Most vacuolar proteases are transported from the endoplasmic reticulum (ER) to vacuoles via the Golgi apparatus. However, higher plants possess a unique papain-type protease, termed KDEL-tailed protease. This protease has a Lys – Asp – Glu – Leu (KDEL) sequence at its C-terminus, which is known as a retention signal of soluble proteins to the ER, although the protease localizes and functions in vacuoles. Investigations on the intracellular trafficking pathway of this unique enzyme have suggested that the protease is transported from the ER to vacuoles by bypassing the Golgi apparatus. In this review, Golgi-dependent vacuolar trafficking of proteases is first explained, then the Golgi-independent vacuolar transport pathway of the KDEL-tailed protease is described.

1 Vacuolar Proteases and Their Trafficking Pathways Along the Endomembrane System

A major function of plant vacuoles is protein degradation and processing, and many vacuolar proteases have been identified as mediators for these proteolytic events. Recent proteomic analysis of vacuoles isolated from Arabidopsis rosette leaves indicated that at least 23 kinds of proteases, including cysteine, serine and aspartic proteases, exist in vacuoles (Carter et al. 2004).

Papain-type proteases (EC3.4.22), which possess a cysteine residue at the active site forming a catalytic triad and generally show broad substrate specificity, are thought to be the most abundant proteases in plant vacuoles (Rawlings and Barrett 1994). Cysteine proteases are initially synthesized as larger precursor proteins with *N*-terminal prodomains of approximately 120 amino acid residues. The prodomain functions as an inhibitor of the protease by occluding the active site and is essential for correct folding of the protein (Vernet et al. 1995; Carmona et al. 1996). Papain superfamily proteases are widely distributed among eukaryotes (Rawlings and Barrett 1994; Betri and Storer 1995) and are localized in lytic compartments (vacuoles or lysosomes) or secreted.

In plant cells, it is known that vacuolar transport signals of soluble proteins can be divided into three categories: sequence specific vacuolar sorting signals (ssVSS), *C*-terminal signals (csVSS), and physical structural signals

(psVSS; Matsuoka and Neuhaus 1999; Vitale and Raikhel 1999). The typical tetra sequence of ssVSS is an Asn – Pro – Ile – Arg (NPIR) motif on the *N*-terminal prosequence of vacuolar proteins. Vacuolar targeting via the NPIR motif was first identified on the polypeptide of sporamin, a storage protein of sweet potato (Matsuoka and Nakamura 1991), and papain-type proteases also contain the NPIR motif on the *N*-terminal prosequence (Matsuoka and Neuhaus 1999). Barley aleurain is the best characterized papain-type protease in terms of the molecular mechanism of vacuolar trafficking. Holwerda et al. (1992) revealed that the NPIR sequence on the *N*-terminal prosequence of aleurain functions as a vacuolar targeting signal, and a putative receptor, termed BP-80, for the vacuolar sorting signals was isolated from maturing Pea cotyledons (Paris et al. 1997).

Homologues of BP-80 have been isolated in pumpkin (PV72; Shimada et al. 1997), Arabidopsis (AtELP; Ahmed et al. 1997) and mung bean (VmVSR; Tsuru-Furuno et al. 2001), and they are called vacuolar sorting receptors (VSRs). VSRs are type I integral membrane proteins consisting of three epidermal growth factor motifs and a protease-associated region at the luminal domains (Mahon and Bateman 2000). It has been shown that BP-80 and AtELP bind in vitro to aleurain and AtALEU, an Arabidopsis aleurain, respectively, via the NPIR sequence (Paris et al. 1997; Ahmed et al. 2000), and that they localize in the Golgi apparatus and prevacuolar compartment (Ahmed et al. 2000). Recently, the effects of heterologous expression of the luminal region of a PV72 tagged with an ER-retention signal, HDEL, on the intracellular transport of AtALUE were observed (Watanabe et al. 2004). The heterologous expression of PV72-HDEL in Arabidopsis resulted in accumulation of the proform of AtALUE in leaf cells of the transgenic plants, suggesting that the VSR mislocalized in ER trapped the proform of AtALUE, and that VSR functions as a receptor for the protease in vivo as well as in vitro. Through these investigations, it has been suggested that papain-type protease possessing ssVSS is transported from the ER to vacuoles via the Golgi apparatus, and that the NPIR sequence recognized by VSR at the Golgi apparatus is a determinant for the vacuolar traffic.

Aspartic proteases (EC3.4.23) are also major proteases found in vacuoles (Elpidina et al. 1990; Runeberg-Roos et al. 1994; Hiraiwa et al. 1997; Ramalho-Santos et al. 1997; Mutlu et al. 1999), and the majority of plant aspartic proteases belong to the A1 family (Rawlings and Barret 1999; Simoes and Faro 2004). It has been revealed that aspartic proteases are widely distributed among vertebrates, plants, yeast, nematodes, fungi and virus (Davies 1990), and that plant enzymes show sequence similarities to their animal counterparts (Runeberg-Roos et al. 1991; Codeiro et al. 1994; Hiraiwa et al. 1997). However, it is noteworthy that plant aspartic proteases possess an inserted sequence of approximately 100 amino acid residues between the *N*- and *C*-terminal regions of the mature enzyme, and that this inserted sequence is not found in animal counterparts. The plant specific insert (PSI) is highly

similar to that of saposins, which are known as lysosomal sphingolipid-activating proteins that appear to be required for hydrolysis of sphingolipids by specific lysosomal hydrolases (O'Brien and Kishimoto 1991; Weiler et al. 1995). Analysis of the crystal structure of the proform of phytepsin, a barley aspartic protease, indicated that the PSI is expected to form an external loop on the surface of the molecules and to have a putative membrane binding region, suggesting the possibility that it plays a role in vacuolar transport via membrane-associated receptors (Kervinen et al. 1999). Direct evidence of the involvement of PSI in vacuolar trafficking was obtained from observations of intracellular localization of phytepsin and PSI-deleted phytepsin heterologously expressed in tobacco cells. Tormakangas et al. (2001) reported that deletion of PSI results in secretion of mutant protease, whereas intact phytepsin continued to localize in vacuoles in the tobacco cells. In addition, phytepsin is known to be glycosylated and acquires complex oligosaccharides, which occurs in the Golgi apparatus (Costa et al. 1997). From these various studies, it has been suggested that aspartic proteases are transported to the vacuoles via the Golgi-complex using PSI as a transport signal, although the possible receptor for PSI has yet to be identified.

2
Golgi-Independent Vacuolar Transport of a KDEL-Tailed Protease

2.1
KDEL-Tailed Proteases

Eukaryotic cells are divided into distinct subcellular compartments or organelles enclosed by one or more membranes. Because protein synthesis occurs mainly in the cytosol, proteins of subcellular compartments have intracellular localization signals that determine their final destinations. A transient signal peptide allows co-translational entry into the lumen of the ER. The ER is the starting compartment for vesicular trafficking to the Golgi apparatus, vacuoles and cell surface along the secretory pathway. Because secretion following a route mediated by the Golgi apparatus is the default destination for proteins introduced into the ER, proteins localizing in the ER, Golgi apparatus or vacuoles must have additional signals. Most soluble ER residents have a permanent *C*-terminal KDEL or HDEL tetrapeptide sequence, which constitutes an ER retention signal (Munro and Pelham 1987; Pelham 1989). The tetrapeptide is recognized by an ERD2-KDEL receptor in the Golgi apparatus, resulting in retrieval of H/KDEL proteins from this compartment back into the ER. The H/KDEL system is conserved through mammals, plants and yeasts (Denecke et al. 1992; Napier et al. 1992; Lee et al. 1993). Besides ER residents found in other eukaryotes, higher plants have unique papain-type proteases that possess KDEL-tails at the *C*-terminus, termed KDEL-tailed proteases (Akasofu et al.

Table 1 KDEL-tailed proteases and their expression sites

Enzyme	Plants	Organs showing expression	*C*-terminal sequences	Ref./accession no.
SH-EP	Mung bean	Germinated seeds	KDEL	Akasofu et al. (1989)
CysEP	Castor bean	Germinated seeds (endosperm)	KDEL	Schmid et al. (1998)
Proteinase A	Vetch bean	Germinated seeds	KDEL	Becker et al. (1997)
EP-C1	French bean	Senescing leaves and pods	KDEL	Tanaka et al. (1993)
TPE4A	Pea	Immature ovules	KDEL	Cercos et al. (1999)
SEM102	Day lily	Senescing leaves and flowers	RDEL	Valpuesta et al. (1995)
SEN11	Day lily	Senescing leaves and flowers	KDEL	Guerrero et al. (1998)
O141	Orchid	Mature ovules	RDEL	Nadeau et al. (1996)
REP-A	Rice	Germinated seeds	KDEM*	Shintani et al. (1997)
CysP1	Soybean	Germinated seeds	KDEL	Ling et al. (2003)
CysP2	Soybean	Germinated seeds	KDEL	Ling et al. (2003)
EP-A	Barley	Germinated seeds	TDEL*	Z97023
PRT5	Sandersonia	Senescing leaves	KDEL	AF133839
AtCEP1	Arabidopsis	—**	KDEL	At5g50260
AtCEP2	Arabidopsis	—**	KDEL	At3g48530
AtCEP3	Arabidopsis	—**	KDEL	At3g48540

* It is unknown whether the *C*-terminal of KDEM or TDEL sequence functions as an ER retention signal.
** The expression site of Arabidopsis KDEL-tailed proteases is unknown.

1989; Tanaka et al. 1993; Valpuesta et al. 1995; Becker et al. 1997; Guerrero et al. 1998; Schmid et al. 1998; Cercos et al. 1999).

In spite of the universal distribution of papain-type proteases, KDEL-tailed proteases appear to be restricted to the plant kingdom. KDEL-tailed proteases have been identified in at least 12 plant species, and most are expressed in senescing organs such as germinated cotyledons, senescing leaves and pods (Table 1). One such KDEL-tailed protease, designated SH-EP, was first isolated from cotyledons of mung bean seedlings as the enzyme responsible for degradation of storage proteins accumulated in protein storage vacuoles of cotyledon cells (Mitsuhashi et al. 1986). SH-EP is synthesized on membrane-bound ribosomes as a 43-kDa precursor through co-translational cleavage of the signal peptide, and the precursor is processed into a 33-kDa mature enzyme via 39- and 36-kDa intermediates during or after transport to the vacuoles (Mitsuhashi and Minamikawa 1989; Okamoto and Minamikawa 1998). Interestingly, regardless of the presence of the KDEL-tail on the polypeptide, it has been shown that mature SH-EP localizes in vacuoles (Okamoto et al. 1994). In the next section, the vacuolar transport pathway of this unique protease is explained.

2.2
Packing of KDEL-Tailed Protease into KDEL-Vesicles at the ER

Immunogold-labeling of cotyledon cells from mung bean seedlings with an antibody against mature SH-EP revealed that a large amount of SH-EP is localized in a vesicle with a diameter of 200–500 nm (Fig. 1A; Toyooka et al. 2000). Here, the large vesicle containing KDEL-tailed protease is called the KDEL-vesicle. Accumulation of a large amount of SH-EP was observed at the edge or middle region of the ER, and KDEL-vesicles were frequently observed close to the ER (Fig. 1A,B). In addition, the ER often terminated in a small vesicle surrounded by a ribosome-attached membrane (Fig. 1C). This data suggested that SH-EP synthesized in the ER is packed into KDEL-vesicles formed at the edge or middle area of the ER, and that budding of the vesicle from the ER is the first step of SH-EP transport from the ER to vacuoles. Questions regarding whether SH-EP exists in KDEL-vesicles as proforms or mature enzyme, and whether the KDEL-tail of SH-EP is removed when the enzyme is packed into the KDEL-vesicles from ER thus arise. Dense labeling of KDEL-vesicles with antibodies against the *N*-terminal propeptide of SH-EP and *C*-terminal KDEL sequence suggested that SH-EP accumulates in the vesicles as enzymatically inactive proenzyme, and that the KDEL-tail of SH-EP is not removed during transport of proSH-EP from the ER to KDEL-vesicles.

KDEL-vesicles, termed ricinosomes, have been detected in endosperm cells of castor bean seedlings and in cells of senescing petals from day lily (Schmid et al. 1999). Moreover, Schmid et al. (2001) successfully isolated KDEL-vesicles from endosperms of castor bean seedlings, and further ana-

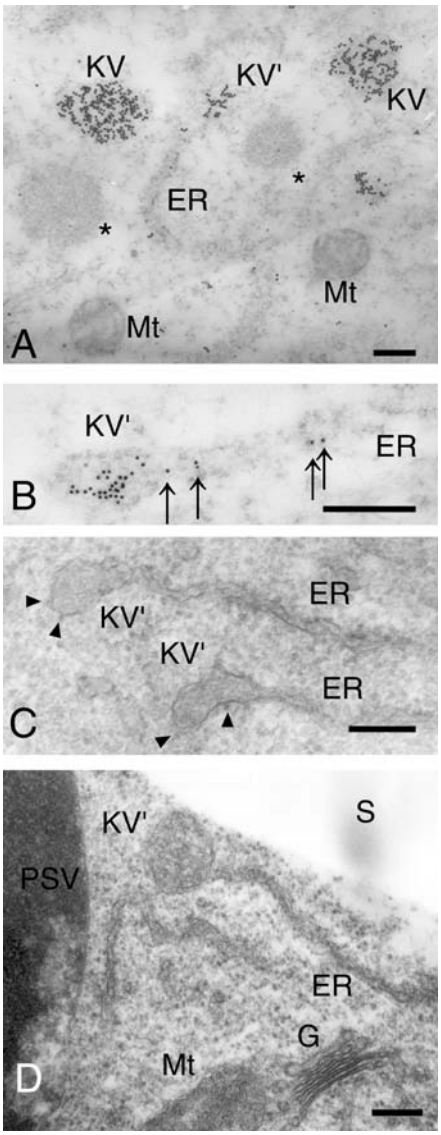

Fig. 1 Electron photographs showing the budding of KDEL-vesicles from ER. **A** Accumulation of SH-EP at the edge of ER (KV′). The area where SH-EP localized was swollen. Two KDEL-vesicles existed close to the ER. **B** Magnified image of the accumulation of SH-EP at the edge of the ER (KV′). SH-EP in the lumen of the ER (*arrows*) seemed to be moving toward the edge of the ER where a large amount of SH-EP was accumulated (KV′). **C** Ultrastructure of cotyledon cells, showing ER terminates in a small vesicle (KV′). *Arrowheads* indicate ribosomes. **D** Ultrastructure of cotyledon cells, showing the existence of vesicles similar to KDEL-vesicles (KV′) adjacent to the ER. ER, endoplasmic reticulum; G, Golgi apparatus; KV, KDEL-vesicle; PSV, protein storage vacuole; S, starch granule; Mt, mitochondrion; Asterisk, unidentified cell compartment, *Bars*, 200 nm

lyzed the composition of matrix proteins in the vesicles by SDS-PAGE and subsequent protein sequencing of major proteins in the gel. Surprisingly, more than 90% of the proteins in the KDEL-vesicles were occupied by the proform of castor bean KDEL-tailed protease, designated CysEP. This indicates that KDEL-tailed proteases are highly concentrated in KDEL-vesicles, and that the vesicles might function as a "battery" of proteases in plant cells.

2.3
KDEL-Vesicles Bypass the Golgi Apparatus

Involvement of the Golgi apparatus in vacuolar transport of KDEL-tailed proteases has been observed in detail, since vacuolar proteins are generally transported along the secretory pathway via the Golgi apparatus, from which vacuolar and secretory proteins are separately sorted. Despite the immunogold-labeling of ER and KDEL-vesicles in cotyledon cells of mung bean seedlings with anti-SH-EP antibody, the Golgi apparatus was never labeled with this antibody (Fig. 2A,B). On the other hand, when an antibody to another vacuolar protease, an asparaginyl endopeptidase, was employed for immunogold labeling, the Golgi apparatus and vacuoles were labeled, but the KDEL-vesicle was not (Fig. 2C). This selective labeling of KDEL-vesicles and Golgi apparatus with two kinds of vacuolar proteases indicates that cotyledon cells use two sorting pathways to transport proteolytic enzymes from the ER to vacuoles, a Golgi-mediated route for asparaginyl endopeptidase and a KDEL-vesicle mediated route for SH-EP. Moreover, a complex glycan antibody did not label KDEL-vesicles, but it did label the Golgi apparatus (Fig. 2D), indicating that proteins from the Golgi apparatus do not contribute to the content or formation of the vesicles. These immuno-cytochemical studies have shown that KDEL-vesicles bypass the Golgi apparatus and directly fuse with protein storage vacuoles (Fig. 2E,F). Bypassing the Golgi apparatus by KDEL-vesicles will probably be consistent even when organelle/vesicle sizes are compared, since the diameter of KDEL-vesicles (200–500 nm) is probably too large for discharge of their content into the Golgi apparatus without extreme structural consequences to this organelle.

2.4
Involvement of the *C*-Terminal KDEL Sequence in Formation of KDEL-Vesicles and in Efficient Vacuolar Transport

Although most plant papain-type proteases are transported to vacuoles or secreted along the general secretory pathway, KDEL-tailed proteases are packed into KDEL vesicles, which directly fuse with vacuoles through a Golgi apparatus-independent pathway. The most notable difference between normal papain-type protease and KDEL-tailed protease is the presence of a KDEL sequence at the *C*-terminus, suggesting that the KDEL-tail is involved in this

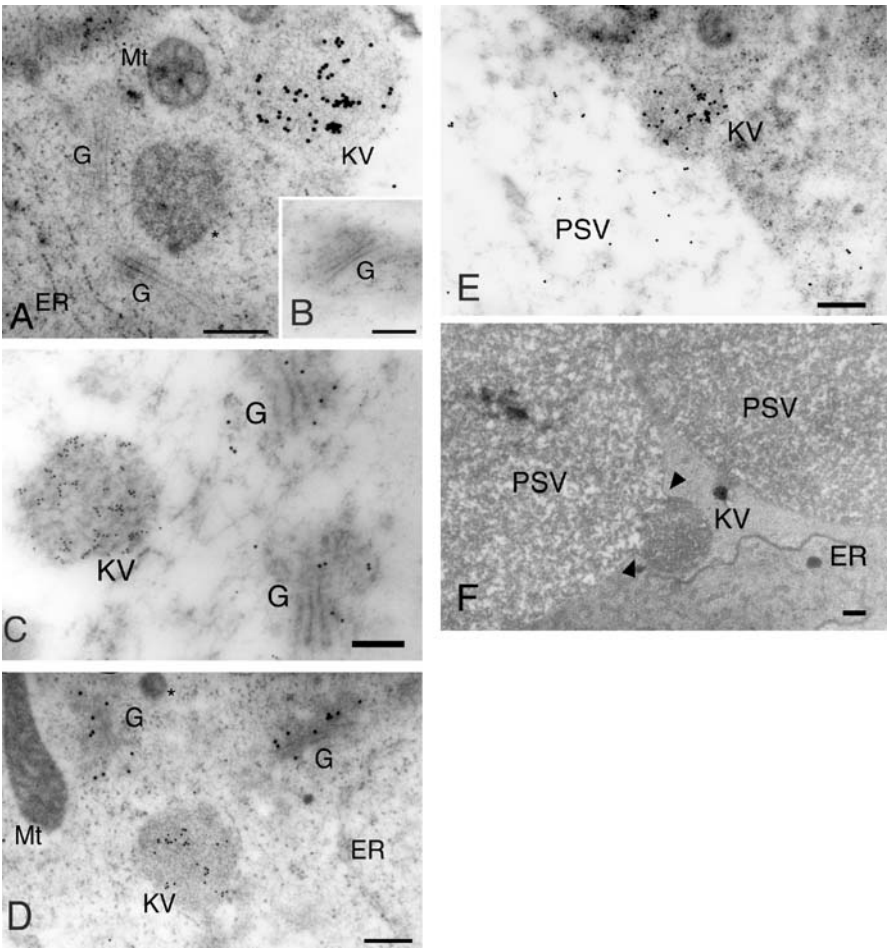

Fig. 2 Electron photographs showing the transport of SH-EP via KDEL-vesicles by bypassing the Golgi apparatus. **A**,**B** The anti-SH-EP antibody did not immunogold-label the Golgi apparatus, although the KDEL-vesicle was labeled. **C** Antibodies to SH-EP (10-nm particles) and an asparaginyl endopeptidase (15-nm particles) selectively labeled the KDEL-vesicle and the Golgi apparatus, respectively. **D** The anti-complex glycan antibody (15-nm particles) and anti-SH-EP antibodies (10-nm particles) specifically immunogold-labeled the Golgi-apparatus and KDEL-vesicle, respectively. **E** KDEL-vesicle immunogold-labeled with the anti-SH-EP antibody fused with a protein storage vacuole (PSV). Gold particles localized in the PSV as well as the KDEL-vesicle. **F** Ultrastructural photograph showing that a vesicle similar to the KDEL-vesicle fuses to PSV. *Arrowheads* indicate the region where the KDEL-vesicle merges with the PSV membrane. ER, endoplasmic reticulum; G, Golgi apparatus; KV, KDEL-vesicle; Mt, mitochondrion; PSV, protein storage vacuole. *Bars*, 200 nm

unique vacuolar trafficking pathway. To address the possibility, SH-EP and its KDEL-deletion mutant (SH-EPΔKDEL) were heterologously expressed in Arabidopsis and intracellular localizations of these proteins were monitored. In cells from stems, cotyledons, rosette leaves and flowers of transformed plants expressing SH-EP, the enzyme accumulated in vesicles with diameters between 200–700 nm (Fig. 3A,B; Okamoto et al. 2003). These vesicles possibly correspond to KDEL-vesicles, since their size and the accumulation of KDEL-tailed protease are characteristic of KDEL-vesicles in cotyledons of mung bean seedlings. In addition, KDEL-vesicles in transgenic plants appeared to fuse with vacuoles (Fig. 3A). In contrast to heterologous expression of intact SH-

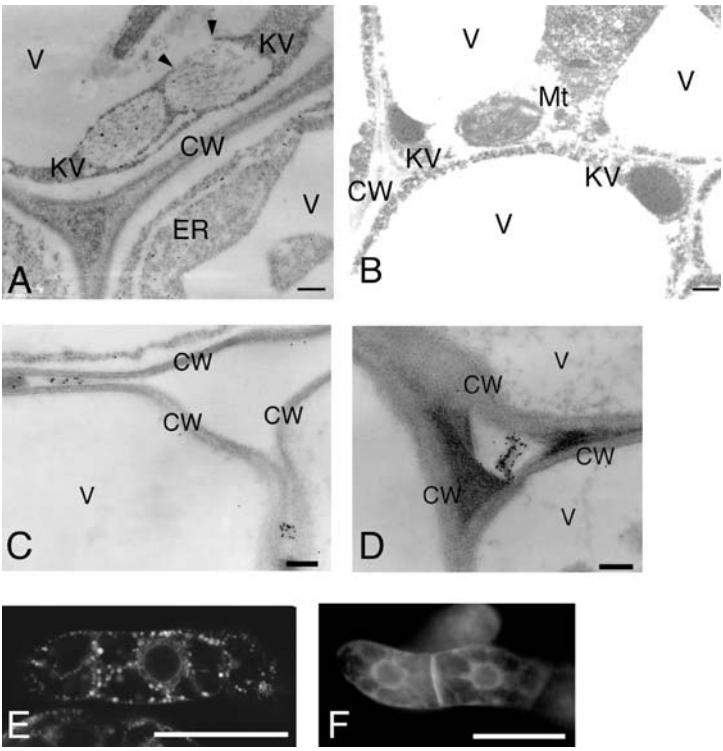

Fig. 3 Heterologous expression of SH-EP (**A**,**B**,**E**) and SH-EPΔKDEL (**C**,**D**,**E**) in Arabidopsis (**A**–**D**) and tobacco BY2 cells (**E**,**F**). **A**,**B** Electron micrographs showing the development of KDEL-vesicles in stem (**C**) and cotyledon cells (**D**) of transgenic Arabidopsis expressing SH-EP. A KDEL-vesicle fused with a vacuole (*arrowheads* in panel **A**). **C**,**D** Electron photographs showing secretion of SH-EPΔKDEL from the cells of rosette leaves of transgenic Arabidopsis. Gold particles from the anti-SH-EP antibody were found at extracellular spaces and possible air spaces. **E**,**F** Intracellular localization of signal peptide (SP)-GFP-SHEP (**E**) or SP-GFP-SHEPΔKDEL (**F**) in tobacco BY-2 cells. Small foci derived from GFP fused with SH-EP were observed in the cells of panel **E**, however such signal was not detected in the cells of panel **F**. *Bars*, 200 nm (**A**–**D**) and 50 μm (**E**,**F**)

EP, formation of KDEL-vesicle was not detected in cells of transformed plants expressing SH-EPΔKDEL. Furthermore, SH-EPΔKDEL was mainly secreted and localized at the extracellular spaces and possible air spaces (Fig. 3C,D), indicating that the mutated protease was not packed into KDEL-vesicle but was secreted. The KDEL-tail of the protease will be essential for formation of KDEL-vesicles and subsequent vacuolar transport via the vesicles.

Additional confirmation of involvement of the KDEL-tail in formation of KDEL-vesicles was from GFP-labeling of the vesicles by heterologous expression of GFP-fused SH-EP or SH-EPΔKDEL in tobacco BY-2 cells. When signal peptide (SP)-GFP-SHEP was expressed, strong fluorescence was detected in small vesicles (Fig. 3E), and the diameter of the GFP-labeled small vesicles appeared to be similar to that of KDEL-vesicles, which were detected in cells of transformed Arabidopsis expressing SH-EP (Fig. 3A,B). In the case of SP-GFP-SHEPΔKDEL expression in tobacco cells, such a small vesicle was not observed, suggesting that deletion of the KDEL-tail from SH-EP resulted in loss of formation of the KDEL vesicle.

2.5
Golgi-Independent Vacuolar Transport of Non-KDEL-Tailed Proteases

In addition to KDEL-tailed proteases, Arabidopsis RD21, a papain-type protease, and γVPE, an asparaginyl endopeptidase, are known to be vacuolar proteases which are transported to vacuoles in a Golgi-independent manner. RD21 and γVPE are localized in spindle-shaped vesicles, which are derived from ER ranging in size from 0.1–10 μm, and the vesicles, termed ER-bodies, are known to fuse with vacuoles via a Golgi apparatus-independent pathway as for the KDEL-vesicles (Hayashi et al. 2001; Hara-Nishimura et al. 2004). Matsushita et al. (2003) successfully identified a β-glucosidase possessing a KDEL-tail (PYK10) as the predominant protein component of ER-bodies (see also Hara-Nishimura in this volume). In addition, strong expression of PYK10 protein in cells appears to be needed for formation/induction of ER-bodies. Although involvement of the KDEL-tail of PYK10 in the formation of ER-bodies remains to be clarified, aggregation of PYK10 at the ER where ER-bodies are formed might occur via its KDEL-tail. RD21 and γVPE are considered to be trapped in ER-bodies during aggregate formation of abundant PYK10 in the ER, resulting in the direct ER-vacuole transport of these non-KDEL-tailed proteases via ER-bodies (Hara-Nishimura et al. 2004).

3
Conclusions and Prospects

The vacuolar trafficking pathways of the major plant vacuolar proteases, papain-type and aspartic proteases, are summarized in Fig. 4. Proforms of

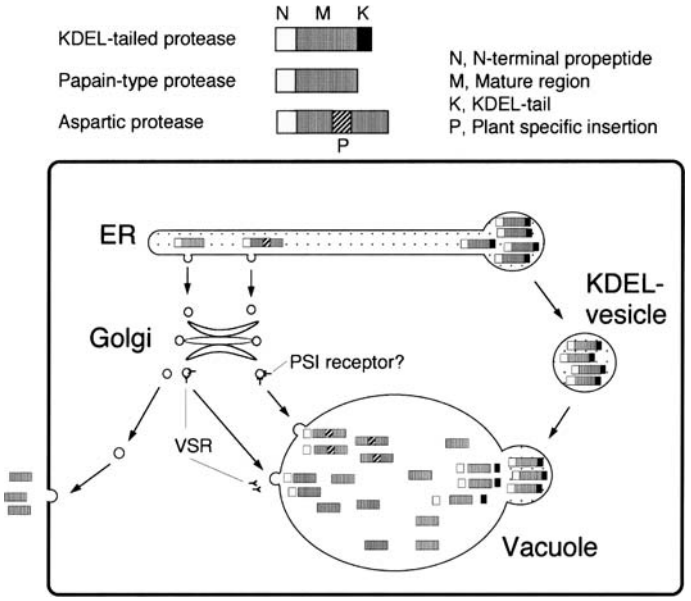

Fig. 4 A model of the intracellular transport pathways for papain-type and aspartic proteases in plant cells. Proforms of non-KDEL papain-type proteases are transported from ER to vacuoles via ssVSS signal (NPIR motif), which is recognized by VSR existing on the Golgi apparatus. Proforms of aspartic proteases are also transported from ER to vacuoles through the Golgi apparatus, in which the proenzymes are recognized by putative receptor for PSI. Proforms of KDEL-tailed papain-type proteases synthesized in the lumen of ER are packed into KDEL-vesicles probably via the KDEL-tail. KDEL-vesicles filled with the proenzymes bud off from the ER, bypass the Golgi apparatus and fuse with vacuoles, resulting in the release of proenzymes into the inside of the vacuoles. When the *C*-terminal KDEL sequence was deleted from the KDEL-tailed protease, mutant protease is secreted probably along the endomembrane system. All vacuolar proteases described in the figures are activated by proteolytic processing after or during the transport of proenzymes to the vacuoles

normal papain-type (without KDEL-tail) and aspartic proteases are first transported from the ER to Golgi apparatus, and then they are recognized by VRS and putative receptor for PSI on the Golgi apparatus, respectively, resulting in transport of the proenzymes to vacuoles. After or during transport to the vacuoles, the proenzymes are converted to mature enzymes through proteolytic processing. In the case of KDEL-tailed proteases, proenzymes are accumulated at the ER and packed into KDEL-vesicles without removal of the KDEL-tail. These KDEL-vesicles, in which KDEL-tailed proteases are predominantly concentrated, bypass the Golgi apparatus and directly fuse with vacuoles. In the vacuoles, proenzymes are converted into active form by the removal of the *N*-terminal prosequence through possible autocatalysis of the enzyme. *C*-terminal propeptide containing the KDEL-tail will be removed by

other vacuolar proteases (Okamoto et al. 2001). With deletion of the KDEL-tail from the protease, mutant proteins are not packed into KDEL vesicles, but are mainly secreted possibly along the endomembrane system.

Why do plant cells utilize KDEL-vesicles for vacuolar transport of the protease? It has been reported that secretion of the KDEL-tailed protease (SHEPΔKDEL) results in growth defects of transformed plants through possible degradation of proteins existing in the extracellular space (Okamoto et al. 2003). In general, KDEL-tailed proteases are expressed at extremely high levels in senescing cells in which massive turnover and recapture of cellular materials occur. Since the vacuolar trafficking pathway via the Golgi apparatus appears to be saturable (Frigerio et al. 1998), plant cells may utilize the KDEL-tail for aggregation of highly expressed dangerous enzymes, such as papain-type proteases, at the ER. In addition to efficient and massive vacuolar transport of protease by large KDEL-vesicles, packing them into KDEL-vesicles may be a way to escape saturation of the vacuolar trafficking route along the endomembrane system and saturation-induced miss-sorting of the vacuolar protease. Induction/formation of KDEL-vesicles in cells of most tissues of transgenic plants by heterologous expression of KDEL-tailed proteases is an example of the enormous plasticity of the ER in plant cells.

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Induction of Specialized Compartments from the ER

Ikuko Hara-Nishimura (✉) · Tomoo Shimada

Graduate School of Science, Kyoto University, Sakyo-ku, 606-8502 Kyoto, Japan
ihnishi@gr.bot.kyoto-u.ac.jp

Abstract The endoplasmic reticulum (ER) differentiates to generate various types of compartments, each of which has its own function. The ER-derived compartments are classified into two types according to their contents. The first type is found in maturing seeds and accumulates seed storage proteins. The second type of compartment accumulates a hydrolytic enzyme. Here we focus on two typical ER-derived compartments: precursor-accumulating (PAC) vesicles as a storage protein type, and ER bodies as a hydrolytic enzyme type. PAC vesicles mediate the mass transport of storage protein precursors directly from the ER to protein storage vacuoles in maturing seeds. A vacuolar sorting receptor of storage protein is localized in the membrane of the PAC vesicles. The vesicles provide a clue to the molecular mechanism of vacuolar sorting of storage proteins. In contrast, ER bodies accumulate a large amount of β-glucosidase with an ER retention signal. They are distributed in the epidermal cells of seedlings and roots. Wounding and chewing by insects induce many ER bodies in rosette leaves, which have no ER bodies under normal conditions. The ER bodies might therefore play a role in a defense strategy of plants. Most of the ER-derived compartments are induced in specific tissues in response to internal and external signals. Hence, the induction of ER-derived compartments is controlled in a sophisticated way by the conditions under which plants grow.

1 Introduction

The membrane surface of the endoplasmic reticulum (ER), which is composed of an extensive network of tubules and cisternae, occupies nearly half the total area of membrane in a cell. The ER membrane is the place where almost all the proteins and lipids for various compartments of the endomembrane system are produced. The endomembrane system includes the ER, Golgi apparatus, endosomes, prevacuolar compartments, vacuoles, lysosomes, secretory vesicles, and the plasma membrane. All compartments are connected by both forward and backward traffics, which are driven by various types of transport vesicles with diameters of about 0.05–0.1 μm. Among the transport vesicles, COPII vesicles are known to be derived from the ER and to be responsible for the initial step of the membrane traffic in eukaryotic cells including yeast, mammals, and plants.

In addition to COPII vesicles, plant cells have the ability to generate much larger compartments from the ER. The diversity of ER-derived compartments has been getting increased attention in plant cell biology in recent

years (Chrispeels and Herman 2000; Hara-Nishimura and Matsushima 2003; Hara-Nishimura et al. 2004). Figure 1 shows an illustration of typical ER-derived compartments, with a standard bar to give an appreciation of their size differences. These ER-derived compartments have sizes ranging from 0.1 to 10 μm (Larkins and Hurkman 1978; Li et al. 1993; Hara-Nishimura et al. 1998; Schmid et al. 1998; Toyooka et al. 2000; Hayashi et al. 2001).

Despite this variety in size and shape, all the ER-derived compartments that have been identified so far share two features: (1) they accumulate a large amount of a single protein or a few different proteins—this feature contrasts markedly with that of COPII vesicles, which export a broad range of proteins; and (2) they only appear in a distinct tissue at a particular time in the life cycle of plants. These features suggest that each ER-derived compartment fulfills its own specific function.

ER-derived compartments accumulate an enormous amount of proteins that are actively synthesized on the ER (Staehelin 1997; Chrispeels and Her-

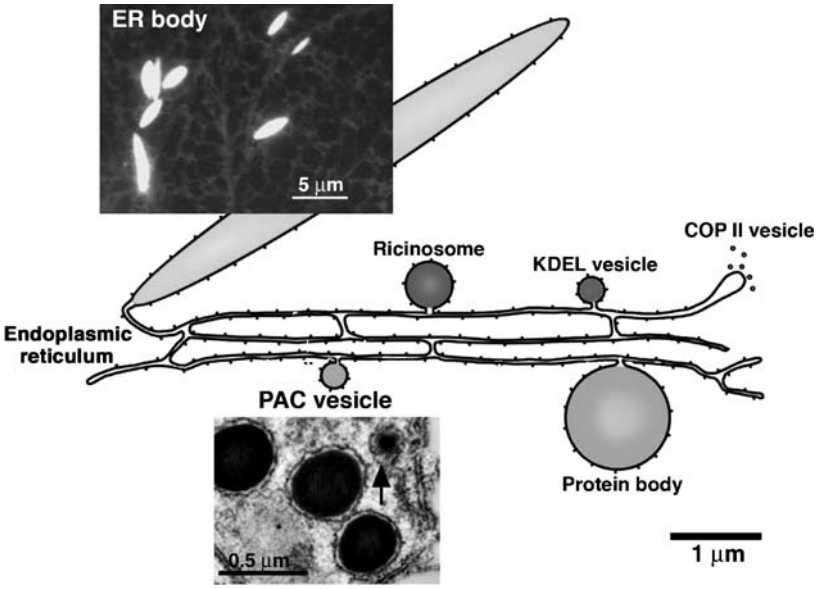

Fig. 1 ER-derived compartments in plants. ER bodies (~ 10 μm long and ~ 1 μm wide) are the largest compartment derived from the ER. Fluorescent spindle-shaped ER bodies in seedlings of transgenic *Arabidopsis* plants expressing green fluorescent protein (GFP) fused with an ER retention signal. PAC vesicles (0.3–0.5 μm) are transport vesicles for storage protein precursors from ER to protein storage vacuoles in maturing pumpkin and castor bean seeds. Protein bodies (1–2 μm) accumulating insoluble storage proteins in maize and rice seeds. KDEL vesicles (0.2–0.5 μm) accumulating a cysteine proteinase (SH-EP) in germinating black gram cotyledons. Ricinosomes (0.2–0.5 μm) accumulating SH-EP in senescing endosperm of castor bean. COPII vesicles (0.05–0.1 μm) are transport vesicles from the ER to the Golgi complex

man 2000). The ER appears to have an ability to generate various types of compartments in response to active synthesis of a distinct protein(s). The compartments are classified into two types according to their contents: storage protein type and hydrolytic enzyme type. The former type accumulates seed storage proteins in large quantities. The latter type accumulates hydrolytic enzymes such as cysteine proteinase and β-glucosidase.

Storage protein types include precursor-accumulating (PAC) vesicles with diameters of 0.3–0.5 μm and protein bodies (diameters $\phi = 1–2$ μm). During seed maturation, a large amount of storage protein is synthesized on the ER as larger precursors. PAC vesicles play a central part in the efficient transport of insoluble aggregates of storage protein precursors directly to protein storage vacuoles in maturing seeds of pumpkin (Hara-Nishimura et al. 1998), castor bean (Hiraiwa et al. 1993), and rice (Takahashi et al. 2005). Protein bodies that are found in cereal seeds of rice and maize accumulate the alcohol-soluble storage proteins prolamin and zein, respectively.

On the other hand, hydrolytic enzyme types include ER bodies of *Arabidopsis* (~ 1 μm diameter and ~ 10 μm long) (Matsushima et al. 2002, 2003b), KDEL vesicles (diameter $\phi = 0.2–0.5$ μm) of germinated black gram seeds (Toyooka et al. 2000), and ricinosomes (diameter $\phi = 0.2–0.5$ μm) of castor bean (*Ricinus communis*) (Schmid et al. 1998). All of these compartments accumulate hydrolytic enzymes with an ER retention signal, KDEL. ER bodies accumulate KDEL-tailed β-glucosidase, while KDEL vesicles and ricinosomes accumulate KDEL-tailed cysteine proteinase. Thus, the ER is the most multitalented and adaptable compartment in plant cells (Staehelin 1997). The mechanisms underlying protein accumulation and the induction of these compartments are discussed for two ER-derived compartments: PAC vesicles and ER bodies.

2
PAC Vesicles

2.1
PAC Vesicles Responsible for Efficient Transport of Storage Proteins

PAC vesicles were originally discovered and isolated from maturing pumpkin seeds (Hara-Nishimura et al. 1991) and accumulate precursors of storage proteins, 2S albumin and 11S globulin, to be transported to protein storage vacuoles in maturing seeds of pumpkin (Hara-Nishimura et al. 1993b) and castor bean (Hara-Nishimura et al. 1998). How do the vesicles accumulate the precursors?

Proprotein precursors of storage proteins are synthesized on the ER and translocated into the lumenal side of the ER in maturing seeds. Figure 2 shows an electron micrograph of maturing pumpkin seeds. Some storage proteins

form core aggregates in maturing pumpkin seeds (indicated by arrows in Figs. 1 and 2). Active synthesis of storage proteins facilitates formation of protein aggregates within the ER. The protein aggregates develop into the electron-dense cores of the PAC vesicles and then leave the ER. A number of PAC vesicles appear at the middle and late stages of maturing pumpkin seeds (Fig. 2). PAC vesicles mediate a transport pathway for insoluble aggregates of storage protein precursors directly to protein storage vacuoles. The aggregate sorting via PAC vesicles to protein storage vacuoles bypasses the Golgi apparatus. This Golgi-independent aggregate sorting is presumed to have evolved for the efficient transport of proteins in some types of maturing seeds that synthesize a large amount of storage proteins.

The final step in the aggregate sorting is the incorporation of the storage protein precursors into a protein storage vacuole. There are two possible modes of incorporation: one is autophagy by the protein storage vacuoles and the other is membrane fusion between PAC vesicles and the vacuoles. Autophagic engulfment by protein storage vacuoles has been observed in maturing seeds of pea (Robinson et al. 1995) and in seedlings of mung bean (van der Wilden et al. 1980). Autophagy should be accompanied by the incorporation and breakdown of membrane proteins of protein storage vacuoles (Maeshima et al. 1994; Inoue et al. 1995; Strzalka et al. 1995). If such autophagy were to mediate the incorporation of PAC vesicles into the protein storage vacuoles, some of the tonoplast should remain inside the vacuoles.

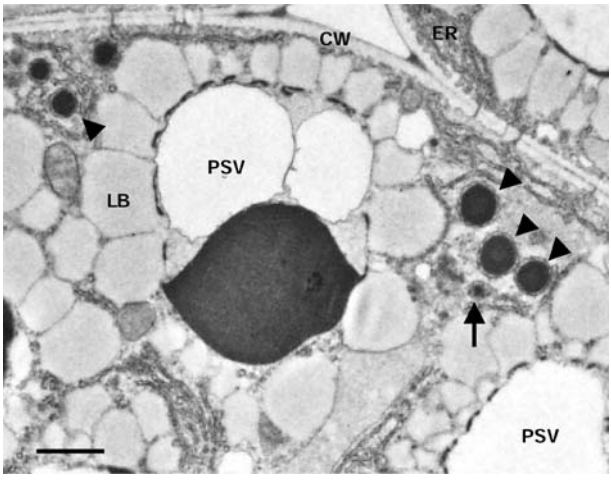

Fig. 2 Electron micrograph showing cotyledon cells at the middle stage of maturing pumpkin seeds. Numerous PAC vesicles (*arrowheads*) about 300 to 400 nm in diameter are visible in the cell. PAC vesicles contain an electron-dense core that is surrounded by an electron-translucent layer. An electron-dense aggregate (*arrow*) is seen within the ER. PSV, protein storage vacuole; ER, endoplasmic reticulum; CW, cell wall; LB, lipid body. *Bar* = 0.5 μm

Detailed immunocytochemical studies about tonoplast should answer the question of whether autophagic engulfment is involved in the incorporation of storage proteins into the protein storage vacuoles.

Most proprotein precursors of storage proteins are converted into their respective mature forms by the action of vacuolar processing enzyme (VPE) after their arrival at protein storage vacuoles (Hara-Nishimura et al. 1993a, 1995; Shimada et al. 2003b). VPE-deficient *Arabidopsis* seeds exhibit a defect of proper processing of storage proteins (Shimada et al. 2003b). Inactive precursor of VPE is also localized in PAC vesicles in maturing castor bean endosperm (Hiraiwa et al. 1993). The VPE precursor is self-catalytically converted to an active enzyme within the vacuoles (Hiraiwa et al. 1997, 1999; Kuroyanagi et al. 2002). PAC vesicles are involved in the transport of not only storage proteins but also a precursor of a membrane protein, MP73, of protein storage vacuoles of pumpkin (Mitsuhashi et al. 2001). The MP73 precursor is also converted into the mature form within the vacuoles.

2.2 Vacuolar Sorting Receptor on the Membrane of PAC Vesicles

Storage protein precursors are selectively taken into the PAC vesicles in two ways: one is by aggregate sorting and the other is through receptor-dependent sorting. The former sorting mechanism is advantageous to maturing seeds that actively synthesize a large quantity of storage proteins as described above. The latter ensures proper delivery of the storage proteins by avoiding the mis-sorting of escaped molecules. What molecular mechanism is involved in the uptake of storage protein precursors into the PAC vesicles?

A proteomic analysis of the isolated PAC vesicles from maturing pumpkin seeds identified a type I integral membrane protein, PV72, which is composed of a large lumenal domain and a transmembrane domain followed by a short cytosolic tail that contains a potential tyrosine-based motif (Shimada et al. 1997). PV72 is the next most abundant protein after the storage protein precursors in the PAC vesicles. PV72 is detected on the membrane of the PAC vesicles (Shimada et al. 1997), and is specifically and transiently accumulated in association with the synthesis of storage proteins. PV72 has been shown to bind to the 2S albumin precursor (pro2S albumin) via the *C*-terminal region including a vacuolar targeting signal (Shimada et al. 2002) and to the vacuolar targeting signal (Asn-Pro-Ile-Arg) of a vacuolar protein, AtAlue (Watanabe et al. 2004). The biochemical and cell biological analyses implied that PV72 acts as a vacuolar sorting receptor (VSR).

PV72 has Ca^{2+} binding domains and the binding of Ca^{2+} stabilizes the receptor–ligand complex even under acidic conditions of pH 4.0. The association and dissociation of PV72 with the ligand is modulated by the Ca^{2+} concentration (EC_{50} value = 40 μM) (Watanabe et al. 2002). If PV72–pro2S albumin complex were delivered to protein storage vacuoles, PV72 could not

release pro2S albumin at the vacuoles because of high Ca^{2+} concentrations inside the vacuoles. The questions are where PV72 traps pro2S albumin and where PV72 releases it. Subcellular fractionation has revealed that PV72 is localized not only to the PAC membrane but also on the Golgi apparatus (Shimada et al. 2002). PV72 might be recycled between PAC vesicles and the Golgi apparatus for recruiting the storage protein precursors from the Golgi apparatus to the PAC vesicles.

For an in vivo demonstration, a reverse-genetic approach was applied to an *Arabidopsis* mutant lacking the homologous gene to PV72. *Arabidopsis* has seven homologues of PV72, which are designated as AtVSR1 to AtVSR7 (*Arabidopsis thaliana* vacuolar sorting receptor). Among seven *atvsr* mutant seeds, only the *atvsr1* mutant seeds abnormally accumulate the precursors of storage proteins 2S albumin and 12S globulin, together with the mature forms of these proteins (Shimada et al. 2003a). The *atvsr1* mutant mis-sorts storage proteins by secreting them from cells, resulting in an enlarged and electron-dense extracellular space in the seeds (Fig. 3). The *in planta* analysis strongly suggests that AtVSR1/PV72 is a sorting receptor that sorts storage proteins for protein storage vacuoles.

How does the sorting receptor function? Most of the storage protein precursor molecules synthesized on the ER form an aggregate within the ER. However, inevitably, some molecules are not incorporated into the aggregates

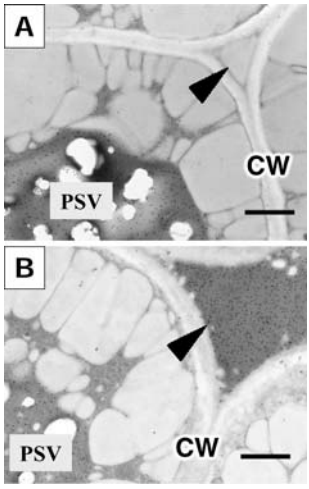

Fig. 3 *atvsr1* mutant mis-sorts storage proteins by secreting them from cells, resulting in an enlarged and electron-dense extracellular space in the seeds. Ultrastructures of seed cells of the wild type (**a**) and *atvsr1-1* mutant (**b**). The extracellular space of the *atvsr1-1* seeds is abnormally enlarged and filled with electron-dense material (*arrowhead*). Immunogold analysis with antibody against 2S albumin. 2S albumin is distributed in the PSVs and the electron-dense extracellular space of the *atvsr1-1* seeds. PSV, protein storage vacuole; CW, cell wall. *Bars* = 1 μm

and remain free within the ER lumen. The free molecules leave the ER for the Golgi apparatus, where they are trapped by the VSR and recruited to the PAC vesicles (Hara-Nishimura et al. 2004). After releasing the precursor molecules, the VSR should be recycled to the Golgi apparatus.

2.3 Induction of PAC Vesicles in Vegetative Tissues

PAC vesicles are specific to maturing seeds, because they accumulate seed storage protein precursors. Unexpectedly, however, PAC vesicles can be induced in vegetative tissues (Hayashi et al. 1999). Figure 4a and b shows electron micrographs of the rosette leaves of transgenic *Arabidopsis* plants, which overexpress a fusion protein composed of a small subunit of pumpkin 2S albumin and a selectable marker enzyme, phosphinothricin acetyltransferase. The leaves develop PAC vesicles together with chloroplasts (Fig. 4).

The induced PAC vesicles exhibit a similar density (1.27 g/cm^3) to those of PAC vesicles from maturing seeds and accumulate a proprotein precursor form of the fusion protein, as do PAC vesicles in maturing seeds. The ER has an ability to produce PAC vesicles not only in seed cells but also in vegetative cells, showing the flexible nature of the ER.

Despite the accumulation of the fusion protein with phosphinothricin acetyltransferase, the transgenic *Arabidopsis* plants are sensitive to phosphinothricin. Compartmentalization of phosphinothricin acetyltransferase in

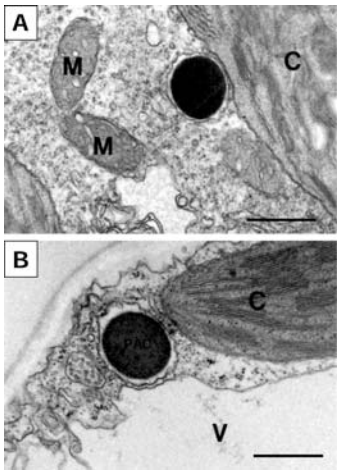

Fig. 4 **a** Electron micrograph of transgenic *Arabidopsis* plants expressing a fusion protein composed of a small subunit of pumpkin 2S albumin and a selectable marker enzyme, phosphinothricin acetyltransferase. **b** Electron micrograph of rosette leaves. PAC vesicles are found in the vegetative cells. C, chloroplast; V, lytic vacuole; M, mitochondria; PAC, PAC vesicles. *Bars* = 0.5 μm

the PAC vesicles does not permit the detoxification of this herbicide. Phosphinothricin acetyltransferase molecules are bound in the aggregates within the PAC vesicle, which obviously causes the loss of enzyme activity. The transgenic plants will become resistant to phosphinothricin if a mutation of a gene results in an alternative subcellular localization of the fusion protein. Such mutants should provide a clue to understanding the molecular mechanism involved in the biogenesis of the PAC vesicles from the ER.

3
ER Bodies

3.1
ER Bodies that Accumulate KDEL-Tailed β-glucosidase

Green fluorescent protein (GFP) from luminescent jellyfish allows us to visualize various organelles in living cells and in real time. GFP fusion proteins have provided new insight into novel subcellular compartments and their dynamic changes. For in vivo observation of the ER, GFP should be fused with a signal peptide at the *N*-terminus and with an ER retention signal (H(K)DEL, Lys/His-Asp-Glu-Leu) at the *C*-terminus. When the ER-targeted GFP is expressed in *Arabidopsis*, a number of bright fluorescing spindle-shaped organelles ($\sim 1\ \mu$m diameter, $\sim 10\ \mu$m long) are conspicuous against a background of a green ER network in the epidermal cells of cotyledons (Haseloff et al. 1997; Kohler 1998; Ridge et al. 1999), as shown in Fig. 1.

The GFP-fluorescing organelles had been referred to as proplastids in a widely used textbook "Essential Cell Biology" (Alberts et al. 1998) and were described as "mystery organelles" by Gunning (1998). In 2001, immunogold analysis with anti-GFP antibodies (Hayashi et al. 2001) showed that the unidentified GFP-fluorescing organelles correspond to dilated cisternae of the ER, which was first observed in radish root cells by electron microscopy in 1965 (Bonnett and Newcomb 1965). This indicates that the GFP-fluorescing organelles are derived from the ER. Thus, the GFP-fluorescing organelles were designated as ER bodies (Hayashi et al. 2001). The ER bodies have a characteristic fibrous pattern inside and are surrounded with ribosomes (Fig. 5a).

Electron microscopy has demonstrated similar compartments in 46 other species of Brassicaceae, seven species of Capparaceae, and four species of other families (Iversen 1970; Behnke and Eschlbeck 1978; Bones et al. 1989). However, a physiological function and the fate of the compartments have long been unknown. GFP-labeled ER bodies have made it possible for the study of the mysterious bodies to progress rapidly.

Transgenic *Arabidopsis* plants expressing GFP-HDEL show that ER bodies develop in epidermal cells of the seedlings but not in rosette leaf cells (Hayashi et al. 2001) (Fig. 6a,c). An *Arabidopsis* mutant whose seedlings hardly have

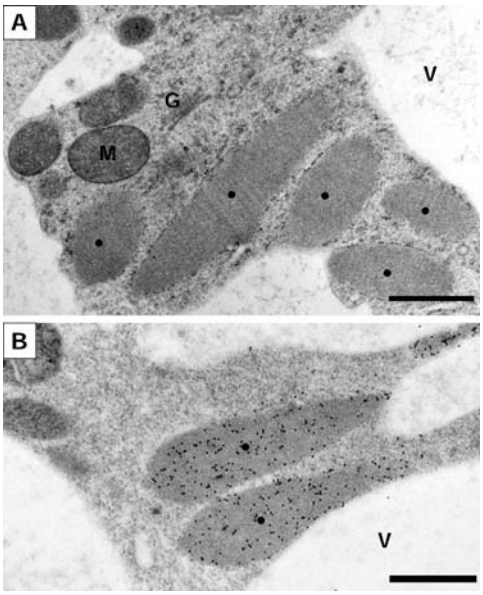

Fig. 5 Spindle-shaped bodies that are derived from ER-accumulated ER-targeted GFP in the epidermal cells of *Arabidopsis* cotyledons. **a** Electron micrograph showing that the ER bodies (*large dots*) have a characteristic fibrous pattern inside and are surrounded by ribosomes. **b** Immunocytochemistry with anti-PYK10 antibodies showing that the ER bodies accumulate PYK10. M, mitochondrion; G, Golgi complex; V, lytic vacuole. *Bars* = 0.5 μm

any ER bodies was isolated by inspecting the seedlings with a fluorescence microscope and was designated as *nai1* (Matsushima et al. 2003a). The *nai1* mutant has no ER bodies throughout the cells of seedlings or rosette leaves, although it has fluorescing ER networks. The *nai1* mutation affects the formation of ER bodies, but not the formation of the ER networks. The *nai1* mutant is a powerful tool to unravel the protein component and physiological function of ER bodies. ER bodies are concentrated in the 1000-*g* pellet fraction. Comparative proteomic analysis with the 1000-*g* pellet fractions between wild-type and *nai1* seedlings showed that the most abundant protein in ER bodies is a β-glucosidase, PYK10 (Matsushima et al. 2003a). Immunogold analysis with anti-PYK10 antibodies shows that PYK10 is localized in the ER bodies (Fig. 5b). PYK10 has an ER retention signal, KDEL, at the *C*-terminus. PYK10 is more selectively accumulated in the ER bodies than GFP-HDEL (Matsushima et al. 2003a). By forming aggregates in the ER lumen, PYK10 molecules may contribute to the formation of the ER bodies. This is supported by the result that PYK10 easily forms a large aggregate (Nagano et al. 2005).

The *NAI1* gene encodes a transcription factor that has a basic–helix–loop–helix (bHLH) domain (Matsushima et al. 2004). Transient expression of NAI1 induces formation of ER bodies in the *nai1-1* mutant (Matsushima et al.

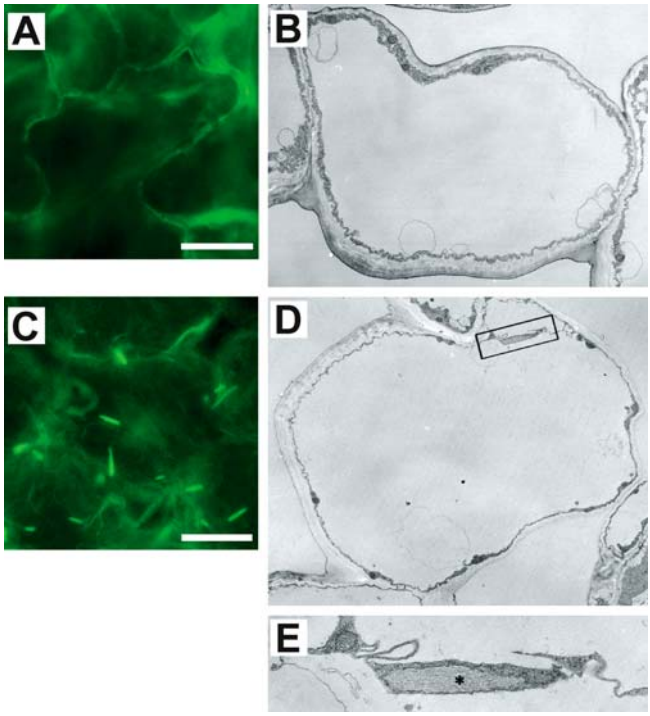

Fig. 6 Induction of ER bodies in rosette leaves treated with MeJA. Fluorescent images of rosette leaves treated with water (**a**) and 50 μM MeJA (**b**). The rosette leaves have no ER bodies. Water has no effect on the ER network or the development of ER bodies (**a**). GFP-fluorescing ER bodies are induced in the rosette leaves by MeJA (**b**). Electron micrographs showing that rosette leaves of nontransgenic *Arabidopsis* induced ER bodies when treated with MeJA. Rod-shaped ER bodies were induced in the epidermal cells of rosette leaves treated with MeJA (**d**). An ER body with a characteristic fibrous pattern in the *boxed area* of **d** is magnified (**e**). An *asterisk* indicates the ER body. No ER body was detected in the epidermal cells of wild-type rosette leaves treated with water (**c**). *Bars* = 20 μm

2004). The transcription factor regulates the formation of the ER bodies. *nai1* expresses no PYK10. The absence of ER bodies in *nai1* mutants is due to the loss of a transcription factor (NAI1) that functions upstream of a major component (PYK10) of ER bodies.

The defense function of β-glucosidases against pathogens is accomplished by producing toxic compounds. PYK10 and the specific substrate might be stored separately. When plant tissues and organelle membranes are injured, the enzyme and substrate come into contact and produce toxic compounds. ER bodies start to fuse with lytic vacuoles when the cells are stressed with a concentrated salt solution (Hayashi et al. 2001). Under stressed conditions, ER bodies might release their contents into the compartments, which accumulate endogenous substrate, to produce the toxic compounds.

The question is why the cotyledons, especially their epidermal cells, have a large number of ER bodies and rosette leaves do not (Hara-Nishimura and Matsushima 2003). ER bodies are not essential for the fundamental process of plant life. Therefore, rosette leaves may save the costs for the synthesis of PYK10 and formation of ER bodies, and induce them as the need arises. On the other hand, cotyledons, which are most sensitive to wounding and chewing by insects, may pay the costs for defense until the cotyledons are senesced. Consistently, the glucosinolate–myrosinase defense system in Indian mustard cotyledons declines during seedling development (Wallace and Eigenbrode 2002).

3.2 Induction of ER Body

The GFP image shows unique and limited distribution of ER bodies in *Arabidopsis* plants. The ER bodies appear after seed germination and disappear during senescence of the tissues (Matsushima et al. 2002). A large number of ER bodies are detected in the epidermal cells of whole organs including cotyledons, hypocotyls, and roots of transgenic *Arabidopsis* seedlings (Matsushima et al. 2002). The epidermal cells are most sensitive to environmental stresses in the plant life (Hayashi et al. 2001). This implies that the development of ER bodies is linked with environmental stresses (discussed above).

In contrast to the seedlings, the rosette leaves have no ER bodies at all under normal conditions. ER bodies are induced throughout the epidermal cells, when the rosette leaves are treated with a plant hormone, methyl jasmonate (MeJA), which mediates plant defenses against mechanical wounding and chewing by insects (Matsushima et al. 2002) (Fig. 6b). Electron microscopy shows the induction of the ER bodies within the rosette leaf cells (Fig. 6d, e). ER bodies are also induced with mechanical wounding (Matsushima et al. 2002).

MeJA-insensitive *coi1* mutant (Feys et al. 1994; Xie et al. 1998) induces no ER bodies. Another plant hormone, ethylene, which has no ability to induce ER bodies, suppresses the effect of MeJA on the induction of ER bodies in rosette leaves. In ethylene-insensitive *etr1-4* mutant (Chang et al. 1993), MeJA induces them even in the presence of ethylene. The induction of ER bodies is strictly linked to the signal transduction of MeJA and ethylene, both of which have an antagonistic effect. The induced ER bodies in the rosette leaves might have a defense function against chewing insects and be in some way related to other wound stresses. ER bodies might play a role in cells that are most exposed to stresses. The ER body formation is a novel type of endomembrane system in the response of plant cells to environmental stresses.

The ER bodies are inducible in *nai1-1* rosette leaves that have been treated with MeJA, although they exhibit irregular shapes (Matsushima et al. 2004). MeJA treatment induces the expression of *NAI1* and *PYK10* genes (Mat-

sushima et al. 2004). MeJA-induced NAI1 is needed for the formation of normal ER bodies.

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Synthesis of Structural and Storage Lipids by the ER

Matthew J. Hills[1] (✉) · Thomas J. Roscoe[2]

[1]John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK
matthew.hills@bbsrc.ac.uk

[2]Laboratoire Genome et Développement des Plantes, CNRS UMR5096,
Université de Perpignan, 52 Avenue de Villeneuve, 66860 Perpignan, France
roscoe@univ-perp.fr

Abstract The endoplasmic reticulum (ER) in plants plays a key role in the synthesis of a wide range of lipids which are essential structural components of all cellular membranes. Lipids also represent the major form of storage carbon in the seeds, pollen and fruit of many plant species and in some cases over 75% of the dry mass of these tissues has been metabolised by the ER. The world vegetable-oils market is worth over $30 billion per year and is of great importance to the agricultural economy. There is therefore particular interest in aspects of ER function relating to triacylglycerol synthesis. In the epidermis, lipids made by the ER are exported to form the cuticular barrier protecting the plant against water loss, biotic and abiotic stresses. In addition, ER-derived glycerolipids, sphingolipids and sterols have essential roles as components of signal transduction pathways. This chapter describes the biochemical pathways of membrane and storage lipid synthesis in the plant ER and charts progress in the identification and characterisation of the genes involved.

1
The Glycerolipid Synthesis Pathway

1.1
General Context

Lipid biosynthesis in plant cells is a highly coordinated process as a consequence of the compartmentalisation between the synthesis of fatty acids in the plastid and glycerolipids. In some species such as Arabidopsis, a significant proportion (> 35%) of the newly synthesised acyl chains are retained by the chloroplast and incorporated into membrane components such as galacto- and sulpho-lipids (Ohlrogge and Browse 1995). The remaining fatty acids (mainly the monounsaturated oleic acid—18 : 1) are exported from the chloroplast as acyl-CoAs and incorporated into phospholipids in the endoplasmic reticulum (ER). The 18 : 1 may be desaturated by the ER to form the polyunsaturated linoleic (18 : 2) and linolenic (18 : 3) fatty acids. A proportion of the lipids containing these fatty acids are returned from the ER to the chloroplast and incorporated into the thylakoids and envelope membranes. Many species retain little *de novo* synthesised fatty acid in the chloroplast

and in these plants almost all of the acyl chains apparently pass through the ER lipid pathway before being returned to the chloroplast (Mongrand et al. 1998). The ubiquitous glycerol-3-phosphate pathway localised in the ER (the plant eukaryotic pathway) ensures the synthesis of phosphatidic acid (PA) for the production of phosphatidylinositol (PtdIns) and phosphatidylglycerol (PtdGro) and the synthesis of diacylglycerol (DAG) for the production of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn, reviewed in Browse and Somerville 1991; Ohlrogge and Browse 1995). The glycerolipid acyltransferases associated with the Kennedy pathway belong to gene families, raising the possibility that a functional specialisation exists among these acyltransferases, each isoform perhaps participating in reactions that are unique to particular tissues or contributing to additional pathways that are associated with the production of PA destined for the production of specialised lipids.

1.2
Synthesis of PA by Microsomal Acyltransferases

As mentioned above, fatty acids are exported from the plastid in the form of acylCoA thioesters and transferred to the endomembrane system for modification and assembly into glycerolipids. The fatty acids are first esterified to glycerol-3-phosphate by the sequential action of glycerol-3-phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA) followed by 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (LPAT) to form PA (Fig. 1). The microsomal GPAT has a modest preference for palmitoyl-CoA whereas the microsomal LPAT has a strong preference for unsaturated C18 acyl groups. As a consequence of the substrate preferences of the ER acyltransferases (Frentzen 1998), the PA produced by the eukaryotic pathway in the ER is enriched in C18 fatty acids at the *sn*-2 position compared to the chloroplast with some C18 : 0 at *sn*-1 and *sn*-2 and with palmitoyl groups, if present, restricted to the *sn*-1 position.

Homology searches using a yeast sequence coding for a protein possessing GPAT activity identified a gene family of seven members encoding putative microsomal GPATs in the genome of *Arabidopsis thaliana* (Zheng et al. 2003). Transformation of a GPAT-deficient strain of yeast by each of the putative GPATs and assays performed with cell lysates in the presence of labelled G3P indicated that five of the seven candidates possessed GPAT activity; however the isoform(s) associated with the Kennedy pathway has not yet been identified. Abrogation of the GPAT1 gene leads to arrest of pollen development and the fatty-acid composition of the seed oil was altered with small decreases in the content of 16 : 0, 18 : 3 and 20 : 1, compensated by increases in 18 : 1 and 18 : 2 (Zheng et al. 2003). Interestingly, no change in seed oil content was observed, suggesting that other GPAT activities compensated in controlling flux through the pathway. The data

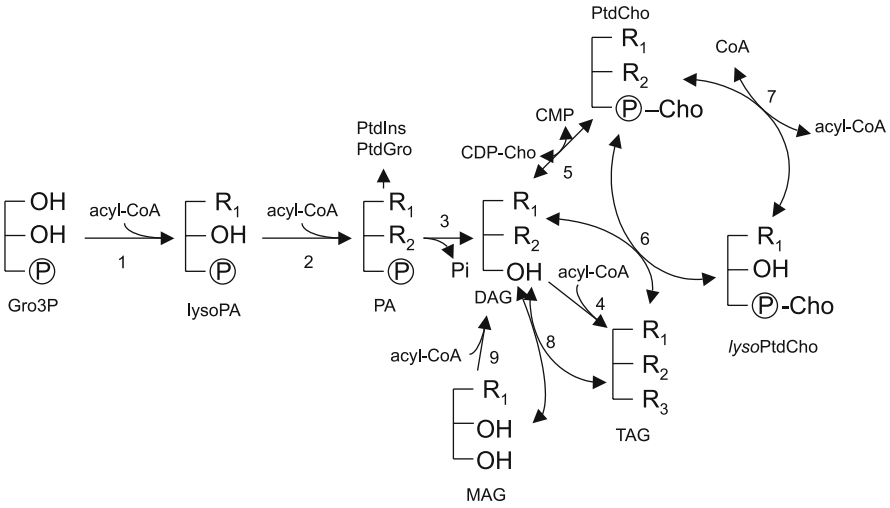

Fig. 1 Pathway of glycerolipid synthesis in the plant ER. Membrane phospholipids and storage triacylglycerols are synthesised from Gro3P and long-chain acyl-CoAs. More detail on phospholipid synthesis is presented in Figs. 2 and 3. 1—glycerol-3-phosphate acyltransferase (GPAT); 2—*lyso*phosphatidic acid acyltransferase (LPAT); 3—phosphatidic acid phosphatase (PAP); 4—diacylglycerol acyltransferase (DGAT); 5—CDP-choline:DAG cholinephosphotransferase (CPT); 6—phospholipid:diacylglycerol transacylase (PDAT); 7—*lyso*phosphatidylcholine acyltransferase (LPCAT); 8—diacylglycerol:diacylglycerol acyltransferase (DDAT); 9—monoacylgylcerol acyltransferase (MGAT)

are an indication of the potential for tissue-specific expression of individual acyltransferases.

In a similar approach, a gene family encoding proteins homologous to LPATs were identified in the *Arabidopsis thaliana* genomic sequence (Maisonneuve et al. 2003). At least five of the LPAT isoforms, including the plastidial isoform, are able to restore growth to an *E. coli* mutant (plsC-) strain deficient in LPAT activity and are thus likely to represent authentic LPATs. A *Brassica napus* cDNA, sharing 96% similarity with the protein encoded by the Arabidopsis LPAT candidate gene, At3g57650, has been verified to code for a protein possessing a 1-acylglycerol-3-phosphate acyltransferase activity by complementation of an LPAT-defective *E. coli* mutant and by enzymatic analyses where oleoyl-CoA was the preferred substrate. The rapeseed cDNA shares strong homology with the cDNAs encoding authentic LPATs of maize (Brown et al. 1994) and *Limnanthes* (Brown et al. 1995) and is expressed ubiquitously. Immunolocalisation has confirmed the association of the *Limnanthes* leaf- and seed-expressed isoform with the microsomal fraction (Brown et al. 2002). Taken together, these data suggest that the rapeseed, maize and *Limnanthes* proteins correspond to the ubiquitous LPAT enzyme of the Kennedy pathway.

1.3
Synthesis of Diacylglycerol in the ER

The majority of the phosphatidic acid produced in the ER is hydrolysed to DAG and inorganic phosphate by the action of phosphatidic acid phosphatase (also known as lipid phosphate phosphatase or LPP). The enzyme acts at a central point in the transition between membrane and storage lipids and has been proposed to control a potentially rate-limiting step in triacylglycerol biosynthesis (Stymne and Stobart 1987). In animals and yeast, distinct isoforms exist associated either with the ER, ensuring glycerolipid biosynthesis, or with the plasma membrane and involved in signalling. In plants, the LPP from avocado has been purified and the biochemical characterisation suggests that this enzyme is a good candidate for the eukaryotic pathway enzyme (Pearce and Slabas 1998). The maintenance of a high PA:LPA ratio by this enzyme suggests that metabolic channelling operates for the Kennedy pathway. Two cDNAs encoding LPPs of *Arabidopsis thaliana* that use both PA and diacylglycerol pyrophosphate as substrates have been well characterised by expression in yeast (Pierrugues et al. 2001). One of these LPPs (At2g01180) is likely to be targeted to the plastid (Beisson et al. 2003) and the second LPP (At1g15080) may be located in the ER but it is not clear whether it functions either in the Kennedy pathway for glycerolipid synthesis or lipid signalling. A third probable mitochondrial LPP (At3g02600) was identified in this report but not characterised.

2
Phospholipid Synthesis

A large proportion of plant phospholipids are synthesised by the ER from the Kennedy pathway intermediates PA and DAG (Fig. 1), although chloroplasts synthesise their own PtdGro and mitochondria have the capacity to metabolise some phospholipids as well. Each phospholipid has a different fatty-acid composition leading to scores of individual lipid components in any one tissue. The recent development of electrospray ionisation mass-spectrometry methods now allows for the quantification of many individual lipid molecules from plants (Welti and Wang 2004). These methods will facilitate the detailed analysis of gene knockout mutants and characterisation of the roles of the large number of genes that have been assigned putative functions in lipid metabolism (Beisson et al. 2003). Much of the work establishing the biochemical pathways of phospholipid synthesis and analysis of the enzymes involved has been reviewed in detail previously (Harwood 1989; Kinney 1993).

2.1
The CDP-Aminoalcohol Pathway for the Synthesis of the Major Phospholipids

2.1.1
Provision of CDP-Choline and CDP-Ethanolamine

PtdEtn and PtdCho are synthesised through the CDP-aminoalcohol pathway beginning with the activation of ethanolamine to phosphoethanolamine (P-Etn, Fig. 2), which forms the branch point between synthesis of the two lipids. The pathway from P-Etn to PtdEtn is direct via CDP-Etn (Fig. 2) but the pathways for PtdCho synthesis in plants vary depending on the tissue (Kinney 1993). The committed step of PtdCho synthesis is considered to be the methylation of P-Etn catalysed by P-Etn methyltransferase (Fig. 2, step 3) and the phospho-base methylation route via P-Cho and CDP-Cho is thought to carry the majority of the flux, at least in leaves (McNeil et al. 2000). PtdEtn is not itself methylated in plants but PtdmonomethylEtn (PtdMME) and PtddimethylEtn (PtdDME) may be methylated to form PtdCho (Fig. 2). Methylation of the free ethanolamine base has only been reported in endosperm of castor (Prudhomme and Moore 1992). The synthesis of P-Cho from P-Etn can be catalysed by a single phosphoethanolamine

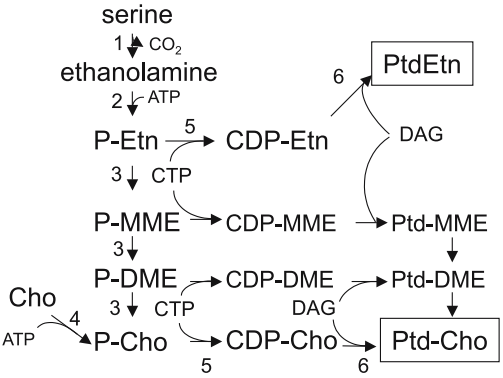

Fig. 2 Pathways of PtdCho and PtdEtn biosynthesis in plant ER. PtdCho and PtdEtn are mainly synthesised by the so called diacylglycerol pathway in plants. The Cho and Etn head groups are transferred to DAG from CDP-Cho and CDP-Etn (step 6). The CDP-Cho and CDP-Etn are synthesised by enzymes located in the cytosol, (steps 1–4) and one (step 5) whose activity is regulated by transfer between the cytosol and ER. The basic structure for PtdCho is shown in Fig. 1. For other phospholipids replace Cho with appropriate headgroup. 1—serine decarboxylase; 2—ethanolamine kinase; 3—phosphoethanolamine methyltransferase (methyl groups supplied from S-adenosylmethionine); 4—choline kinase; 5—CTP:phosphocholine cytidyltransferase; 6—DAG:CDP-aminoalcoholphosphotransferase. MME—monomethylethanolamine; DME—dimethylethanolamine

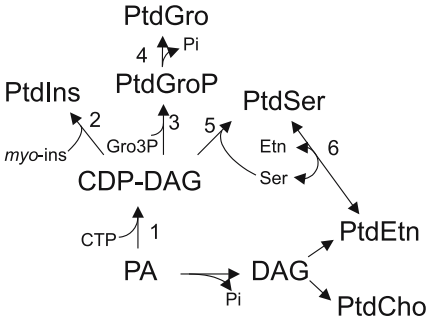

Fig. 3 Pathways of PtdIns, PtdGro and PtdSer biosynthesis in the ER of plants. These lipids are synthesised by the CDP-DAG pathway. 1—CDP-DAG synthase; 2—phosphatidylinositol synthase; 3—phosphatidylglycerolphosphate synthase; 4—phosphatidylglycerolphosphate phosphatase; 5—phosphatidylserine synthase; 6—phospholipid base exchange enzyme. The basic structure of a phospholipid (PtdCho) is shown in Fig. 1. For other phospholipids replace Cho with appropriate head group

N-methyltransferase (PEAMT or NMT1) protein which catalyses all three methylations using S-adenosylmethionine as the source of methyl groups (Bolognese and McGraw 2000; Nuccio et al. 2000). The spinach PEAMT lacked detectable activity on PtdEtn or Etn (Nuccio et al. 2000). As yet, the enzyme(s) that methylate the PtdMME and PtdDME have not been identified. Three putative PEAMT genes have been identified in Arabidopsis and a deletion of one of them (At3g18000) leads to profound effects on root architecture and a reduction in the content of PtdCho in the roots (Cruz-Ramirez et al. 2004). The mutant phenotype can be reversed with either P-Cho or choline, confirming the importance of the P-base methylation pathway in PtdCho synthesis in plants. This also showed that sufficient choline kinase (CK) activity is present for PtdCho synthesis from free choline (Cruz-Ramirez et al. 2004). In another study, silencing of PEAMT expression caused the development of pale leaves, hypersensitivity to salt stress and temperature-sensitive male sterility, although some PEAMT expression remained in these plants (Mou et al. 2002). Indeed, the synthesis of choline by this route is important in some species for the synthesis of the osmoprotectant glycinebetaine (Nuccio et al. 2000). Phosphocholine can also be synthesised from free choline by CK (Fig. 2, step 4) and two CK cDNAs cloned from soybean have been expressed and characterised (Monks et al. 1996). Four putative CK genes have been identified in Arabidopsis but as yet not characterised, and it is likely that one or more of these will code for the ethanolamine kinase required for step 2 in Fig. 2. Phosphocholine is activated to CDP-choline by CTP:phosphocholine cytidylyltransferase (CCT) and this enzyme is considered to be important in controlling flux through the PtdCho synthesis pathway in animals. Plant CCT, like its animal counterpart, is found in both soluble (inactive) and membrane (active) fractions in cell extracts (Kinney and Moore 1987). The CCTs con-

tain a C-terminal amphipathic alpha helix that, in animals at least, has been shown to be involved in binding of the protein to membranes, thus controlling CCT activity (Jackowski and Fagone 2005). However, little work on this aspect of regulation of the plant enzyme has been reported. cDNAs encoding CCTs were cloned from Brassica (Nishida et al. 1996), Arabidopsis (Choi et al. 1997) and pea (Jones et al. 1998) and CCT activity confirmed by expression of the Arabidopsis and pea cDNAs in yeast and *E. coli* respectively. Two CCT genes have been identified in Arabidopsis and expression of one of them is induced strongly by low temperatures (Choi et al. 2001; Inatsugi et al. 2002). The amount of membrane PtdCho increases when plants are subjected to low temperatures (Yoshida 1984; Uemura et al. 1995) and it appears that the synthesis of PtdCho is strongly regulated by expression of the CCT and PEAMT genes.

2.1.2 The Aminoalcoholphosphotransferases

PtdCho and PtdEtn, the two most abundant phospholipids are largely synthesised by the integral membrane aminoalcoholphosphotransferases (AAPTs) from *sn*-1,2 DAG and either CDP-choline or CDP-ethanolamine, respectively (Fig. 2, step 6). In these reactions CMP is displaced from CDP-choline or CDP-ethanolamine by the *sn*-3 hydroxyl of *sn*-1,2 DAG. Biochemical evidence that the same enzyme is responsible for catalysing both reactions (Lord 1975; Sparace et al. 1981) was confirmed once genes encoding AAPTs from soybean (Dewey et al. 1994), Arabidopsis (Goode and Dewey 1999) and *Brassica napus* (Qi et al. 2003) were cloned and the proteins expressed and characterised. The Arabidopsis AAPT proteins share 85% sequence identity and both contain ER membrane retention signals. Although all AAPTs characterised were shown to use both CDP-choline and CDP-ethanolamine as substrates, the AAPT2 from Arabidopsis and AAPT1 from Brassica preferentially use CDP-choline. Interestingly, the AAPT from *Chlamydomonas reinhardtii*, an organism which does not synthesise PtdCho but only PtdEtn, is capable of synthesising both PtdCho and PtdEtn if the substrates are provided (Yang et al. 2004). The AAPTs are activated by Mg^{2+} or Mn^{2+} and inhibited by Ca^{2+} and CMP (Goode and Dewey 1999; Qi et al. 2003). Strong expression of the soybean AAPT in tobacco leaves did not cause any change in the fatty-acid composition of phospholipids (Goode and Dewey 1999). On the other hand expression of the AAPT from *Brassica napus* in Arabidopsis caused a small but significant increase in the polyunsaturated fatty-acid content (Qi et al. 2003). The AAPTs in microsomal membranes isolated from developing seeds from safflower and oilseed rape showed no specificity for the acyl chain composition of the *sn*-1,2-DAG, suggesting that this enzyme draws on DAG molecules in the pool surrounding the enzyme in the membrane (Vogel and Browse 1996). However, the AAPTs from pea leaves and germinating soybeans

were all shown to prefer 1-palmitoyl-2-linoleoyl DAG as lipid substrate (Justin et al. 1987). A detailed study of the substrate selectivity of purified heterologously expressed AAPT enzymes in mixed detergent/substrate micelles has yet to be made.

2.2 The CDP-DAG Pathway for the Synthesis of the Minor Phospholipids

Phosphatidylinositol, phosphatidylglycerol and phosphatidylserine are synthesised from PA via CDP-diacylglycerol (CDP-DAG). The enzyme CDP-DAG synthase (CDS) uses PA and CTP as substrates and releases phosphatidylinositol phosphate in addition to the CDP-DAG (Fig. 2, step 1). cDNAs encoding CDP-DAG synthases were cloned from potato and Arabidopsis using knowledge of sequence homology, and CDS activity was proved by expression of cDNA in *E. coli* combined with purification of the protein (Kopka et al. 1997). Arabidopsis is predicted to contain two ER-bound CDS genes, each predicted to contain eight membrane spanning domains, but little analysis of their function in plants has been reported. The head group of the phospholipid is then attached to the lipid by displacement of CMP from CDP-DAG (Fig. 2).

2.2.1 Phosphatidylinositol

PtdIns synthase (PIS) catalyses the reaction between inositol and CDP–DAG-producing PtdIns and CMP. A PIS gene was identified in Arabidopsis by sequence homology and the cDNA expressed in *E. coli* (Collin et al. 1999) which accumulated PtdIns, a phospholipid it normally lacks. The cDNA encoding Arabidopsis PIS was also able to complement the yeast PIS deletion mutant (Xue et al. 2000). The presence of a di-lysine retention signal at the C-terminus of the protein suggests PIS is located in the ER but this is yet to be substantiated. The enzyme was shown to require CDP-DAG and *myo*-inositol as substrates and either Mn^{2+} or Mg^{2+} as cofactor (Collin et al. 1999; Xue et al. 2000). In vitro evidence has been presented that the Arabidopsis PIS may also catalyse exchange of the inositol head group of PtdIns with *myo*-inositol in the presence of low concentrations of CMP (Justin et al. 2002). The acyl chain composition of PtdIns in plants is highly saturated compared to other phospholipids but it is not known how this is maintained. Biochemical analysis of the overexpressed PIS protein suggested that the acyl composition of PtdIns is likely to be determined as much by the localisation of the PIS within subdomains of the ER and the CDP-DAG pool surrounding it as by the substrate selectivity of the enzyme itself (Justin et al. 2003). A second Arabidopsis PIS gene has been identified by sequence homology but not yet characterised (Beisson et al. 2003).

2.2.2 Phosphatidylserine

PtdSer is present at low concentrations in plants. There is biochemical evidence that it can be made both by PtdSer synthase (PSS) which uses CDP-DAG and serine and by head-group exchange of serine with Cho or Etn from PtdCho or PtdEtn respectively depending on the plant species and tissue (Kinney 1993). A cDNA encoding a protein that showed strong PSS activity was cloned from wheat in a screen to identify genes that enhance resistance to aluminium toxicity (Delhaize et al. 1999). However, a recent BLAST search reveals that no homologs of the wheat PSS are identified in any other plant species and that the closest homolog to the wheat PSS is a gene from a cereal fungal pathogen (Gibberella). It now seems likely that the "wheat" clone was isolated from fungus infected plant material. On the other hand, a clear homolog of the mouse PSS is present in the Arabidopsis genome (At1g15110), although the mouse protein synthesises PtdSer by a base-exchange mechanism using PtdEtn or PtdCho rather than transfer of serine to CDP-DAG (Stone et al. 1998). It was later shown that the PSS from mouse is located in the mitochondria-associated membrane, an ER-type membrane that purifies with mitochondria rather than the bulk ER (Stone and Vance 2000). There is as yet no definitive information on the location of the phospholipid base-exchange enzyme in plants. A plant gene encoding a CDP-DAG:serine phosphatidyltransferase has yet to be identified in plants. The plasma membrane of plants is relatively rich in PtdSer and there is evidence that this lipid may be supplied as small, PtdSer-enriched vesicles that are released from the ER in an adenosine triphosphate (ATP)-dependent process (Sturbois-Balcerzak et al. 1999; Vincent et al. 2001). However, the plasma membrane itself has been shown to have high phospholipid head-group serine exchange activity suggesting that this membrane obtains its PtdSer from two sources (Vincent et al. 1999).

2.2.3 Phosphatidylglycerol

The synthesis of phosphatidylglycerol (PtdGro) from CDP-DAG and Gro-3-phosphate via PtdGro-3-phosphate has been demonstrated in microsomal membranes from a number of species (Kinney 1993). A gene from Arabidopsis encoding an ER PtdGroP synthase (PGPS), which catalyses the first step of PtdGro synthesis (Fig. 2), was identified and characterised by expression in yeast and purification (Muller and Frentzen 2001). The ER PGPS requires Mn^{2+} for activity and is inhibited by Mg^{2+} and Ca^{2+}, unlike the bacterial proteins, which are more active with Mg^{2+} (Muller and Frentzen 2001). Very little is known about PGP phosphatase, required to dephosphorylate PGP to Ptd-

Gro in plants and no candidate genes have yet been identified for any of the cellular compartments in Arabidopsis.

3
Fatty-Acid Desaturation in the ER

3.1
Role of Polyunsaturated Fatty Acids

A large proportion of the fatty acids in plant membrane lipids are polyunsaturated, which helps to maintain membrane fluidity and cell integrity, particularly at low temperatures (Wolter et al. 1992). The critical importance of ER-synthesised polyunsaturated fatty acids to the plant's ability to resist exposure to low temperatures is shown by the *fad2* mutant of Arabidopsis, which lacks the capacity to make polyunsaturated fatty acids in the ER. When grown at 22 °C *fad2* mutant plants are almost indistinguishable from wild type. If transferred to 6 °C, however, *fad2* plants show signs of stress through accumulation of anthocyanins, leaf necrosis and death (Miquel et al. 1993) whereas wild type are unaffected. The appearance of symptoms in the *fad2* mutant is relatively slow compared to plant species that are known to be chill-sensitive plants where rapid membrane disruption is evident. Evidence suggests that the decreased supply of polyunsaturated fatty acids from the ER to the mitochondrion leads to changes in lipid:protein ratios and micro-viscosity of the mitochondrial membranes and modifies mitochondrial function in the mutant (Caiveau et al. 2001). Linolenic acid, which contains three double bonds, is also used as a substrate for the production of oxylipin signalling molecules such as jasmonic acid (Feussner and Wasternack 2002). Triple *fad3*, *fad7*, *fad8* mutant Arabidopsis plants are unable to synthesise any 18 : 3 and are subsequently unable to synthesise jasmonic acid and are very sensitive to attack by pests (McConn et al. 1997). The pathways of fatty-acid desaturation were identified by a classical biochemical genetic approach using Arabidopsis mutants (Browse and Somerville 1991; Miquel and Browse 1998; Wallis and Browse 2002). As mentioned in the introduction, a significant proportion of polyunsaturated fatty-acid synthesis is catalysed by the fatty-acid desaturases of the endoplasmic reticulum (Browse and Somerville 1991), and in many species of higher plants this proportion is very high (Mongrand et al. 1998).

3.2
The Desaturases of the ER

In the ER, oleic acid (18 : 1) is first desaturated to linoleic acid (18 : 2) by the action of oleate desaturase (FAD2) (Okuley et al. 1994). FAD2 desaturates oleic acid esterified to both the *sn-1* and – 2 positions of PtdCho (Sperling et al. 1993).

Linoleate, esterified to PtdCho may be further desaturated to linolenate (18 : 3) by linoleate desaturase (FAD3) (Arondel et al. 1992). Both of the ER desaturases use cytochrome b_5 and cytochrome b_5 reductase to transfer electrons from nicotinamide adenine dinucleotide (NADH) and both use molecular oxygen (Smith et al. 1990; Kearns et al. 1991). Based on in vitro reconstitution studies, a model of microsomal electron transfer for Arabidopsis was proposed where NADH-cytb_5 reductase and nicotinamide adenine dinucleotide phosphate (NADPH)-cytP450 reductase both reduce cytochrome b_5 using NADH and NADPH respectively (Fukuchi-Mizutani et al. 1999). A direct transfer of electrons from NADPHcytP450 reductase to the desaturase was also suggested as a possibility in this model where cytP450 mono-oxygenase functions as a terminal acceptor. The desaturases contain eight histidines in tripartite arrangements which coordinate with two iron atoms at the catalytic centre of the desaturase (Shanklin and Cahoon 1998). Expression of affinity-tagged FAD2 and FAD3 in tobacco BY-2 cells combined with immunofluorescence demonstrated that both N and C termini reside on the cytoplasmic face of the ER (Dyer and Mullen 2001). A model of the topology of FAD2 and FAD3 based on animal structure predictions show the three catalytic His boxes extend from the cytosolic side (Dyer and Mullen 2001). Since the cytochrome b_5 also projects into the cytosol, it is likely that the desaturation reactions take place out of the plane of the ER membrane (Okuley et al. 1994). The affinity tagging study also confirmed the view that both desaturases are localised to the ER (Dyer and Mullen 2001). FAD2 and FAD3 are co-translationally inserted into the ER using the SRP/Sec61 translocon machinery, but have different ER membrane protein-retention signals (McCartney et al. 2004). Whilst FAD3 has a dilysine motif that is common to many ER membrane proteins from a wide range of eukaryotes, FAD2 was found to use a novel motif (ΦXXK/R/D/EΦ, where Φ = large hydrophobic residue) that was also shown to occur in a number of other putatively ER-retained proteins (McCartney et al. 2004).

A number of divergent FAD2 enzymes from a range of species have been identified that catalyse the synthesis of hydroxy, epoxy, acetylenic and conjugated fatty acids in the seeds (Broun et al. 1998; Lee et al. 1998; Iwabuchi et al. 2003). Relatively few changes in the protein sequence are required to alter the activity of a desaturase to convert it to a hydroxylase (Broun et al. 1998). Sterculic acid, a cyclopropane fatty acid, is also derived from oleic acid esterified to PtdCho. Cyclopropane synthase, which catalyses this reaction, is microsomally bound and uses S-adenosylmethionine as the methyl donor, but is not *FAD2*-related (Bao et al. 2003).

3.3 Regulation of the Desaturases

The mechanisms that regulate of expression of the ER desaturases are not well understood, though it is thought that the amount of *FAD3* transcript present

in an Arabidopsis leaf is important in controlling the synthesis of 18 : 3. In the *ife* mutant of Arabidopsis, a twofold increase in the 18 : 3/18 : 2 ratio is associated with an 80% increase in *FAD3* transcript (Shah et al. 1997). Close linkage of the *ife* mutation to *FAD3* suggests the lesion lies within a region that controls the *FAD3* transcript level. A study of transgenic *FAD3::LUC* Arabidopsis plants showed that the regulation of *FAD3* is quite complex, depending on the developmental stage of the plant and interactions between the concentrations of auxins and cytokinins (Matsuda et al. 2001). A screen for mutations in genes that regulate the polyunsaturated fatty acids (PUFA) content (*rfc*) of Arabidopsis roots at loci other than the desaturases themselves identified four new loci (Horiguchi et al. 2001). However, once cloned, *RFC3* was found to encode a plastid ribosomal protein suggesting the change in PUFA content in *rfc3* roots was a pleiotropic effect in that case. The remaining *RFC* genes have yet to be identified. Post-transcriptional regulation of desaturase activity may also be important in response to changes in temperature. Exposure of wheat roots to low temperature caused an increase in the 18 : 3 content of their lipids from 22% to 55% and this coincided with a seven fold increase in TaFAD3 protein content of the ER. An increased association of Ta*FAD3* mRNA with polyribosomes was found at low temperature, suggesting that the increase in FAD3 protein content was due to a higher rate of message translation (Horiguchi et al. 2000).

3.4
Transport of Polyunsaturated Fatty Acids from the ER to the Chloroplast

In an Arabidopsis leaf about half of the polyunsaturated fatty acids synthesised by the ER are returned to the chloroplast, where they are incorporated into the membranes of the thylakoids and inner envelope (Browse and Somerville 1991). The mechanism of transport of lipid between the ER and plastid is not well understood (Bessoule and Moreau 2004). Pulse-chase experiments with leek seedlings suggest that lyso-PtdCho may be the lipid transferred from the ER to the chloroplast envelope (Mongrand et al. 2000). Pea leaf chloroplasts can acylate phospholipids in an acyl-CoA-dependent manner, a reaction required if a *lyso*-lipid is the transferred lipid molecule (Kjellberg et al. 2000). This study also provided evidence that the chloroplast envelope has a discreet subdomain of the ER attached to it, which is proposed to be analogous to the mitochondria-associated membranes mentioned earlier in this chapter. The conversion of PtdCho in the plastid outer envelope to DAG by a cytosolic phospholipase D has also been demonstrated recently (Andersson et al. 2004), a reaction that may be required before import of lipid into the chloroplast can occur. Studies of a mutant in galactolipid metabolism identified a permease-like protein of the chloroplast outer envelope that is involved in the import of glycerolipids (Xu et al. 2003). It is hoped that an extension of this genetic approach will facilitate identification of other proteins involved in the process of lipid transfer.

3.5
Synthesis of Very-Long-Chain Fatty Acids in the ER

Plants contain very-long-chain fatty acids (VLCFA), which are characterised by chain lengths of 20 or more carbon atoms and are either saturated or monounsaturated. VLCFAs form components of epidermal lipids, sphingolipids or seed storage lipids. VLCFA are synthesised from 18 : 0- or 18 : 1-CoA by a process of elongation whereby two carbon atoms are added to the carboxyl end of the acyl chain. The elongase complex comprises four dissociable subunits, each possessing a unique enzymatic activity: 3-ketoacyl-CoA synthase (KCS), 3-ketoacylCoA reductase (KCR), 3-hydroxyacylCoA dehydratase and trans 2,3-enoyl-CoA reductase (ECR). Acyl-CoA elongase has been purified from microsomal preparations of several species and tissues, including leek epidermis (Agrawal and Stumpf 1985) and developing rapeseed (Domergue et al. 2000). The KCS catalysing the condensation step in developing Arabidopsis seeds is encoded by the fatty acid elongation 1 (*FAE1*) gene (James et al. 1995). Ectopic expression of *FAE1* in Arabidopsis caused the synthesis of VLCFA in all tissues, suggesting the other three elongase subunits are constitutively expressed. This implies that the tissue- and species-dependent synthesis of VLCFA is controlled by KCS expression (Millar and Kunst 1997). The *FAE1* elongases possess a cysteine active site and are distinct in sequence from the yeast Elo-type elongases and from mammalian polyunsaturated fatty-acid elongases. In maize, the *GLOSSY8* gene was identified after characterisation of a leaf wax mutant and encodes a KCR component of the ER acylCoA elongase (Xu et al. 2002). Another Arabidopsis gene, At3g55360, which potentially encodes the ECR subunit has been identified on the basis of its ability to complement the yeast Tsc13 mutant. Tsc13 is deficient in ECR activity and is characterised by a reduced content of VLCFA in sphingolipids (Gable et al. 2004). When the Arabidopsis ECR was co-expressed in yeast cells with either of the yeast Elo2 or Elo3 KCSs, it co-immunoprecipitated with the Elo proteins solubilised from microsomal fractions, indicating a physical association between these proteins in the ER. *FAE1* is a member of the large KCS gene family in Arabidopsis, reflecting the numerous requirements for fatty-acid elongation pathways in different plant tissues. In contrast, the reductase subunits of the elongase are encoded by one or two genes, confirming the hypothesis that it is the expression of the KCS subunit paralog that confers the ability to synthesise VLCFA in a particular cell or tissue, and the other subunits participate in all elongase reactions.

4
Triacylglycerol Biosynthesis

In addition to their essential housekeeping function in the synthesis of intermediates for the production of membrane lipids, the acyltransferases of the

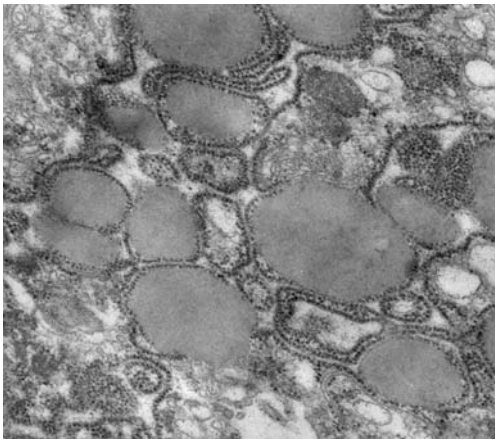

Fig. 4 Transmission electron micrograph of developing pollen grains from *Brassica napus*. Pollen grains from a large number of plant species accumulate triacylglycerols in the cytoplasm, as they do in seeds. The triacylglycerols in pollen are thought to supply lipids for the synthesis of the pollen tube. The ER and associated oil bodies derived from the ER are very clearly shown in this image, which was generously provided by JE Ross and DJ Murphy

Kennedy pathway in the ER also provide the precursor DAG for storage lipid biosynthesis in developing seeds and fruits and pollen. An example of extensive development of ER and deposition of TAG in oil bodies of pollen is shown in Fig. 4.

In seeds, the developing zygote has a requirement for fatty-acid biosynthesis and membrane lipid production for cell division and expansion during the early stages of embryo development. Seeds that accumulate large quantities of triacylglycerol as a reserve have an additional requirement for fatty-acid synthesis to support triacylglycerol production during the maturation phase of embryogenesis. Plant-specific aspects of TAG biosynthesis and the reserve deposition pathway were recently reviewed (Beaudoin and Napier 2004). Phospholipid bilayer membranes can accommodate up to about only 3 mol % of TAG, above which it partitions (Hamilton 1989; Lacey et al. 1999). As TAG is synthesised by the ER it accumulates as a separate phase and is secreted as droplets coated with oleosins. These droplets combine to form storage oil bodies within the cytoplasm of the seed (Murphy 2001). The functions of the highly unusual oleosin proteins and the control of their targeting to the ER are described in detail in chap. by Huang, this volume.

4.1 The Acyl-CoA-Dependent Pathway of Triacylglycerol Synthesis

DAG synthesised by the Kennedy pathway may be further esterified with an acyl-CoA at the *sn*-3 position via the action of diacylglycerol acyltrans-

ferase (DGAT). This reaction redirects DAG from membrane lipid synthesis to the production of TAG. A sequence homology search with mammalian DGAT permitted the cloning of a cDNA encoding DGAT1 from Arabidopsis and enzymatic assays demonstrated that the recombinant protein possessed DGAT activity (Hobbs et al. 1999). Arabidopsis *tag1* mutants, characterised by a reduction in seed oil content and altered seed fatty-acid composition also had reduced DGAT activity (Katavic et al. 1995; Routaboul et al. 1999) which mapped to the *TAG1* locus. Complementation of the *tag1* mutant alleles with the wild-type gene confirmed the functional identity of DGAT1 (Zou et al. 1999). The DGAT1 gene is expressed in developing seeds and pollen but also in germinating seeds and the protein and associated DGAT activity is also present in roots and shoots of seedlings and the stem of the inflorescence (Lu et al. 2003). Thus it is possible that triacylglycerol synthesis plays an important role in lipid metabolism in non-storage tissues. A second Arabidopsis DGAT was identified by homology to a fungal DGAT gene that was cloned following protein purification (Lardizabal et al. 2001). Unfortunately, there are no reports on the function of the Arabidopsis DGAT2 in knockout mutants. A bifunctional wax ester synthase/DGAT was cloned from the bacterium *Acinetobacter calcoacetcus* (Kalscheuer and Steinbuchel 2003). The Arabidopsis genome contains 11 putative homologs of this bifunctional enzyme (Beisson et al. 2003), but there are currently no reports on the characterisation of any of these genes. The DGAT enzyme(s) is thought to possess a broad substrate preference, similar to that of the microsomal GPAT, such that the fatty acid incorporated at positions *sn*-1 and *sn*-3 of TAG depends on the composition of the fatty acyl-CoA pool (Frentzen 1998). The microsomal LPAT exhibits a strong selectivity for unsaturated C18 oleoyl and linoleoyl groups (Brown et al. 2002), leading to oils where saturated acyl groups are almost absent from position *sn*-2.

4.2
Incorporation of Modified Fatty Acids from Membrane Phospholipids into TAG

De novo synthesised oleate may be modified by desaturation reactions catalysed by FAD2 and FAD3 or other modifying enzymes depending on whether the species accumulates unusual fatty acids (Sect. 3.2). The 18 : 2 and 18 : 3, or otherwise modified fatty acids may then be transferred from PtdCho and the acyl-CoA pool (Fig. 1, step 7) by a freely reversible exchange reaction catalysed by acyl-CoA:*lyso*-PtdCho acyltransferase (LPC-AT); the basic biochemistry is reviewed in (Frentzen 1993). The released polyunsaturated or modified acyl-CoA may be incorporated into triacylglycerol by the acyltransferases of the Kennedy pathway. As yet, a gene encoding acyl-CoA:LPC-AT has not been identified from any organism. The development of LPC photoaffinity probes gave hope that these proteins might be identified directly (Tumaney and Rajasekharan 1999) but this approach has not yet borne fruit.

It seems most likely that acyl-CoA:LPC-AT genes will be identified through detailed analysis of the LPAT homologs in Arabidopsis described in Sect. 1.1.

The important role that LPC-AT plays in linking pools of acyl substrates has become evident from attempts to manipulate polyunsaturated fatty-acid biosynthesis in yeast and plants. In yeast that was engineered to produce arachidonic acid (20 : 4), an accumulation of highly unsaturated fatty acids in PtdCho indicated that synthesis of acylCoA thioesters was limiting the production of docosahexanoic acid (22 : 6). These studies suggested that the LP-CAT, controlling the release of delta-6 desaturated 18 : 3 from *sn*-2 of PtdCho and its transfer to the acyl-CoA pool to allow elongation, is rate-limiting (Domergue et al. 2003). In yeast, whereas the transfer from acyl-CoA to PtdCho was very efficient, the rate of the reverse reaction was slow, suggesting the existence either of different isoenzymes for each reaction or a strong and different substrate preference for the forward and reverse reactions (Domergue et al. 2003). Similarly, analysis of seed lipids from transgenic plants expressing the appropriate elongases and desaturases indicated that the yield of polyunsaturated fatty acids in TAG was also limited by the transfer of delta-6 saturated fatty acids from PtdCho to the acyl-CoA pool (Abbadi et al. 2004). The delta-6 unsaturated 18-carbon polyunsaturated fatty acids were directly and rapidly incorporated into TAG, preventing transfer to the acyl-CoA pool, and permitting elongation for the production of C20 polyunsaturated fatty acids (Abbadi et al. 2004).

PtdCho can also contribute modified fatty acids to TAG synthesis by the donation of its DAG moiety catalysed by the reverse reaction catalysed by CPT described in Sect. 2.1. This permits the accumulation of DAG containing polyunsaturated fatty acids, to provide a precursor for TAG synthesis (Stymne and Stobart 1987). The relative importance of the CPT and LPC-AT routes in providing modified fatty acids for TAG is unclear and may vary according to the plant species.

4.3 Additional Pathways Leading to Triacylglycerol Synthesis in the ER

Metabolic labelling studies using microsomal preparations from safflower and castor bean showed that extensive acyl-CoA independent transacylation between membrane lipids, DAG and TAG is possible (Mancha and Stymne 1997; Stobart et al. 1997). In one reaction, fatty acids are rapidly transferred from the *sn*-2 position of PtdCho to DAG, generating TAG and *lyso*PtdCho (Fig. 1, step 6). A candidate yeast gene encoding this activity was identified by homology search using the logic that a lecithin:cholesterol acyltransferase-related enzyme could be responsible for the transfer of fatty acids from PtdCho to TAG, (Dahlqvist et al. 2000). The encoded protein partially complemented the corresponding yeast knockout mutant and the activity was described as phospholipid:diacylglycerol acyltransferase (PDAT). In yeast, this

enzyme plays an important role in triacylglycerol synthesis, however, after characterisation of the Arabidopsis PDAT enzyme, the authors now question the significance of PDAT in controlling lipid composition or redistribution of acyl groups between lipids (Stahl et al. 2004). Thus, the physiological role of PDAT in Arabidopsis is unclear. However, since it is involved in the breakdown of major membrane lipids to form lysophospholipids, PDAT may have a role in signalling or in membrane lipid repair (Stahl et al. 2004). The characterisation of an Arabidopsis PDAT T-DNA insertion mutant confirmed the existence of a diacylglycerol:diacylglycerol transacylation (DDAT) activity (Fig. 1, step 8), which contributes to triacylglycerol synthesis or remodelling but is normally masked by PDAT activity (Stahl et al. 2004). DDAT activity was first reported from microsomal preparations of safflower seeds (Stobart et al. 1997), and a similar activity was observed in sunflower seed microsomes (Frazer et al. 2000). These membrane preparations possess activities that catalyse the interconversion of mono-, di-, and triacylglycerols.

In addition to producing TAG, the DDAT reaction generates monoacylglycerol (MAG) but the mechanism for reincorporation of this lipid into Kennedy pathway intermediates was not clear (Stobart et al. 1997). Recently, the gene encoding MAG acyltransferase (MGAT) that catalyses the synthesis of the DAG has been cloned from mammals where the enzyme plays an important role in the resynthesis of TAGs in the intestine (Yen et al. 2002). The MGAT shares sequence homology with members of the DGAT2 gene family (Lardizabal et al. 2001). Although the existence of a peanut MGAT enzyme has been confirmed, the gene has not yet been cloned (Tumaney et al. 2001). The significance of MGAT and DDAT activities for storage lipid synthesis in plants may be related to their ability to enrich TAGs with polyunsaturated or unusual fatty acids and as well as to enhance the accumulation of TAG content in seeds.

4.4
Quality Control of Membrane Lipids in the ER

The TAGs in edible oil crops contain the same fatty acids as those present in cytoplasmic membrane lipids since both storage and membrane lipids are produced by the same pathway. However, many plant species synthesise storage lipids that contain unusual fatty acids (Voelker and Kinney 2001). The chemical reactivity and physical properties of these diverse, unusual fatty acids are incompatible with the structural integrity and physiological functioning of the lipid bilayer of membranes. In developing seeds, storage and membrane lipid biosynthesis occur simultaneously in the endoplasmic reticulum and share common enzymes and intermediates. Nevertheless, unusual fatty acids are excluded from the membrane lipids during seed development, which indicates an efficient segregation between triacylglycerol and membrane lipids (Millar et al. 2000). This discrimination is

remarkable since, as described above, in many cases the modification reaction occurs when the fatty acyl groups are esterified to the membrane lipid PtdCho. A fundamental problem of plant lipid biology therefore relates to how plants are able to control the fatty-acid composition of membrane and storage lipids.

4.4.1
Segregation of Unusual Fatty Acids into TAG Biosynthesis

The seeds of species that accumulate unusual fatty acids in their triacylglycerols possess additional microsomal acyltransferases, in particular, LPAT and DGAT. These enzymes exhibit a wide variation in substrate preference for both donor acyl groups and acceptor glycerolipids to facilitate channelling of unusual fatty acids into TAGs. In coconut, a seed-specific LPAT isoform is present that prefers medium-chain saturated fatty acids, allowing the synthesis of a trisaturate TAG (Knudson et al. 1995). Similarly, the synthesis of trierucin by *Limnanthes douglasii* requires a seed-specific 22 : 1-CoA-preferring LPAT isoform (Hanke et al. 1995). These tissue specific paralogs are absent from species that synthesise oils with common fatty acids at the *sn*-2 position of the triacylglycerol. The seeds of *Cuphea lanceolata* synthesise TAG that is rich in 10 : 0 mediated by the substrate selectivities of the acyltransferases (Bafor et al. 1990). The microsomal GPAT of *C. lanceolata* incorporates either medium- or long-chain fatty acids into position *sn*-1 of the Gro3P. When caprate is present in LPA, a LPAT isoform that prefers 10 : 0-CoA catalyses acylation at the *sn*-2 position. The *C. lanceolata* DGAT exhibits a strong selectivity for DAG containing 10 : 0 and uses 10 : 0-CoA to acylate position *sn*-3 (Bafor et al. 1990). The DGATs of other species that accumulate unusual fatty acids also exhibit a selectivity for them (Wiberg et al. 1994). Since the DAG and PtdCho pools are in equilibrium, DGAT therefore plays an important role in the removal of these fatty acids from PtdCho by the channelling of the DAG moiety released by the action of CPT into TAG. As mentioned in Sect. 2.1, the CPT does not itself have strong substrate selectivity for DAG species (Vogel and Browse 1996) and it was concluded that CPT does not influence channelling to triacylglycerol.

4.4.2
Compartmentalisation of TAG Biosynthesis in the ER

Since segregation of intermediates for storage or membrane lipid synthesis does not seem to be based on substrate discrimination by the diacylglycerol metabolising enzymes, Vogel and Browse (1996) hypothesised that channelling of unusual fatty acids to TAGs may require the spatial separation of distinct pools of DAGs containing normal or unusual fatty acids in the endoplasmic reticulum. The discovery that genes encoding enzymes of

the Kennedy pathway are represented by gene families (Maisonneuve et al. 2003; Zheng et al. 2003) fulfils the requirement of the hypothesis for distinct, and possibly tissue-specific, isozymes. That enzymes of membrane and storage lipid synthesis are co-localised to the same ER subdomains in sunflower (Lacey et al. 1999) does not preclude the possibility that distinct subdomains or metabolons exist for triacylglycerol synthesis in species that accumulate unusual fatty acids. Indeed the separation of a low-density subfraction derived from the ER of rapeseed that was greatly enriched in the enzymes of TAG synthesis compared to phospholipid synthesis supports this possibility (Lacey and Hills 1996). The protein composition of the low-density membrane fraction was quite different to that of the bulk ER (Lacey and Hills 1999). However, that the modification of fatty acyl groups occurs when these are esterified to phosphatidylcholine argues against separation of phospholipid and triacylglycerol synthesis in the endomembrane system unless all acyl exchange occurs through the acyl-CoA pool.

4.4.3
Editing of Phospholipids in the ER

Another proposed mechanism for removing unusual fatty acids from membrane lipids and sequestering them in TAGs is by active and selective removal or editing. This process could be mediated either by the action of specific phospholipase activities or by acyltransferases and transacylases. For instance, a phospholipase A2 (PLA2) activity has been shown to remove medium-chain fatty acids from PtdCho in the developing seeds of *Ulmus*, (Stahl et al. 1995). Similarly, *Ricinus*, *Cuphea* and *Euphorbia* species possess PLA2 activities that are selective for specific, unusual fatty acyl groups. The action of specific phospholipases would reduce contamination of membrane lipids and produce unusual fatty acids that could re-enter the acyl-CoA pool as substrates for acyltransferases for TAG synthesis. A PLA2 from *Ulmus* was purified and a cDNA cloned, but the protein was strongly predicted to be targeted through the secretory pathway, and the role of this particular class of PLA2 is not clear (Stahl et al. 1998, 1999).

In an alternative mechanism, the PDAT activity (see Sect. 4.3) was shown to transfer unusual fatty acids from the *sn*-2 position of PtdCho to DAG, forming TAG and *lyso*PtdCho (Fig. 1, step 6). In this manner, unusual fatty acids may be removed from PtdCho directly into TAG and prevented from accumulating in DAG and the acyl-CoA pool (Dahlqvist et al. 2000). The specificity of PDAT for the type of acyl group transferred is thought to vary among species and reflect the capacity for the synthesis of unusual fatty acids accumulated in seed TAGs. Although the contribution of the Arabidopsis PDAT to the control of lipid acyl composition is unclear (Stahl et al. 2004), the closest homolog of AtPDAT among the six members of the Arabidopsis gene family is expressed in a seed-specific manner and therefore this isoform

may be more suited to the role of an editing/channelling enzyme in seeds. Since the ability to synthesise unusual fatty acids has evolved independently among plant families it is probable that several editing mechanisms involving a number of enzymes are responsible for the removal of unusual fatty acids from membrane lipids, the relative importance of which varies among species (Banas et al. 2000).

The mechanisms controlling the expression of genes involved in storage oil synthesis are not yet understood although microarray analysis shows strong coordination in developing Arabidopsis seeds (Ruuska et al. 2002). Recent evidence shows that a particular AP2/EREB domain protein plays a role in transcriptional regulation of the pathway (Ruuska et al. 2002; Cernac and Benning 2004).

5
Minor Membrane Components

In addition to the main structural glycerophospholipids, the ER also synthesises a number of other lipid components including the sphingolipids and sterols. Although these are relatively minor in terms of proportion compared to the phospholipids, they can have significant impact on membrane structure and function. In animals, sterols and sphingolipids have been shown to function in the formation of lipid rafts in the plasma membrane but only recently has evidence for their existence in plants been presented (Mongrand et al. 2004; Borner et al. 2005). The sterols and sphingolipids are structurally complex groups of molecules and the pathways of their synthesis contain many steps compared to the phospholipids. For detailed descriptions of these pathways, the reader is referred to recent reviews, which cover each topic in much greater depth (Benveniste 2004; Schaller 2004; Dunn et al. 2004).

5.1
Sterols

Plants make a wide range of sterols, which are isoprenoid-derived molecules that are synthesised by the ER. Sterols have roles in the regulation of membrane fluidity in conjunction with polyunsaturated phospholipids and sterol derivatives (brassinosteroids). They also regulate a number of important plant processes including cell division and differentiation and elongation (Clouse 2002). The main sterol found in many plants is sitosterol but campesterol and stigmasterol are also found in significant amounts. However, the complex structure of sterols leads to the potential for a wide range of individual molecules depending on the position of methyl groups and double bonds. Since sterols may occur as free molecules within the membrane, be esterified to fatty acids or be glycosylated, a very large number of minor sterol com-

ponents have been identified that vary depending on the plant species and tissue (Benveniste 2004). Sterols are a group within the structurally diverse isoprenoids, which also include carotenoids, chlorophylls, quinines, terpenes and terpenoids. Some of these molecules such as the hemiterpenes, monoterpenes, and diterpenes are synthesised by a pathway in the chloroplast. Sterols are synthesised in the ER from cytosolic acetyl-CoA via HMG-CoA, mevalonate and isopentenyl pyrophosphate and 2,3-oxidosqualene. HMG-CoA reductase, which catalyses the rate-limiting step of isoprenoid synthesis, is located in the ER (Campos and Boronat 1995). Overexpression of HMGR was found to cause accumulation of sterol esters (Schaller et al. 1995) whereas mutation of one of the Arabidopsis genes (HMGR1) led to reduced sterol levels, dwarfing and male sterility (Suzuki et al. 2004). Evidence suggests that two mRNAs of different lengths are transcribed from one HMG-CoA reductase gene and the proteins translated from these messages are localised to different subdomains of the ER (Campos and Boronat 1995; Denbow et al. 1996; McCaskill and Croteau 1998). A highly conserved *N* glycosylation residue in the *N* terminus of the long-form protein is thought to control the differential location of this isoform (McCaskill and Croteau 1998). It is suggested that the short isoform synthesises mevalonate that is channelled through the sterol synthesis pathways whereas the long, glycosylated, isoform produces mevalonate for sesquiterpenoids required for plant defence (McCaskill and Croteau 1998). More recently it has been shown that the short (housekeeping) isoform may not only be located in the ER, but is mainly contained in small vesicles derived from the ER (Leivar et al. 2005).

The key, plant-specific step in sterol synthesis is catalysed by cycloartenol synthase which converts 2,3-oxidosqualene to cycloartenol. The gene encoding this enzyme was first identified from Arabidopsis using a chromatographic screen and a yeast sterol mutant (Corey et al. 1993). A number of sterol methylases, desaturases and demethylases are required for the further metabolism of sterols, leading to a network of potential pathways (Benveniste 2004). However, one or two main routes through the potential network have been identified through the careful analysis of Arabidopsis mutants, biochemistry using microsomal membranes isolated from maize seedlings and the heterologous expression of plant sterol genes in yeast (Diener et al. 2000; Clouse 2002; Benveniste 2004; Schaller 2004). The Arabidopsis mutants have also revealed the crucial role played by sterols in the very early stages of cellular growth and development of the embryo; e.g. *cephalopod*—sterol methyl transferase 1 (Schrick et al. 2002), *fackel*—$\Delta^{8,14}$ sterol Δ^{14} reductase (Schrick et al. 2000), *hydra1*—Δ^{8}–Δ^{7}-sterol isomerase (Souter et al. 2002). Brassinosteroids are important plant growth regulators derived from campesterol, which control many aspects of plant growth and development (Li et al. 1996). The main phenotype of mutants in brassinosteroid synthesis is dwarfism and the *dwf* mutants have been used to clone the genes involved (Clouse 2002).

5.2 Sphingolipids

Sphingolipids are a complex group of ER-synthesised membrane components that have received relatively little attention in plants (Dunn et al. 2004). Sphingolipids are thought to function as structural membrane components and as signalling molecules involved in the regulation of a number of cell processes. These include sphingosine-1-phosphate in signal transduction from changes in abscisic acid concentration to alterations in guard cell turgor (Ng et al. 2001) mediated by the heterotrimeric G protein (Coursol et al. 2003) and regulation of apoptosis (Brodersen et al. 2002; Spassieva et al. 2002). Little quantitative analysis of sphingolipids has been made in plants but they are known to be relatively abundant in the plasma membrane and tonoplast. Identification of the plant genes involved in the sphingolipid synthesis pathway has relied heavily on the use of yeast mutants (Dunn et al. 2004). The pathway begins with the production of sphinganine from serine and palmitoyl-CoA via 3-ketosphinganine (Fig. 5). Sphinganine, a long-chain base, can be hydroxylated, phosphorylated or acylated by a very-long-chain fatty acid to form ceramide (Fig. 5). The long-chain base may also be desaturated by Δ^4 and Δ^8 long-chain-base desaturases (Sperling et al. 1998; Ternes et al. 2002). There is a great deal of structural diversity within the sphingolipids with respect to the long-chain base and fatty-acid composition.

Although the sterols, sphingolipids and PtdSer are synthesised by the ER they are generally found to accumulate in the plasma membrane and tonoplast. In general, phospholipids from the ER are thought to be transferred to the plasma membrane and tonoplast through the secretory pathway. However, as mentioned earlier in the chapter, ER isolated from leek was found to

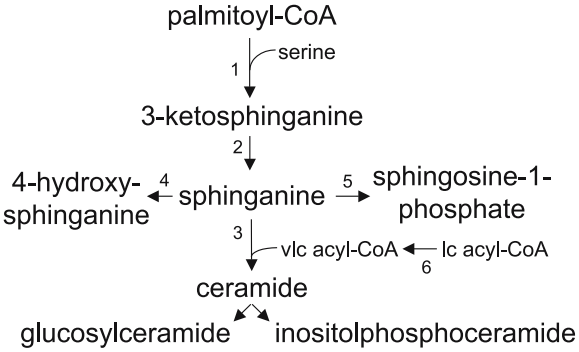

Fig. 5 Outline of the pathways of sphingolipid synthesis in plant ER. 1—serine palmitoyltransferase; 2—3-ketosphinganine reductase; 3—sphinganine acyltransferase; 4—sphinganine hydroxylase; 5—long-chain base kinase; 6—fatty-acid elongase; (v)lc—(very-)long-chain acyl-CoA

form PtdSer-enriched vesicles (70–80 nm) in the presence of ATP (Sturbois-Balcerzak et al. 1999), which it was proposed may be transferred to the plasma membrane. The kinetics of transport of sterols from the ER is similar to that of PtdSer (Moreau et al. 1998) and suggests both lipids are transported by the same vesicles. Specific inhibition of sterol synthesis in leak seedlings led to accompanying decreases in PtdSer and glucosylceramide, indicating that the regulation of the various biosynthetic pathways of these co-transported lipids is coordinated (Hartmann et al. 2002). Reciprocally, inhibition of the secretory pathway by brefeldin A led to the inhibition of the sterol synthesis pathway and an accumulation of sterol precursors (Merigout et al. 2002).

6
Conclusions and Future Prospects

Although good progress has been made in our understanding of lipid synthesis in the ER, there are a number of fundamental questions that remain to be answered. How does the plant cell maintain the required amount of lipid? How is membrane lipid composition maintained with respect to lipid type and acyl chain composition? The compensation by the eukaryotic pathway for the loss of the prokaryotic membrane lipid synthesis in the *act1* mutant of Arabidopsis (Kunst et al. 1988) has revealed the extent to which membrane synthesis is coordinated between the plastid and the ER. However, the embryo lethality of the *act2* knockout mutation (Yu et al. 2004) shows the limits of this cooperation, whereby the signalling mechanism of this homeostasis remains obscure.

Insight into the mechanisms that regulate the coordination of lipid and protein synthesis in the biogenesis of membranes is beginning to emerge from the characterisation of the ER stress response in seeds. The *floury-2* (*fl-2*) mutation in maize, is characterised by abnormal storage-protein accumulation, and resembles a phenotype similar to that seen in mammalian and yeast cells during ER stress, the unfolded protein response, reviewed by (Chapman et al. 1998). A link between phospholipid synthesis and the ER stress response is also apparent from the effects of overexpression of integral membrane proteins. These are seen to lead to an increase in phospholipid biosynthesis, a proliferation of the ER and an induction of the unfolded protein response, and seem to represent a response to a perceived need for an increased membrane surface area. The *fl-2* mutation causes an altered protein body composition and morphology and an up-regulation of several enzymes of phospholipid synthesis. The resulting increase in phospholipid and triacylglycerol biosynthesis in maize kernels led to the hypothesis that the ER stress response plays an important role integrating protein and lipid metabolism but also coordinating protein and lipid reserves during seed formation (Shank et al. 2001). The regulatory elements of the signalling ki-

nase:endoribonuclease (IRE1) and transcriptional regulation (XBP1) leading to the induction of PtdCho synthesis and ER biogenesis in fibroblasts (Sriburi et al. 2004) constitute a possible portal for understanding plant membrane biogenesis.

Sequencing of the Arabidopsis genome has led to the annotation of a large number of putative ER lipid genes (Beisson et al. 2003), the majority of which belong to gene families, allowing for the possibility of differential expression and additional levels of cellular regulation of the synthesis of specific lipids or pathways. Therefore, much remains to be done to characterise the biochemical activities of the enzymes and in the assignment of biological functions of individual lipid genes within the growth and development of the whole plant.

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Oleosins and Endoplasmic Reticulum in Seeds and Anthers

Anthony H. C. Huang

Center for Plant Cell Biology, Department of Botany and Plant Sciences,
University of California, Riverside, CA 92521, USA
Anthony.Huang@UCR.edu

Abstract Three types of related subcellular oil-rich particles are present in plants: storage oil bodies in seeds for gluconeogenesis during germination, storage oil bodies in pollen providing acyl moieties for membrane synthesis in the pollen tube, and tapetosomes in the anther tapetum for delivering lipids and proteins to the maturing pollen surface. Each of these oil-rich particles has a basic structure of an oil body, which consists of a triacylglycerol matrix enclosed by a layer of phospholipids and the structural protein oleosins. All components of an oil body are synthesized and assembled in endoplasmic reticulum (ER), from which a budding oil body is released. An oleosin molecule has a highly conserved central domain of 72 uninterrupted hydrophobic residues flanked by variable amphipathic *N*- and *C*-terminal segments. Its unique central domain is presumed to have evolved from a transmembrane segment of an ER protein. An oleosin molecule does not have an *N*-terminal ER-targeting signal and is targeted to the signal recognition particle and then ER via its central hydrophobic domain. Targeting studies of oleosin molecules that have been modified by adding a *N*-terminal ER-targeting signal, shortening the central hydrophobic stretch and eliminating the *N*- or *C*-terminal amphipathic stretch, have provided a model delineating the mechanism of oleosin targeting to ER and oil bodies. A tapetosome possesses numerous oleosin-coated oil bodies associated ionically with abundant membranous vesicles, both of which are assembled in and then detached from ER.

1 Introduction

Eukaryotes and prokaryotes contain neutral lipids in subcellular structures for food reserves and other purposes. These lipid particles are present in the seeds, flowers, pollen, and fruits of higher plants; the vegetative and reproductive organs of lower plants, algae, fungi, and nematodes; mammalian organs/tissues such as mammalian glands and adipose tissues; and bacteria. Oil bodies (OBs) in seeds are the most prominent and best studied of all of these lipid particles.

Seeds of most plant species store oils (triacylglycerols [TAGs]) as a food reserve for germination and postgerminative growth. The TAGs are present in small spherical OBs of approximately 0.5–1 μm in diameter. Each OB has a matrix of TAGs surrounded by a layer of phospholipids (PLs) and structural proteins termed oleosins. The small size of OBs provides a large surface

area per unit TAG, which facilitates lipase binding and lipolysis during germination. OBs inside the cells of mature seeds or in isolated preparations are highly stable and do not aggregate or coalesce. This stability is in contrast to the instability of artificial liposomes made from amphipathic and neutral lipids; the liposomes gradually coalesce after formation. Also, lipid particles in yeast and special mammalian cells, as well as extracellular lipoproteins in mammals and insects, are unstable because they undergo dynamic metabolic fluxes of their surface and matrix constituents. Seed OBs are stable because their surface is shielded by a layer of oleosins, which, for firm anchorage, has a long hydrophobic stretch that is absent in proteins on lipid particles of other organisms. In maturing seeds, TAGs, PLs and oleosins are synthesized in endoplasmic reticulum (ER), from which budding OBs are released.

Research into seed OBs and oleosins has been reviewed in the past by Huang (1992; which reviewed earlier literature), Frandsen et al. (2001), Galili et al. (1998), Hsieh and Huang (2004), Murphy (2001) and Napier et al. (1996). This article emphasizes recent studies, including the findings of a novel organelle, the tapetosome, in the tapetum cells of floral anthers.

2
Distribution and Structure of Oleosins

Oleosins in seeds are small proteins of about 15–26 kDa. They completely cover the surface of a subcellular OB (Fig. 1). They can be abundant in seeds with a high proportion of oils and small OBs (therefore more OB surface area). For example, *Arabidopsis* seeds have more than 40% (wt/wt) oils and small OBs of $\sim 0.5\ \mu m$ diameter, and 10% of the seed proteins are oleosins.

More than 200 genes encoding oleosins have been identified, and oleosins are restricted to plants alone. Recently, genes encoding oleosins on the storage OBs in Arabidopsis pollen (Kim et al. 2002) and tropical cacao seeds (Guilloteau et al. 2003) have been described. These findings negate a proposal of an alternative subcellular mechanism for stabilizing the OBs in pollen and another proposal explaining the short lifespan of tropical seeds such as cacao on the basis of their having unstable OBs. The transcript of a gene encoding an oleosin in the moss *Physcomitrella* can be found in an EST database; this is the most primitive plant known to contain oleosins. Whether algae contain oleosins is not known. Arabidopsis has 17 genes encoding oleosins: nine (eight in tandem) on chromosome 5 that are active in the tapetum cells, five active in seeds, and three active in both seeds and pollen (Kim et al. 2002). Minor proteins present in isolated OB fractions of some seeds have been termed caleosin and steroleosin (Frandsen et al. 2001). They do not have a long hydrophobic sequence, even though they have a short sequence similar to, but much less conserved than, the proline knot sequence in oleosins (to be described). Their mode of association with OBs should not be similar to that

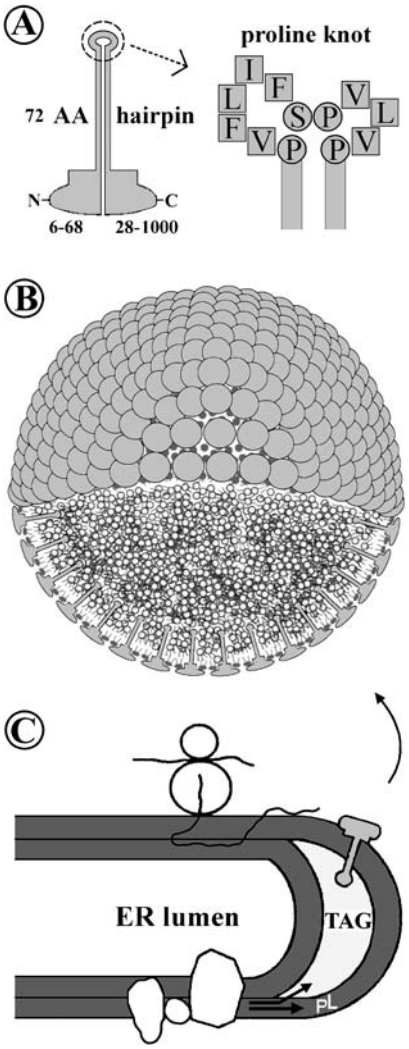

Fig. 1 Models of an oleosin molecule, a seed oil body, and the synthesis of an oil body on endoplasmic reticulum. **a** The three portions of an oleosin molecule, showing the *N*-terminal hydrophilic/amphipathic portion, the central hydrophobic hairpin (and residues at the turn, including the proline knot of three proline residues and one serine residue), and the *C*-terminal hydrophilic/amphipathic portion. The number of residues and their ranges in the three portions in all 17 Arabidopsis oleosins are shown. **b** An OB having oleosins (*medium grey*) and PLs (*dark*) enclosing the matrix TAGs (*light grey*). All molecules are drawn approximately to scale, whereas the diameter of the OB has been reduced 24 times to magnify the surface structure. **c** A budding OB being produced on RER. The ER lumen, the two PL layers (*dark*), the sequestered TAGs (*light grey*) in a budding OB, a ribosome with an mRNA synthesizing an oleosin polypeptide (*dark line*, of an unknown configuration), and enzymes (*irregular circles*) for the synthesis of TAGs and PLs are shown (modified from Hsieh and Huang 2004)

of oleosins, and the possibility of their being contaminants or remnants of ER after biogenesis needs to be explored.

An oleosin molecule can be divided into three portions according to its amino acid sequence (Fig. 1a). The *N*-terminal portion can be short or long (e.g., 6–68 residues in Arabidopsis) and hydrophilic or amphipathic. The central portion is a long hydrophobic stretch of 72 residues. The *C*-terminal portion can be short or very long (e.g., 28–1000 residues in Arabidopsis oleosins), and its ~ 30 residues adjacent to the central hydrophobic stretch can form an amphipathic α-helical structure that interacts horizontally with the charged phosphate and choline groups of the PL layer on the OB surface. The *C*-terminal portions of most Arabidopsis oleosins consist of fewer than 100 residues; a few have 100–150 residues; one has 403; and another has 1000 residues. Each of the longer *C*-terminal portions contains many repeats of short peptides, which are not conserved among oleosins and may not have any functional significance. Some of the repeated short peptides have several glycine residues. Oleosins in the tapetum are sometimes called glycine-rich proteins, although such a terminology does not describe the important characteristics of the proteins.

The central hydrophobic stretch of 72 uninterrupted hydrophobic residues is the hallmark of an oleosin. No other protein in any organism has such a long, or even half as long, hydrophobic stretch. Proteins on the surface of extracellular or intracellular lipid droplets, such as apolipoproteins, perilipin, adipophilin, and caveolin in mammals, phasin in bacteria and lipid-associated protein (PAP) in plastids, do not have such a long hydrophobic stretch; their polypeptides run parallel to the surface of the lipid droplets rather than penetrate into them. The 72-residue hydrophobic stretch of an oleosin is long enough (a transmembrane [PL bilayer] peptide has 20–25 residues) to form a hairpin that penetrates the surface PL monolayer of an OB into the matrix (Fig. 1b).

The center of the hydrophobic stretch has three proline residues and one serine residue that could interact to form a "proline knot" (Fig. 1a). This proposal is based on the presence of the less hydrophobic proline and serine residues among the other more hydrophobic residues and TAGs in the matrix of an OB, as well as on the fact that proline residues on polypeptides are breakers or turners of α-helical and β-structures. The formation of the proline knot could permit creation of a hairpin structure of the whole hydrophobic stretch, with two arms of 30 residues joined by a turn of 12 residues. The 72 residues of the hydrophobic stretch are conserved in terms of hydrophobicity among oleosins of diverse species, and the conservation is higher at the proline knot and its immediate vicinity ($-\,PX_5SPX_3P\,-$). All oleosins contain the three proline and one serine residues at identical locations in the center of the hydrophobic stretch.

All researchers agree that the central hydrophobic stretch forms a hairpin structure with a proline knot at the turn, but disagree on the secondary struc-

tures of the two hairpin arms. Earlier, it was proposed that the two arms have an antiparallel α-helical structure on the basis of an algorithm prediction (actually no database for predicting secondary structures of polypeptides in a hydrophobic environment exists) or an antiparallel β-structure on the basis of symmetry of residues between the two arms (Huang 1992). If the two arms had an antiparallel β-structure, they could bend at several locations where pairing of small glycine residues occurs; the bending would create more interactions among residues and thus offer higher stability. Two laboratories used circular dichroism and Fourier transform infrared spectroscopy to determine the secondary structures of the oleosin hairpin in its imitated native conditions. They arrived at the opposite conclusions of either an α-helical structure (Alexander et al. 2002) or β-structure (Li et al. 2002). The controversy underlines the difficulties in measuring the uniquely long hydrophobic polypeptide in a neutral-lipid environment. In addition, the arms or even the turn could interact with those in adjacent oleosins in the OB matrix. Such interactions could provide higher stability to the oleosin hairpin, in which the peptide bonds are relatively hydrophilic, in the hydrophobic environment. In maize, oleosins of two isoforms coexist in a 1 : 1 ratio, and an interaction between the pair is likely (Lee and Huang 1996). Furthermore, the *N*- and *C*-terminal portions of an oleosin, even though on the OB surface, may play a role in maintaining the hairpin in a special configuration. The *N*- and *C*-terminal portions of an oleosin on the surface of a seed OB may act as receptors for the binding of lipase during germination. This possibility can be tested by using a seed lipase protein derived from a cloned lipase gene, the cloning being facilitated by currently available genomics and proteomics technologies.

3
Synthesis of Oils, Oleosins, and Oil Bodies in Endoplasmic Reticulum

Oil bodies, including their constituent TAGs, PLs, and oleosins, are synthesized on ER. Diacylglycerol acyltransferase (DAG AT), the last enzyme and the only one unique to the synthesis of TAGs, as well as enzymes for the synthesis of precursor DAGs and PLs, are associated with rough ER (RER). An alternative TAG-synthesizing enzyme, which can transfer the acyl moiety from PLs instead of acyl-CoA to DAG, is also located in ER. The presence of these enzymes in ER is not surprising in view of the hydrophobicity of TAG and its metabolic precursors. TAGs synthesized in ER are sequestered in the hydrophobic region (i.e., the acyl region of the PL bilayer). Continuation of TAG accumulation at a domain of ER forms a budding OB, which is enclosed by a single layer of PLs (Fig. 1c). This budding OB, covered with a PL monolayer, is stabilized by inclusion of oleosins to its surface.

Ribosome-mRNA with a nascent oleosin peptide can be guided to ER via the signal-recognition particle (SRP) pathway. mRNA for synthesis of oleosin

is associated with RER. Translation of oleosin mRNA in an in vitro synthesis system is retarded or enhanced, respectively, when SRP or microsomes are added (Abell et al. 2002; Beaudoin and Napier 2002; Loer and Herman 1993; Thoyts et al. 1995). The findings suggest that the translation of oleosin mRNA pauses after binding of SRP to the nascent peptide and accelerates when the newly synthesized oleosins incorporate into ER. In addition, stable incorporation of in vitro-synthesized oleosin (commercial in vitro synthesis systems usually contain SRP) into microsomes is inhibited when the SRP receptor (SRP-60) on microsomes is removed beforehand through proteolysis. This inhibition can be restored with reconstituted SRP receptor. Yeast transformed with an oleosin gene synthesizes and targets oleosin to OBs (Ting et al. 1997). When the transformed yeast strains are mutants defective in SRP components, oleosin is not targeted to OBs, and the nontargeted oleosin is proteolytically degraded (Beaudoin et al. 2000).

Targeting of oleosin to ER in vitro can occur with the use of SRP components and microsomes from yeast, mammals, or plants. Thus the unique aspect of the targeting mechanism is the targeting signal(s) in the oleosin molecule. Modified oleosins produced via gene recombination can be tested for their stable insertion into microsomes in vitro or into ER in vivo (Abell et al. 1997, 2002; Beaudoin and Napier 2002). The *N*- and *C*-terminal portions are relatively unimportant in targeting oleosin to ER. Rather, the long hydrophobic stretch of oleosin is the predominant factor for targeting. No specific signal sequence in the hydrophobic stretch is required. Instead, any of the multiple and probably overlapping sequences along the hydrophobic stretch can target the protein to ER. Significantly, the highly conserved proline knot is not important for the in vitro targeting of oleosin to microsomes, because replacement of the three proline residues with leucine residues does not affect the targeting. The finding that multiple peptides along the hydrophobic stretch can be the targeting signals is consistent with the knowledge that the hydrophobic pocket of an SRP can recognize a diverse array of hydrophobic ER-targeting peptides at the *N* termini or interior of many proteins.

The nascent oleosin polypeptide synthesized or being synthesized on ER assumes a topology on the basis of its hydrophobic and hydrophilic interactions with the PL bilayer. The hydrophilic/amphipathic *N*- and *C*-terminal portions interact with the PL layers on the cytosolic side of ER (Fig. 1c), whereby the central hydrophobic stretch buries itself in the hydrophobic acyl portion of the PL bilayer. Much evidence from in vivo and in vitro experiments exists for such a topology for the nascent oleosin (Abell et al. 1997, 2002; Beaudoin et al. 2002). The *N*- and *C*-terminal portions, but not the hydrophobic stretch, of oleosin in isolated microsomes are susceptible to proteolysis by exogenously added proteases; this observation is similar to that of oleosins on mature OBs. The secondary structure of the 72-residue hydrophobic stretch in the hydrophobic portion of the PL bilayer is unknown but likely differs from that in a mature OB. The matrix of a mature OB, but not the hy-

drophobic region of ER, provides an excess of hydrophobic volume for the hydrophobic stretch to assume its presumably most stable hairpin configuration. The hydrophobic stretch of oleosin within the hydrophobic region of ER could assume a bended hairpin structure or an extended structure with or without coiling, running parallel to the PL bilayer (Fig. 1c). An additional consideration is the actual thickness of the hydrophobic region of the PL bilayer. While ER is synthesizing oleosins, it also produces massive amounts of TAGs, which will be temporarily sequestered in, and thus enlarge, the hydrophobic region of the PL bilayer. Thus, the hydrophobic region of the PL bilayer may have more room for the hydrophobic stretch of a nascent oleosin than that defined by the length of the two acyl chains.

Both the newly synthesized oleosins and the temporarily located TAGs on ER diffuse to budding OBs. This movement is made possible in accordance with the fluid mosaic model of membrane action and thermodynamic considerations. TAGs and the oleosins will be more stable in the hydrophobic environment of a budding OB. A native oleosin stably inserted into ER diffuses to the budding OB, but a stably inserted, artificially modified oleosin may not. The mechanism of this oleosin movement has been studied in vivo through using modified oleosins and measurements of oleosins recovered in ER and OB fractions (Abell et al. 1997, 2002, 2004; Beaudoin and Napier 2002). Strictly speaking, this approach measures not just targeting success per se, but also the stability of the modified oleosins in OBs. Modified oleosins that can diffuse to the budding OBs may be unstable there and removed by endogenous proteolysis. The molecular requirements for oleosin to diffuse successfully to, and incorporate stably into, OBs are similar to those for targeting the protein to ER; however, more are required. The proline knot in the hydrophobic stretch is also essential, presumably for stable anchoring of oleosin on OBs. A modified oleosin without the proline knot (e.g., having the three proline residues replaced with leucine residues) can probably insert into ER and also diffuse to the budding OB but would be unstable there and thus eliminated by endogenous proteolysis. In addition to the need for the proline knot, decreased length or elimination of the *N*- or *C*-terminal portions or decreased length of the hydrophobic stretch all lead to a reduced recovery of the modified oleosin in OBs.

Oleosins must be only on the cytosolic side of ER to be able to diffuse to the budding OB. Attempts to insert the whole oleosin molecule into the luminal side of ER have been unsuccessful. An *N*-terminal ER targeting peptide from a nonoleosin protein attached to the *N* terminus of an oleosin, produced via gene recombination, can pull the *N*-terminal portion of the oleosin but not the hydrophobic stretch (with or without the *C*-terminal portion) into the ER lumen (Abell et al. 2002, 2004). Apparently, the hydrophobic interaction between the long hydrophobic stretch and the acyl moieties of the PL bilayer (with or without the added hydrophilic interaction between the *C*-terminal portion and the PL layer on the cytosolic side) is too strong for the oleosin

to leave the PL bilayer and insert into the lumen. This modified oleosin can incorporate into ER but cannot insert into the budding OB. Obviously, its polypeptide spanning across the whole PL bilayer of ER cannot diffuse to the PL monolayer of a budding OB (Fig. 1c). Even if it could, it would be unstable there.

It is uncertain whether a ribosome-mRNA-oleosin complex can target to ER or the budding OB directly without involvement of the SRP pathway. All the evidence from in vitro experiments shows that the SRP system is involved. A ribosome-mRNA-oleosin complex with the hydrophobic stretch dangling outward in vitro could bind to the hydrophobic pocket of an added SRP, regardless of whether SRP is actually involved in vivo. Certainly, it has been shown that oleosin synthesized in vitro cannot insert into mature OBs co- or posttranslationally (Hills et al. 1993). However, a mature OB is packed with oleosins on its surface and has no extra room for new oleosins. Oleosin synthesized in vitro can insert into artificial OBs whose surface has not been filled completely with oleosins (Chen and Tzen 2001). A ribosome-mRNA-oleosin complex with the hydrophobic stretch dangling outward could theoretically bind to the hydrophobic region of ER or a budding OB whose surface had not been filled completely with oleosins. Nevertheless, the strongest evidence for the need of SRP to guide oleosin to ER has come from in vivo studies with yeast mutants defective in SRP components (Beaudoin et al. 2000). This finding with yeast should be tested with plants. Further, whether oleosin synthesis employs both the SRP system and a direct insertion mechanism has not been evaluated.

As newly synthesized TAGs and oleosins on ER diffuse to and converge at the budding OB, a gradient of enrichment of these two components should exist from the point of synthesis to the budding OB. This concentration gradient can explain the immunocytochemical observation that more oleosins are present in the ER near the budding OBs (Herman 1987). Whether subdomains of ER for TAG and oleosin synthesis are present remains to be documented. In an in vitro study, sunflower seed microsomes supplied with precursors synthesized TAGs and, after this synthesis, were subfractionated by density gradient centrifugation (Lacey et al. 1999). The fraction with the lowest buoyant density contained more TAG, oleosin, and lipid synthesis activity on a per fraction basis. This fraction may represent ER subdomains specialized for TAG and oleosin synthesis, or simply fragments of ER regions originally closest to the budding OBs and thus having more TAGs and a lower buoyant density. In an earlier experiment, when an extract of maturing maize kernel was subfractionated by density gradient centrifugation, DAG AT, the last and unique enzyme for TAG synthesis, was found with cytochrome reductase in RER fragments of diverse buoyant densities (Cao and Huang 1986). The DAG AT was not concentrated in ER fragments with the lowest buoyant densities and therefore most TAGs (or fewest polysomes). Thus, in the maize cells, TAGs are probably synthesized in diverse regions of ER and diffuse to the budding OBs. In the tapetum in

Brassica anthers, oleosin-coated oil droplets are structural analogs of seed oil bodies (see Sect. 4). During synthesis of these tapetum oil droplets, oleosin and the ER chaperone calreticulin were colocalized in extensive regions of the ER network, as seen in situ by immunofluorescence microscopy. Thus, the tapetum oleosins, and perhaps TAGs also, are synthesized in diverse regions of ER rather than in highly restricted ER subdomains.

A budding OB is released from ER as a solitary oil body (Fig. 1c). An early release will generate a smaller OB, and vice versa. The size and shape of an OB are determined in part or completely by the relative amount or rate of synthesis of oils and oleosins. High-oil maize kernels (having a high oil-to-oleosin ratio) generated by breeding have large, spherical OBs, whereas low-oil kernels have small OBs with irregularly shaped surface (Ting et al. 1996). In cells that do not synthesize oleosins, such as those in the fatty mesocarp of fruits, the OBs (lipid globules) become very large (see next paragraph). A special mechanism may exist for the physical release of a budding OB from ER. Oleosins accumulated on the bud surface may interact among themselves to produce a physical force of constriction at the neck of the bud, thereby releasing the OB. Or, the physical release may require specific cytosolic proteins (e.g., dynamins). These possibilities can be tested by screening for Arabidopsis mutants whose seeds have larger or smaller OBs or only budding OBs viewed with a light microscope after lipid staining. Some of these mutants may be defective in the mechanism for physical release of OBs from ER.

In the fatty mesocarp of fruits such as avocado, oil palm, and olive, each cell has one to several large lipid globules, which occupy the bulk of the cell volume. Little or no oleosins are present on these lipid globules. Mesocarp lipids are for attracting animals and serve for seed dispersion and thus are not required to be in small entities such as seed OBs. Mostly likely, TAGs are synthesized in ER, as in seeds, but without a cosynthesis of oleosins (Fig. 1c). As a consequence, the budding OB enclosed only by PLs becomes larger (and/or fuses with adjacent budding OBs) before it is released from ER. This is equivalent to the synthesis of larger OBs in maize kernels having a high oil-to-oleosin ratio. It is possible that the mesocarp cells can be modified to synthesize small OBs instead of large lipid globules if oleosin is allowed to be cosynthesized with TAGs via genetic engineering. Although in theory this genetic engineering project can be easily achieved, in practice it is difficult because the better-known avocado, oil palm, and olive that contain fatty mesocarp are tree crops.

4
Oleosins in Tapetum Cells and the Novel Organelle, Tapetosome

The presence of oleosins in tapetum cells of anthers in Arabidopsis and Brassica was discovered a decade ago from unintended gene cloning results

(deOliverira et al. 1993; Roberts et al. 1994). The finding was unexpected because tapetum cells were not known to contain OBs similar to those in seeds. Subsequently, these oleosins were found to be present in a novel, neutral lipid-containing organelle, which has been termed the tapetosome because of its unique presence in the tapetum of plants (Wu et al. 1997). To date, the presence of tapetal oleosins is limited to species of the insect/self-pollinating Brassicaceae family, especially Brassica and Arabidopsis.

In Arabidopsis, nine genes encode the tapetal oleosins, eight of which are in tandem on chromosome 5 (Fiebig et al. 2004; Kim et al. 2002; Schein et al. 2004). One of these genes is highly expressed to produce an oleosin of 53 kDa, which represents about 70% of all tapetal oleosins. Most of the other Arabidopsis tapetal oleosins are smaller (10–23 kDa), but one has 115 kDa. As expected, Brassica has a similar oleosin gene system (Roberts et al. 1994; Ross and Murphy 1996; Ruiter et al. 1997), and the most active gene (ortholog of the Arabidopsis gene encoding the 53-kDa oleosin) produces a major oleosin of 45 or 48 kDa (from the *B. rapa* AA genome or *B. oleracea* CC genome, respectively). Genes encoding the tapetal oleosins have undergone rapid evolution that altered the *N*- and *C*-terminal regions but not the hairpin regions, as the genes encoding seed oleosins do. Findings of these evolutionary changes reiterate that the *N*- and *C*-terminal regions of oleosins have minimal constraints for protein structures, and thus functions.

The tapetum is a one-cell layer enclosing the anther locule, in which microspores mature to become pollen. Tapetum cells are the only anther sporophytic cells that are metabolically very active and control maturation of microspores. At an early stage of anther development, the tapetum cells are specialized for active secretion and contain abundant RER and secretory vesicles. At a late stage of anther development, at least in Brassicaceae species, the cells become a temporary storehouse of ingredients to be deposited onto maturing pollen as pollen coat. The tapetum cells at this late stage of development are packed with two predominant storage organelles, the elaioplasts and tapetosomes (Owen and Makaroff 1995; Platt et al. 1998; Polowick and Sawhney 1990). The elaioplasts, of 3–4 μm in diameter, are specialized plastids largely devoid of thylakoids but filled with small spherical lipid droplets of steryl esters enclosed by the structural protein PAP. Although elaioplasts of similar morphology can be found in nontapetum cells, such as fruit and petal cells, tapetosomes are unique to the tapetum cells. Each spherical tapetosome, of 2–3 μm in diameter, has oleosin-coated TAG droplets associated with vesicles derived from ER. These oleosin-coated TAG droplets are similar in structure and constituents to seed OBs.

The contents of tapetosomes and elaioplasts are selectively retained and discharged to the anther locule after death of the tapetum cells during the final stage of anther development. Oleosins, but not TAGs, of tapetosomes and steryl esters, and not the structural protein PAP of elaioplasts are selectively retained and transferred to the pollen surface, forming the bulk of pollen coat

(Wu et al. 1997, 1999). The rationale and mechanism for the selectivity are unclear. It is intriguing that in seed OBs, TAGs are the prime ingredient for physiological function and oleosins are the accessories, whereas in tapetosomes, oleosins may be the main element for physiological function (to be described) and TAGs are the accessories. The tapetum TAGs disappear after death of the cells, and their function and metabolic fate are unknown. They may be used as an energy source for active metabolism of the tapetum cells. Their fatty acids could also be used to produce jasmonic acid as a floral maturation hormone, or alkanes as one of the two major lipid constituents (the other being the elaioplast steryl esters) for deposition onto maturing pollen. These possibilities are testable with Arabidopsis mutants defective in tapetum TAG synthesis or degradation.

Although the steryl esters and other lipids on pollen form a useful waterproofing layer, the function of the abundant oleosins there is less clear. In Brassica, the predominant 45/48-kDa oleosin on pollen has been cleaved selectively into two fragments, one containing the *N*-terminal portion and the central hydrophobic stretch, and the other the long hydrophilic *C*-terminal portion (Ross and Murphy 1996; Ting et al. 1998). Whether other smaller oleosins on pollen are cleaved is not known. The cleavage may be fortuitous in mutation and have no physiological relevance. The most abundant oleosin on pollen has a large size (53 kDa in Arabidopsis and 45/48 kDa in Brassica) owing to its possession of numerous repeats of short peptides at its *C* terminus. Each of these repeats possesses several glycine residues, which again makes the protein glycine-rich.

Because of its glycine-rich nature, it has been speculated that this pollen oleosin (and extrapolating to other oleosins) might interact the cell walls of the stigma. Such a speculation should be taken with caution. Oleosins have undergone rapid evolutionary changes, and both tapetal and seed oleosins have repeats of short peptides at their *C* termini; some of these repeats have high glycine contents, whereas others do not. The rapidity and extensiveness of changes at the *C* termini may reflect the minimal structural constraints on this part of the protein to perform functions. The high glycine contents at the *C* termini of oleosins may be fortuitous, and certainly the glycine-rich *C* termini in some seed oleosins do not have an apparent function for interaction with cell walls. In fact, the short repeats at *C* termini of the most abundant tapetum oleosins have not only a high glycine content but also high serine and lysine contents, making the oleosin also serine-rich and lysine-rich (the Arabidopsis 53-kDa oleosin has 26, 16, and 14 mol %, and the Brassica 48-kDa oleosin 21, 16, and 11 mol % of glycine, serine, and lysine, respectively).

An oleosin molecule may serve dual functions on pollen and subsequently on the stigma because of its amphipathic property. Its *N*- and *C*-terminal portions are hydrophilic/amphipathic, and its central portion is hydrophobic. The overall amphipathic oleosin can act as an emulsifying agent to uniformly

coat the pollen with steryl esters, alkanes, flavonoids, and other ingredients. It may also aid in water uptake for germination after the pollen grain has landed on the stigma. Brassicaceae species have dry stigmas, and water must be drawn from the stigma interior to the pollen for germination and tube growth. Steryl esters and other neutral lipids are not amphipathic enough to be able to act as a wick. However, the abundant and amphipathic oleosins (and/or flavonoids) could act in this manner. On the basis of these two proposed functions, the mutational addition of repeats of short peptides, which are all fairly hydrophilic, to the *C* termini and fragmentation of the Brassica 45/48-kDa oleosins into two halves do not affect the function of the oleosins. The proposed functions are also in agreement with the observation that the pollen of an Arabidopsis null mutant in the major pollen-coat oleosin does not hydrate efficiently on the stigma (Mayfield and Preuss 2000). This partial loss of function could have been due to the lack of sufficient oleosins on the pollen to serve as a wick and/or the pollen coat not having been properly emulsified. Overall, the major structural constraints on oleosins to perform the proposed functions are a long hydrophobic stretch to interact with the TAG droplets in tapetosomes (not a function per se but for storage in the organelles) and an amphipathic molecule to emulsify the pollen coat materials and take up water from stigma. All the observed mutational changes on tapetal oleosins have not affected these constraints and are thus extensive because of the lack of selective pressure.

Tapetosomes have a unique morphology (Platt et al. 1998; Wu et al. 1997). Transmission electron microscopy has revealed that in situ each tapetosome has a nonhomogeneous interior whose internal structures cannot be recognized. However, these structures can be observed clearly after the tapetosomes has been isolated and subjected to osmotic swelling. A tapetosome consists of oleosin-coated TAG droplets associated via ionic linkage with ER-derived vesicles (Fig. 2). Isolated tapetosomes, after a high- or low-pH treatment, can be subfractionated into TAG droplets (which contain oleosins and TAGs), and membranous vesicles (which possess ER-derived calreticulin and luminal binding protein).

Tapetosomes are synthesized via a special mechanism, as revealed in a recent study with immunofluorescence microscopy and transmission electron microscopy (Hsieh and Huang 2005). During early development of a tapetum cell, the ER luminal protein calreticulin exists as a network, and contains no oleosins. Subsequently, oleosins appear together with calreticulin in the ER network, which possesses centers with a high ratio of oleosin to calreticulin. Transmission electron microscopy shows that at this stage massive ER cisternae interconnect the numerous maturing tapetosomes in a cell. Finally, the ER network largely disappears, and solitary tapetosomes containing oleosins and calreticulin prevail. These and other (Platt et al. 1998) microscopical studies, along with findings from subcellular fractionation, allow for a model depicting the biogenesis of tapetosomes from RER (Fig. 2). Initially,

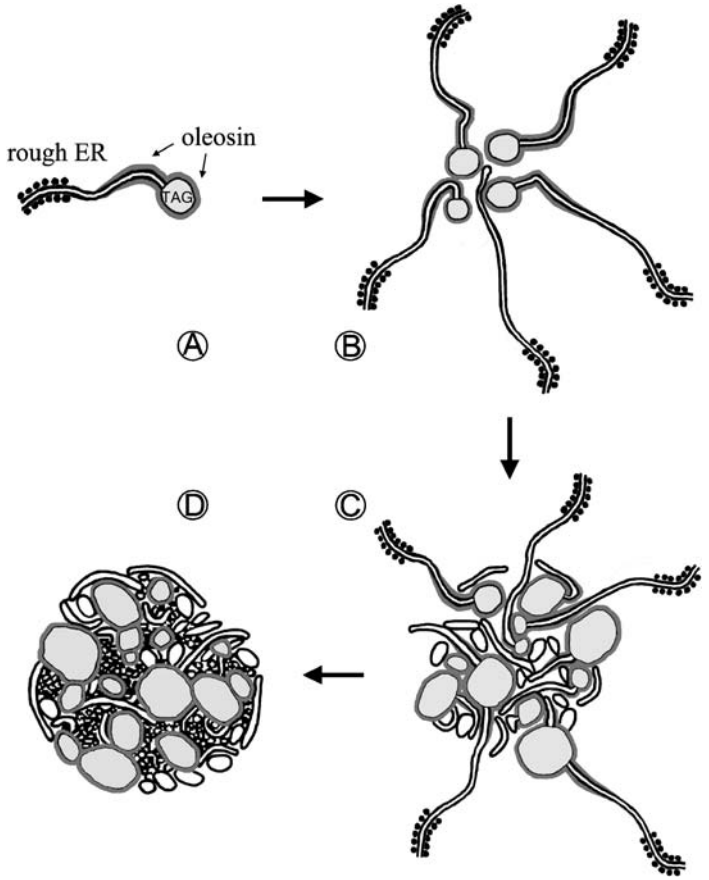

Fig. 2 Model for the synthesis of a tapetosome in Brassica tapetum cells. **a** formation of an oleosin-coated oil droplet from RER by a mechanism similar to that in Fig. 1c. Each oil droplet consists of an oil matrix (*light grey*) enclosed by a layer of PL (*dark*) and oleosins (*medium grey*). **b** Association of several budding oil droplets and ER cisternae. **c** A maturing tapetosome containing detached ER vesicles. **d** A mature tapetosome (modified from Hsieh and Huang 2004)

TAG droplets are produced via an ER-budding mechanism identical to that in maturing seeds. These TAG droplets are covered by oleosins and PLs. As many are produced they converge. More ER cisternae are connected to the droplet clusters and eventually break off as vesicles. As a consequence, a tapetosome is formed. During the peak period of tapetosome formation, all the maturing tapetosomes in the cell are interconnected via ER cisternae.

The function of the abundant ER-derived vesicles in tapetosomes remains to be elucidated. These vesicles possess the same basic constituents of calreticulin and luminal binding protein as the ER cisternae do. The vesicles in tapetosomes may aid in the transfer of oleosins from lysed tapetum cells to

the pollen surface. They may possess proteins that would subsequently exert action on the stigma, such as incompatibility factors and other signaling proteins. They may contain ions such as calcium and boron for the pollen surface; these ions would subsequently modulate the cell wall structures of the stigma. Or, they may contain flavonoids and other secondary metabolites for the pollen surface. Such pollen-surface metabolites are well known, but of uncertain function. Subcellular fractionation and modern microscopy should be used to test the presence of these ingredients in the tapetosome vesicles.

Future studies on the tapetal oleosins and tapetosomes should aim at expanding the existing findings to non-Brassicaceae species, pinpointing the roles of oleosins on pollen, and examining the contents of the ER-derived vesicles in tapetosomes. Working hypotheses exist and are testable. In addition, use of Arabidopsis mutants defective in individual constituents will aid these tests.

5
Evolution of Oleosins, Oil Bodies, and Tapetosomes

Prokaryotes, in general, do not store TAGs as food reserves. A minor exception is *Actinomyces*, which produce TAGs under certain nutritional and other environmental conditions. TAGs were likely to have evolved as efficient food reserves in primitive eukaryotes by the addition of one enzyme, DAG AT, which was evolved from one of the existing acyltransferases. This enzyme diverted DAGs from the ubiquitous PL metabolic pathway to TAGs. Initially, the hydrophobic TAGs were present between the two PL layers of the ER membrane, where DAG AT was. Today, seeds of some species on occasions still have some TAGs present along the hydrophobic region of the PL bilayers in ER (Wanner et al. 1981). The presence of excess TAGs in the ER membrane would interfere with the normal functioning of ER. This problem was overcome by removal of the TAGs from ER via budding to become solitary droplets. The droplets, each containing a TAG matrix enclosed by a layer of PLs originated from ER, would be unstable. In yeasts, the droplets were made more stable through a coat of amphipathic proteins, especially the TAG synthesizing and hydrolyzing enzymes. The semistability would allow the droplets to undergo dynamic metabolic fluxes. In mammals, the droplets were modified to different forms with proteins and membranes, such that they were also semistable and amenable to metabolic fluxes. In plants, the droplets were stabilized by the evolutionary appearance of oleosins, whose long hydrophobic hairpin could stabilize the droplets so effectively that they were amenable to prolonged storage in desiccated seeds. Oleosins and their coated oil droplets have been found in diploid and triploid storage sporophytic cells of seeds of angiosperms and gymnosperms, haploid storage cells of female gametophytes (in seeds) in gymnosperms, haploid cells of male ga-

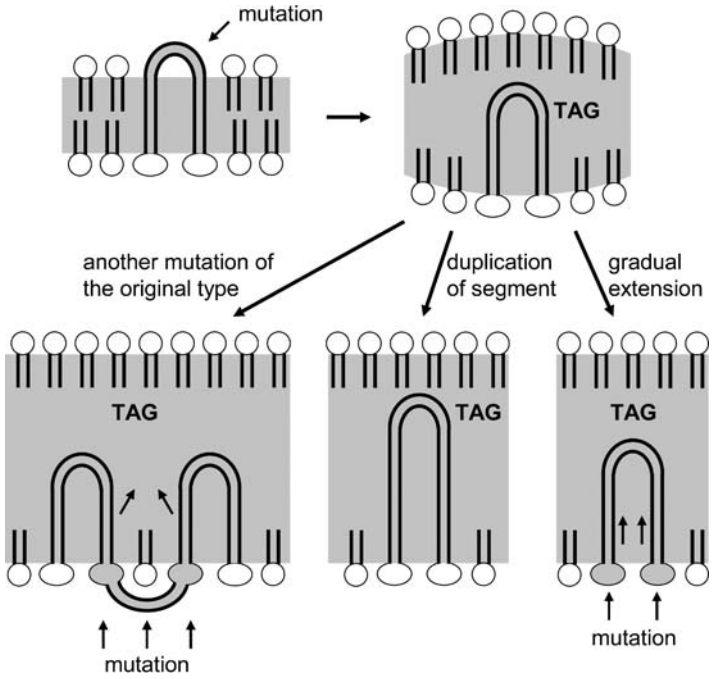

Fig. 3 Model for the evolution of oleosins. The 72-residue hydrophobic segment of an oleosin molecule was viewed as having evolved from the transmembrane segment of an ER protein. The shaded area and *thick lines* represent hydrophobic regions. These include the acyl moieties of PL (*two lines joining a circle*), TAGs, and the transmembrane, hydrophobic portion of an ER protein being evolved to an oleosin hairpin (*enclosed column*). *Unshaded circles* depict hydrophilic portions of PL and proteins (modified from Huang 1996)

metophytes (pollen), the moss *Physcomitrella* (possibly in diploid sporophyte or haploid gametophytes) and the diploid sporophytic cells of floral tapetum.

The hydrophobic stretch of 72 resides in oleosins is the longest, and is actually more than twice as long as any found in any prokaryotic or eukaryotic protein. The mechanism by which it has evolved is intriguing. A hypothesis has been proposed (Fig. 3) on the basis of the following observations (Huang 1996):

1. The length of 72 residues is about four times that of a transmembrane polypeptide ($\sim$ 20 residues)
2. Several relatively hydrophilic residues are present in the middle of both antiparallel stretches
3. A certain degree of residue symmetry exists along the two antiparallel stretches

The hypothesis depicts that the long hydrophobic stretch has evolved from duplications of a transmembrane peptide of an ER protein in a primitive plant or alga. The hypothesis can be tested by comparing the amino acid sequences

of the oleosin hairpins (and the corresponding nucleotide sequences) with those of transmembrane segments of proteins, especially of ER, in the most primitive organisms (currently, the moss *Physcomitrella*). Whereas the hairpin hydrophobic stretch is conserved, the *N*- and *C*-terminal portions have undergone extensive evolutionary changes because of limited structural and functional constraints.

Oleosin-coated oil droplets in diverse plant species can be categorized into two groups according to their structures and functions. The solitary oleosin-coated OBs in seeds and pollen store TAGs for germination and postgerminative growth in the respective organs. The tapetosomes contain clustered oleosin-coated oil droplets associated with ER-derived vesicles and store and deliver materials to the surface of maturing pollen. Whether, during evolution, solitary oleosin-coated oil droplets similar to the modern seed OBs appeared before the complex tapetosomes, or vice versa, is a matter for speculation. The most primitive plant known to contain oleosin is the moss *Physcomitrella*. The moss oleosin is presumably associated with storage OBs in the sporophyte or gametophytes. The moss does not have flowers or tapetum and thus would not have analogs of tapetosomes. Brassicaceae species contain abundant tapetosomes in tapetum, whereas the maize tapetum does not have any. Thus, tapetosomes were likely to have evolved from solitary oleosin-coated TAG droplets similar to the modern OBs in seeds. Initially, these ancestral droplets, solitary or in groups, in tapetum delivered oleosins to the pollen surface. Subsequently, they became associated with vesicles that also contain materials for the pollen surface. Thus, tapetosomes are thought to have evolved to perform the overall function of packaging and storing materials for delivery to the pollen surface.

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Participation of the Plant ER in Peroxisomal Biogenesis

Richard N. Trelease (✉) · Matthew J. Lingard

School of Life Sciences, Arizona State University, PO Box 874501,
Tempe, AZ 85287-4501, USA
trelease.dick@asu.edu

Abstract Diverse and compelling evidence is presented in support of the participation of the ER in the biogenesis of different kinds of plant peroxisomes. New and previous data coupled with interpretations and opinions are embedded within four multistep peroxisome assembly models derived from studies with diverse organisms. The main objective of this Chapter is to compare and contrast the varied involvement of the ER in the biogenesis of peroxisomes within the context of the four general models for peroxisome origination, assembly, maturation, and replication. Two of the models depict a unique participation of the ER in the origin and subsequent maturation of nascent *pre-peroxisomes*. In the third *autonomous* model, the ER is not involved, whereas in the fourth *semi-autonomous* model, ER-derived vesicles contribute to the maturation/differentiation and replication of *Pre-existing peroxisome*. The semi-autonomous model pertains to the biogenesis of plant peroxisomes. Within this scheme, a subset of peroxisomal membrane proteins (PMPs), collectively called group I proteins, e.g. peroxin 16 and ascorbate peroxidase, are synthesized in the cytosol and trafficked *indirectly* to peroxisomes via ER-derived vesicles. Interestingly, current evidence does not predict the origin of new plant peroxisomes directly from domains of the ER. Instead, mature pre-existing peroxisomes apparently replicate via constitutive duplication (fission) in response to the action of one or more isoforms of a peroxin homolog designated as peroxin 11. Nascent daughter organelles acquire membrane phospholipids and PMPs from ER-derived vesicles.

1 Introduction

The focus of this chapter is to examine via published data and intuitive reckoning the extent of ER participation in the biogenesis of plant peroxisomes. However, to do this effectively one needs to consider the breadth and variation of peroxisomal biogenesis in all organisms that have been examined. Within this context, we consider biogenesis in the broadest sense that includes all means of: (a) ontogeny/formation of nascent pre-peroxisomes, (b) maturation (elaboration/differentiation) of pre-peroxisomes and pre-existing peroxisomes, (c) constitutive duplication of pre-existing peroxisomes during normal cell division, and (d) induced proliferation of pre-existing peroxisomes independent of normal cell division. These aspects are included with varying emphases within the numerous recent reviews of peroxisome biogenesis in yeasts, mammals, and plants. Thus, rather than giving a comprehensive coverage of original articles, we have decided to cite these reviews.

Figure 1 is a comprehensive integration of the four generalized (main?) peroxisomal biogenesis models, which have been modified for simplicity of presentation. Conspicuous by their absence are the names and locations of specific membrane and matrix proteins (including names of specific peroxins). Our emphasis is on biogenetic events and trafficking pathways related more to acquisitions of membrane proteins and lipids rather than on the uptake of matrix proteins. In three of the schemes, the ER is shown as the origin of, or significant contributor to, new mature peroxisomes. We have borrowed some key terms from original descriptions and refer to these models in Fig. 1 as follows: the ER-lamellae peroxisome formation pathway (white–black arrows), the ER vesicle-fusion/maturation peroxisome formation/assembly pathway (white arrows), and the ER semi-autonomous peroxisomal growth and division pathway (black arrows). The latter pathway also includes constitutive division and the regulated/induced proliferation of new mature peroxisomes (black arrows). The reader should realize that similar constitutive division and induced proliferation of new mature peroxisomes should also be shown as extensions of the former two pathways (white–black and white arrows), and are not included for the sake of simplicity. The fourth pathway, referred to as the autonomous peroxisomal growth and division pathway (dashed lines), does not invoke participation of the ER. The source of membrane phospholipids in this pathway (discussed later) is portrayed as an elusive small *protoperoxisome*.

All four models portray multistep assembly pathways, which enlist a diverse array of membrane and matrix proteins. All of these proteins are synthesized from nuclear-encoded genes because peroxisomes do not possess their own DNA or protein-synthesizing machinery. Accordingly, all of the compartments shown in Fig. 1 acquire their membrane and matrix proteins post-translationally from the cytosol. Matrix proteins are typically imported *directly* into vesicles, pre-peroxisomes, pre-existing peroxisomes, etc. Details of the cellular and molecular mechanisms for matrix protein import are not described here, but are thoroughly discussed in other reviews (Baker and Sparkes 2005; Erdmann and Schliebs 2005; Mullen 2002). PMPs, including peroxins, enzymes, membrane transporters, etc. may be added from the cytosol *directly* or *indirectly* to the various peroxisome compartments. Indirect trafficking typically involves sorting of a subset of so called group I proteins (PMPs) through the ER to peroxisomes (Titorenko and Rachubinski 2001a,b). Group II PMPs bypass the ER and sort directly to peroxisomes. Even in recent reviews and original articles, indirect trafficking of PMPs through the ER has often been labeled controversial. This is based mostly on historical prejudices against ER involvement in peroxisome biogenesis. This bias is unfortunate because it is misleading; different PMPs may be added directly or indirectly. Interesting and pertinent questions are now whether all PMPs classified as group I or II PMPs in one organism are similarly classified in other organisms, and if not, why have

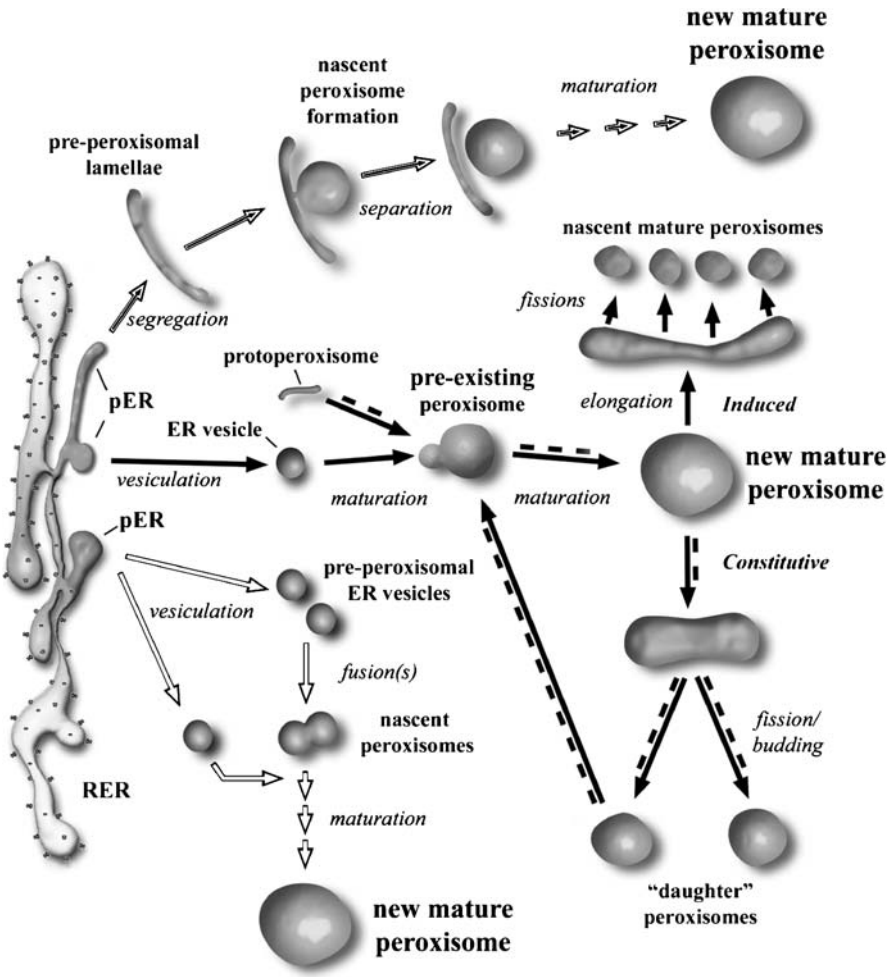

Fig. 1 multistep peroxisomal assembly models/pathways for different cell types and/or organisms. Each model includes a unique pathway that portrays the biogenesis of new mature peroxisomes. The following are names for each pathway that are used for exploring the involvement of the in ER peroxisomal biogenesis. *White–black arrows*—ER-lamellae peroxisome formation pathway; *White arrows*—ER vesicle-fusion/maturation peroxisome formation/assembly pathway; *Black arrows*—ER semi-autonomous peroxisomal growth and division: constitutive and regulated replication pathway; *Dashed lines*—Autonomous peroxisomal growth and division pathway. Note that each of the new mature peroxisomes depicted in each pathway are also perceived to undergo constitutive division and/or induced proliferation. This is not shown in all cases for simplicity. Details of individual events and features are described in the text. RER - rough ER

these differences evolved? Specific examples of group I and II plant PMPs are considered later.

A family of specialized proteins called peroxins are involved by definition in the various aspects of peroxisomal biogenesis. *PEX* genes were identified mostly in mutant studies with yeasts. The different peroxins are denoted as PexNp, which is preceded by two letters referring to their genus/species and are numbered consecutively in accordance with recommendations of a special committee (Distel et al. 1996), e.g. AtPex16p denotes *Arabidopsis thaliana* peroxin 16 protein. Approximately 23 *PEX* genes (17 kinds of peroxin homologs) have been identified in plants (mostly in Arabidopsis), whereas approximately 17 *PEX* genes (14 kinds of peroxin homologs) have been identified in mammals, and 34 in yeasts. Since Charlton and Lopez-Huertas (2002) describe them in detail, we do not attempt here to include descriptions of plant peroxin functions as ascribed to each aspect of peroxisomal biogenesis. Also, as mentioned above, Fig. 1 does not show any of the individual sites of peroxin uptake, or any other protein, within these multistep pathways. Protein import varies among the different model organisms, although certain important consistencies are known, especially relative to so-called early peroxins involved in the early stages of peroxisome origination/formation. In these cases, specific peroxins are described later in this chapter.

A knowledge and understanding of the participation of the ER in peroxisomal biogenesis in non-plant organisms is clearly a prerequisite for this chapter. Relatively few model organisms and systems have been studied extensively. These are mammals (mostly human culture cells) (Purdue and Lazarow 2001), yeasts (five different cellular species) (Veenhuis et al. 2000), trypanosomes (mostly *Trypanosomal brucei*) (Parsons et al. 2001), and the nematode *Caenorhabditis elegans* (Thieringer et al. 2003). However, little data and consequently few working models on peroxisomal biogenesis specifically focus on *plant* ER. Work on plants has been mostly with roots and leaves (cotyledons) of common (crop) species and suspension-cultured cells, namely tobacco BY-2 and Arabidopsis cells (Baker and Graham 2002).

A by-product of this low diversity in study systems is that research groups have generally focused on a particular species, or several species within a common group of organisms. Unfortunately, published work on plant cell organelles has not generally been included in discussions of data obtained on non-plant organisms. An undesirable consequence is that interpretations are often incorporated into biogenesis models touted as universally applicable to all organisms regardless of obvious important differences between mammals, yeasts, and plants. Such generalizations are not always correct and it is important and prudent to point out notable exceptions to the models presented by Titorenko and Rachubinski (2001a,b), Mullen et al. (2001a), van der Klei and Veenhuis (2002), Eckert and Erdmann (2003), and Koch et al. (2004). Hence, it is a challenge to identify and segregate those species-specific features from those that are generally applicable.

The assemblage of four generalized models for peroxisomal biogenesis in one comprehensive figure (Fig. 1) is a result of sorting and sifting of published models. This figure is not universally applicable, nor is it intended to represent four mutually exclusive scenarios within one cell or necessarily within different cells in any one organism. Nevertheless, one can identify main sources for each pathway in the figure. The descriptive title coined for each model/pathway (listed above and in the legend of Fig. 1) are intended to focus on the proposed events and actions, rather than on the model organism(s). At this stage, we do not know whether a certain model applies specifically or solely to one organism or species, even though such implications exist in the literature.

The ER-lamellae pathway comes from ultrastructural electron tomographic analyses of mouse dendritic cells (Geuze et al. 2003; Tabak et al. 2003). The ER vesicle-fusion/maturation pathway is derived from a combination of ER-derived vesicle fusion data obtained with the yeast *Yarrowia lipolytica* (Titorenko and Rachubinski 2001a,b) and vesicle maturation described in *Saccharomyces cerevisiae* (Hoepfner et al. 2005; Kragt et al. 2005; Kunau 2005; Tam et al. 2005). The ER semi-autonomous growth and division of pre-peroxisomes is based largely on data for plant peroxisomes (e.g., Mullen et al. 1999, 2001a; Trelease 2002; Lisenbee et al. 2003; Karnik and Trelease 2006). The latter pathway partially overlaps with the autonomous growth and division pathway championed by Lazarow (Purdue and Lazarow 2001; Lazarow 2003), and is supported by results obtained by Gould's group (Sacksteder and Gould 2000; South et al. 2000) on mammalian cultured cells and *S. cerevisiae*.

A key difference between the semi-autonomous and autonomous models is the source of membrane proteins and phospholipids for pre-existing peroxisomes. In the semi-autonomous scheme, ER-derived vesicles deliver PMPs and phospholipids to growing pre-peroxisomes, whereas in the autonomous scheme membrane remnants or protoperoxisomes, which purportedly are not derived from ER, are envisioned to provide the necessary molecules for self-replicating (autonomous) peroxisomes. Both schemes invoke constitutive formation of daughter peroxisomes prior to, or during, cell division. The portrayal of regulated proliferation comes mostly from numerous contributions with mammalian cells (e.g., Koch et al. 2004) and yeast cells, mostly with *Hansenula polymorpha* (e.g., Veenhuis et al. 2000). Examples of regulated proliferations are reported for plants in diverse publications briefly discussed and cited below.

In the next sections, we outline and discuss the data, interpretations, and opinions related to the generalized working models presented in Fig. 1. Whenever appropriate, we take the opportunity to insert pertinent information related to plants, and to the plant ER. Models describing peroxisomal biogenesis in mammalian cells are considered first for several reasons: (a) because of their historical influence, (b) due to the wealth of pertinent publications, (c) since the adamant conclusions that the ER does not partic-

ipate in autonomous peroxisomal biogenesis is longstanding, and (d) due to new evidence obtained on mouse (and *S. cerevisiae*) cells that challenges and refutes anti-ER concepts and conclusions.

2
Peroxisomal Biogenesis in Mammalian Cells—The Historical Perspective for ER Participation

It became apparent in the 1970s that deficiencies in peroxisomal biogenesis were responsible for certain diseases, e.g. Zellweger syndrome, which is characterized by peroxisomal membrane ghosts in the human cells. These ghosts possessed membrane proteins, but were devoid of matrix proteins. Obviously, a direct relationship between a debilitating disease in humans and the biogenesis of a specific organelle (peroxisomes in this case) stimulated concerted research in this area. As a result, a model known as the ER vesiculation model emerged. It was characterized by the co-translational insertion of nascent proteins to the ER membrane and lumen followed by a *pinching off* of nascent peroxisomes. Electron microscopy studies that showed close associations of peroxisomes with profiles of rough ER in plant, animal, and yeast cells were often cited in support of this universal vesiculation model. However, plant biologists in particular, constantly pointed out that the peroxisome boundary membrane was not in direct continuity with ER membranes in a manner indicative of vesiculation from rough ER. Examples of such images are given in Trelease (2002, Fig. 1). Taken together, these contrary image interpretations and the lack of convincing evidence for co-translational import of proteins into peroxisomes, [discussed extensively at international meetings, see Kindl and Lazarow (1982), and in early reviews, see for example Trelease (1984)] led to this model falling out of favor in the early 1980s.

Lazarow and Fujiki published a most influential review in 1985. They effectively dismantled any remaining support for the ER vesiculation model, citing experimental evidence that peroxisomal proteins were synthesized on free ribosomes and inserted post-translationally into peroxisomes. They proposed a new model, referred to as the autonomous growth and division model, which excluded any participation of ER. This model is depicted using dashed lines in Fig. 1. For the next ten years or so, this model for peroxisome biogenesis became firmly entrenched in the literature encompassing all organisms, not just for mammals from whence the supporting data was derived. However, in 1996 Trelease (Muller and Trelease 1996) and Baker (1996) stated that growth and division was an oversimplification, i.e., that alternative scenarios such as division and growth, division without growth, and growth without division were known. Unfortunately, these two papers had little impact at the time.

3
Autonomous Growth and Division Model—Constitutive and Regulated (Induced) Proliferation Pathways

Recent reviews pertaining to the self-replicating model (Purdue and Lazarow 2001; Lazarow 2003) have included new information on the sequential uptake of specific peroxins, while strongly adhering to basic non-involvement of ER in the process of peroxisomal biogenesis. The basic tenant is that pre-existing peroxisomes are the source for the constitutive, steady-state production of daughter peroxisomes during normal cell division (Fig. 1, dashed lines). Accordingly, pre-existing immature peroxisomes mature/become elaborated through *direct* acquisition of matrix and membrane proteins post-translationally from the cytosol. Although not cited in either of the two reviews, much evidence in support of direct post-translational insertion of matrix and membrane proteins has come from studies with pre-existing plant peroxisomes (e.g. Brickner et al. 1997; Lisenbee et al. 2005; Mullen et al. 1999; Murphy et al. 2003; Pool et al. 1998; Hunt and Trelease 2004; Sparkes et al. 2005). Such acquisitions are clearly a manifestation of plant peroxisomal growth (enlargement). Specific examples are given in Sects. 3.2 and 5.1.

3.1
Constitutive Peroxisomal Division (Proliferation)

Surprisingly, reliable examples for constitutive duplication of peroxisomes prior to or during cell division are few and only rudimentary for mammalian cells. This type of proliferation is considered distinct from regulated (induced) proliferation of peroxisomes; however, the two events are often difficult to distinguish functionally (Koch et al. 2004; Thoms and Erdmann 2005; Yan et al. 2005). These proliferative events are illustrated as separate pathways in Fig. 1 (dark arrows, with and without dashed lines).

The most direct and convincing evidence for constitutive peroxisomal division was obtained with the red alga *Cyanidioschzon merolae*, which possesses one peroxisome per cell. Miyagishima et al. (1999) presented ultrastructural views of the peroxisome undergoing binary fission just prior to cell division. Direct evidence for plant peroxisomes is not available, although recent data derived from analyses of two of the five Arabidopisis Pex 11 homologs are strongly suggestive (Lingard and Trelease 2006). Each of the individually overexpressed isoforms, arbitrarily designated as AtPex11a–e, sort *directly* from the cytosol to pre-existing peroxisomes in BY-2 and Arabidopsis suspension cultured cells. Two of them, namely AtPex11a and -e, promoted duplication of pre-existing peroxisomes that did not first undergo peroxisomal elongation as described for mammalian peroxisomes (Yan et al. 2005). It was therefore surmised that the plant peroxisomes divided via constrictive

fission before, or during, cell division, hence constitutive duplication was not definitively demonstrated.

A good example of peroxisomes becoming partitioned between mother and daughter cells (for several generations) was obtained in the studies of Hoepfner et al. (2001, 2005) on *S. cerevisiae* using time-lapse microscopy. Daughter peroxisomes were produced through active fission processes, and were targeted and segregated in buds under the control of the dynamin-like protein, Vps1p. Another dynamin-like protein, DLP1, has been shown to be necessary for peroxisomal fission in both mammalian (Koch et al. 2003, 2004; Li and Gould 2003) and Arabidopsis cells (Mano et al. 2004), but it is not clear whether these two examples represent constitutive or regulated proliferations. Thus only meager data are available to support the duplication of peroxisomes as portrayed in the autonomous or semi-autonomous growth and division models (Fig. 1).

3.2
Regulated Induction of Peroxisomal Proliferation

Enlarged and usually elongated peroxisomes may be formed from preexisting peroxisomes in several organisms, including plants, under regulated induced conditions such as changes in cell culture medium and density, growth factors, increased irradiance, ureide production, hypolipidemic drugs, fatty acids, increased free radicals (hydrogen peroxide, ozone) (black arrow, Fig. 1). Isoforms of Pex11 proteins and various types of dynamin-like proteins are involved in these induction pathways (Koch et al. 2004; Thoms and Erdmann 2005). Schrader et al. (1998) concluded that certain Pex11 forms promote elongations in the induction pathways, whereas other Pex11 forms stimulate elongation in the constitutive pathway. Although Pex11 proteins induce peroxisomal elongation prior to peroxisomal division, they are themselves not mechanically involved in the peroxisomal fission process.

Lingard and Trelease (2006) found that overexpression of AtPex11c and -d resulted in dramatic peroxisomal elongations in both Arabidopsis and tobacco BY-2 cells (see for example Fig. 2). In contrast to similar situations in mammalian cells, these elongated peroxisomes do not undergo subsequent division(s). Such a maintained elongation is reminiscent of glyoxysome and leaf peroxisome elongation in oilseed cotyledons and greening leaves (Gruber et al. 1972; Kunce et al. 1984). In these cases, elongation was perceived to manifest peroxisome differentiation due to acquisition of specific enzymes to accomplish specialized functions of the different types of peroxisomes. Other examples of induced proliferations are budding and apparent fissions of plant peroxisomes that were exposed to different conditions (described in Trelease 2002). More recent examples are ozone-induced H_2O_2 proliferation in peroxisome numbers in aspen and birch leaves (Oksanen et al. 2004), and budding

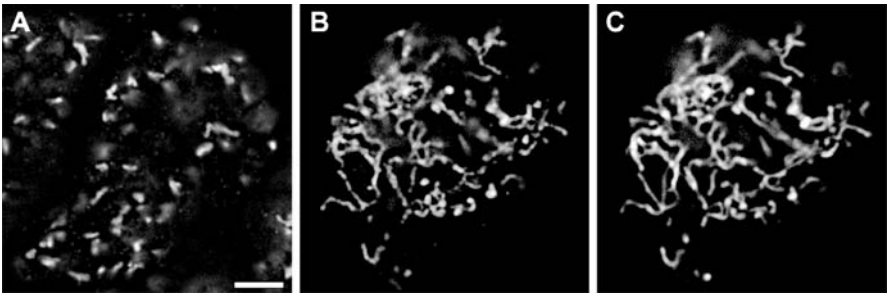

Fig. 2 Epifluorescence images of single Arabidopsis suspension cells illustrating peroxisomal elongation/tubulation induced by overexpression of AtPex11c (At1g01820). **A** Mock-transformed cells with typical peroxisomes labeled with anti-catalase IgGs (1 : 2000, 1 h). **B**,**C** Cells were transformed with mycAt*PEX*11c, fixed in formaldehyde (20 h post bombardment), perforated/digested with pectolyase/cellulase, permeabilized in Triton X-100, and labeled with primary and dye-conjugated secondary antibodies in microfuge tubes. **B** Catalase/CY-2-labeled elongated/tubulated peroxisomes are distributed throughout the cell. **C** The same cell as in **B** shows that mycAtPex11c/rhodamine-labeled elongated peroxisomes are co-localized with CY-2-labeled peroxisomes in **B**. *Bar* = 5 μm

(fission) from stationary (actin anchored) peroxisomes induced via a myosin motor pulling force (Jedd and Chua 2002).

Clearly there is ample evidence, albeit scattered and via different mechanisms, for regulated induced proliferation of plant peroxisomes. However, this would require substantial new membrane biosynthesis. What could be the source of the new membrane components? This is a particularly challenging question for those who adhere to the autonomous peroxisomal growth and division concept.

4
Is the ER the Source of the Membrane Components for Nascent Peroxisomes and/or Peroxisomal Growth and Division?

This obvious question has been the source of debate ever since Lazarow and Fujiki introduced their provocative model 20 years ago. Only recently, may a clear answer have been obtained for nascent peroxisomes. Compelling evidence has been presented for the ER origin and subsequent maturation of peroxisomes in *S. cereviseae* (Hoepfner et al. 2005; Kragt et al. 2005; Tam et al. 2005). Kunau (2005) suggests that that the work of these authors signifies the "end of the debate" for the origin of peroxisomes. The next sections provide background and insights into the lengthy debates on this controversial issue.

4.1
Source and Transfer of Peroxisomal Membrane Phospholipids

Most of the data on the lipid composition of peroxisomal membranes comes from studies with plant peroxisomes (see Trelease 2002, and references therein). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most common phospholipids in plant peroxisomal membranes. Interestingly, nonpolar lipids are also prevalent in plant membranes. Statements are often made that PC and PE are the most prevalent phospholipids in mammalian and yeast peroxisomal membranes, but studies supporting these claims are few and often incomplete.

There is no evidence in any organism that peroxisomes possess the enzymes capable of synthesizing their own phospholipids, hence the most logical source of PC and PE is the ER. Assuming (as most people do) that the ER is indeed the source, an important question is how the lipids are transferred to peroxisomes. Kagawa et al. (1973) have provided direct evidence for the transfer of radiolabeled phospholipids from the ER to castor-bean endosperm glyoxysomes during postgerminative growth. This seems to be the only report for direct transfer from ER in any plant. Chapman and Trelease (1991) examined lipid transfer in cottonseed cotyledons, and surprisingly found that radiolabeled PC and triolein transferred directly from oil bodies to glyoxysomes, but not from the ER. An interesting parallel is that of Wanner et al. (1982) who presented ultrastructural images and proposed that nascent watermelon seedling glyoxysomes were formed from so-called lipid body appendices, which they concluded were derived directly from segments of rough ER. This is not unreasonable since oil bodies (particularly their boundary membrane) are themselves derived from the ER (Huang 1992; see also Huang, this volume).

Phospholipid exchange proteins have long been considered likely candidates for lipid transfer, but are now considered too inefficient for the extensive peroxisomal (membrane) proliferation events shown in Fig. 1. Membrane contact sites (MCSs), at which two organelles come into close apposition, have gained more support recently as a means of transfer of lipid from the ER to organelles such as peroxisomes (Levine 2004). As mentioned earlier, micrographs depicting MCSs between segments of ribosome-free ER and peroxisomes are commonly observed in plant cells. Particularly striking examples of this have been observed in non-rhizobia-infected cells in developing root nodules of four different species (Kaneko and Newcomb 1987, 1990; Newcomb et al. 1985). Peroxisomes were found to undergo a 60 times increase in volume per unit cytoplasm in concert with a dramatic increase in the abundance of tubular ER that arose as extensions from rough ER cisternae. Enlarging peroxisomes were invariably closely associated with tubular ER and were usually almost surrounded by it. Newcomb's group and others (Frederick et al. 1968; Huang et al. 1983; Trelease 1984) postulated that these images were manifesta-

tions of sites where membrane components, especially phospholipids, moved from the ER to pre-existing (differentiating) or forming peroxisomes.

In summary, there is a paucity of reliable data on the lipid composition of peroxisomal membranes in all organisms (e.g. oilseed and yeast glyoxysomes, leaf-type peroxisomes, liver/kidney peroxisomes, mammalian microperoxisomes, trypanosome glycosomes). Nevertheless, the available data, especially from plants, strongly implicate the ER as the source of membrane phospholipids. It is therefore surprising that the actual means of phospholipid transfer from the ER to peroxisomes has not yet been established in any single organism.

4.2
Do Progenitor Membranes (e.g., Peroxisomal Ghosts, Remnants, Protoperoxisomes) Other than ER Vesicles Participate in Nascent Peroxisome Formation?

Studies with yeast and mammalian mutants that abolished peroxisomal membrane protein (PMP)

and/or *early peroxin* import have provided important insights into this aspect of peroxisomal biogenesis (Eckert and Erdmann 2003; Sacksteder and Gould 2000; Sparkes and Baker 2002; Subramani et al. 2000). In most yeast mutants with abrogated uptake of matrix proteins, the normal complement of membrane proteins were incorporated properly into peroxisomal membrane *remnants*, or so-called membrane ghosts. Exceptions were found in *pex3* Δ, *pex16* Δ, and *pex19* Δ mutants, where peroxisomal ghosts were not found and PMPs were mislocalized to the cytosol. These and other results led to the conclusion that these three peroxins (so-called early peroxins) were essential for peroxisomal membrane assembly in mammals and yeasts. Particularly germane to our considerations here were the findings that, upon transformation of these individual mutants with wild-type genes coding for the corresponding mutated peroxin, normal peroxisomes were formed in the transformed cells. The obvious question that arises is: how do peroxisomes reform in these cells in the apparent absence of progenitor peroxisomal membrane ghosts?

This question was answered very recently in three studies with *S. cerevisiae* (Hoepfner et al. 2005; Kragt et al. 2005; Tam et al. 2005). Collectively, cell fractionation and real-time fluorescence microscopy approaches were used to trace variously tagged Pex19p and Pex3p in mutant and wild-type cells. Pex3p was unequivocally observed in the ER at foci, where Pex3p budded off into membrane vesicles and ended up in peroxisomes. The process was Pex19p-dependent and the vesicles matured into functional new peroxisomes. This general scheme is portrayed with the white arrows in Fig. 1. Although Kunau (2005) considered these studies sufficiently profound to "end the debate" on the participation of ER in the formation/creation and maturation of new peroxisomes, the production of nascent plant, mammalian, and other yeast

peroxisomes via this pathway needs to be demonstrated before the ER can be universally considered as the source of new peroxisomes as Kunau has implied for all organisms.

These results are a source of major criticism for the autonomous growth and division scheme because strict adherence to this model would necessitate the de novo formation of peroxisomes in the rescued mutants. One major fault with this model is that the spontaneous assembly of membranes from molecules derived from the cytosol is not generally accepted for the biogenesis of any organelle. Instead, the favored concept is that membranes beget membranes. Curiously, proponents of the autonomous model do not endorse de novo membrane formation, but also do not consider ER vesicles as the source of membrane components for nascent peroxisome formation. At this point, the uninformed reader needs some help to understand this prevailing anti-ER sentiment. It is based on two main factors: (a) a long-term fervent belief in self-replication (autonomy) of mammalian cell peroxisomes, and (b) an overt acceptance of the negative evidence for ER participation in mammalian peroxisomal biogenesis (discussed in more detail below).

This anti-ER sentiment has stimulated researchers to seek alternative membrane progenitors within mutant cells. For this, the reader is directed to interesting scenarios and opinions presented in the reviews of Purdue and Lazarow (2001) and Lazarow (2003). Briefly, they and others believed that the mutant cells possessed tiny, remnant membrane ghosts or protoperoxisomes that were missed in previous investigations due to inadequate detection methodologies. They elaborated on the few instances where "careful and aggressive" searches for such remnant vesicles led to discoveries of protoperoxisomes (also called remnants or pre-peroxisomes) in a *pex19* Δ mutant of *Pichia pastoris* and in *pex3* Δ mutants of three yeast species and mammalian Chinese hamster ovary (CHO) cells. They concluded that similar remnants were missed in other mutants; therefore, invoking de novo membrane synthesis was not necessary to explain reformation of peroxisomes in the mutant transformants. Interestingly, they predicted that existing protoperoxisomes served as peroxisomal progenitors in cells that experienced natural peroxisomal decimation, and not just in mutants.

Accordingly, a protoperoxisome is shown in Fig. 1 as part of the autonomous growth and division scheme (dashed lines). However, some proponents (Koch et al. 2004; Lazarow 2003) of the pathway in mammalian cells entertain the notion that some ER-derived vesicles may fuse with pre-existing peroxisomes to supply some minimal membrane protein(s) and lipid(s). Lazarow (2003) makes it clear, however, that this event is not to be construed as a de novo origin of peroxisomes. Rather, it should be considered as a small (minimal) contribution within a multistep elaboration/maturation pathway for conversion of pre-existing peroxisomes into mature peroxisomes for subsequent fission into daughter (not new) peroxisomes. In our opinion, acquiescence of the existence of membrane progenitors, be they remnants or

ER vesicles, transcends the autonomous model into a semi-autonomous concept. The latter is a prevailing concept for peroxisomal biogenesis in plants and most yeasts, and recently has been applied to mouse dendritic cells modeled in Fig. 1 as the ER-lamellae peroxisome formation pathway (white–dark arrows). Details of this model and ones ascribed to plants are considered below.

As mentioned above, one significant reason for not accepting ER involvement in peroxisome formation/proliferation was that evidence sought to specifically demonstrate the participation of the ER in mammalian and some yeast cells was either not obtained or was deemed inconclusive. Significantly, negative arguments of this nature have not been applied to data obtained on plant cells, if only because results obtained with plant systems are typically overlooked or ignored in most studies/reviews dealing with yeasts and mammals. Notable exceptions are the studies of Titorenko and Rachubinski (2001a,b, 2004) on the yeast *Y. lipolytica*, and two critical, reviews by Eckert and Erdmann (2003) and Sparkes and Baker (2002). We summarize in the following section pertinent results and interpretations dealing with participation of the ER in peroxisome biogenesis, including those performed on plant cells. Details and original references are not given here, but can be found in the reviews cited above and in Mullen et al. (2001a), Lazarow (2003), Purdue and Lazarow (2001), and Trelease (2002).

4.3
Positive/Negative Interpretations of ER-Peroxisome Biogenesis Studies

In an early study, rat PMP50 was found synthesized on ER-bound ribosomes and its human homolog trafficked from rough ER to peroxisomes (Bodnar and Rachubinski 1991). A criticism was that the 50-kD polypeptide was an ER protein, not an authentic peroxisomal protein. Proliferation of the ER was noted in human cells overexpressing HsPex3p (Kammerer et al. 1998). In *S. cerevisiae*, O-glycosylated ScPex15p (a membrane protein) resided in both peroxisomes and ER karmellae (stacked ER sheets) (Elgersma et al. 1997). These authors later reported that overexpression of ScPex15p led to an artificial ER localization. However, Mullen et al. (1999) used the same Sc*PEX15* gene construct as a positive control in their in vitro ER import experiments: ScPex15p and cottonseed peroxisomal ascorbate peroxidase (GhAPX) both imported post-translationally into maize ER microsomal membranes, but not into isolated peroxisomes. *Candida boidinii* PMP47, employed as another positive control, inserted appropriately into isolated peroxisomes, but not into ER microsomal membranes.

Brefeldin A (BFA), a macrocyclic lactone which leads to a collapse of the Golgi apparatus into the ER and the subsequent cessation of ER export (Nebenführ et al. 2002), resulted in the accumulation of three peroxin PMPs in the ER of *Hansenula polymorpha* (Salomons et al. 1997). Following re-

moval of BFA, these three peroxins moved to the peroxisomes. Similar results with BFA treatment were reported for endogenous and transiently expressed GhAPX in tobacco BY-2 cells (Lisenbee et al. 2003; Mullen et al. 1999) and for AtPex16p in Arabidopsis suspension cells (Karnik and Trelease 2006). In contrast, BFA treatments had no effect on the sorting of HsPex3p or HsPex16p to human fibroblast peroxisomes (South et al. 2000), or on AtPex2p and AtPex10p uptake into peroxisomes in tobacco-leaf epidermal cells (Sparkes et al. 2005).

Peroxisomal biogenesis in *Saccharomyces cerevisiae* was unaffected in the double mutants *sec61/ssh1*, which reportedly abolished both co- and post-translational import of lumenal and membrane proteins into ER (South et al. 2001). Incubation of cells at 15 °C, which blocks (slows) exit of proteins from ER, did not adversely affect targeting of HsPex16p to human fibroblast peroxisomes (South and Gould 1999). Different results were obtained in similar cold-treatment experiments with AtPex16p in Arabidopsis cells (Karnik and Trelease 2006). Endogenous or transiently expressed AtPex16p accumulated in ER at 15 °C and upon re-equilibration at room temperature, AtPex16p moved in vesicles to pre-existing peroxisomes.

De novo peroxisome formation from the ER tacitly implies that exit from the ER and movement to peroxisomes of membrane vesicles containing early peroxins would not occur in the presence of inhibitors of coat protein (COP) vesicle formation (see Aniento, Matsuoka, and Robinson, this volume). These inhibitors had no effect on the sorting of three early peroxins to peroxisomes in human fibroblasts (Voorn-Brouwer et al. 2001). Nor did incubations in coat protein I (COPI) and coat protein II (COPII) inhibitors adversely affect reformation of peroxisomes in *pex3*Δ and *pex16*Δ mutants after introduction of wild-type genes (South et al. 2000). On the other hand, COPI and ARF1 have been implicated in the biogenesis of peroxisomes in CHO cells (Passreiter et al. 1998). Similar COP inhibitor experiments have not been done with plants.

Solid support for the participation of the ER in peroxisome biogenesis comes from research with *Y. lipolytica* (Titorenko and Rachubinski 2001a,b). It is interesting that this work was often referred to as a curious or striking exception, and therefore typically had not been challenged or refuted. It is gratifying that the new model derived from research with *Saccharomyces cerevisiae* (Kunau 2005) together with model derived from the biochemical studies on *Y. lipolytica* is now merged into our generalized model (white arrows). Perhaps this work will no longer be considered as an exception, but as representative for yeast peroxisome biogenesis in general.

Amongst the four distinct secretory pathways diverging at the level of the ER in *Y. lipolytica*, there are certain secretory mutants (*sec*) impaired in peroxisomal biogenesis. For example, *sec* mutants lacking signal recognition particles (SRP), and *sec238*, a protein factor involved in protein secretion, exhibited defects in protein exit from ER and reductions in number and size of

peroxisomes. Yl*pex1* Δ and Yl*pex6* Δ mutants showed accumulation of early peroxins, YlPex2p and YlPex6p, in ER. In normal cells, pulse-radiolabeled endogenous YlPex2p and YlPex16p were targeted from the cytosol to ER where they were core *N*-glycosylated and then chased within ER-derived vesicles to peroxisomes, bypassing the Golgi apparatus. Reconstitution experiments with subcellular fractions revealed that nascent mature peroxisomes were formed via a complex multistep assembly process beginning with the exit of ER vesicles (nascent peroxisomes) and possessing a subset of early peroxin PMPs (Titorenko and Rachubinski 2001a,b). As mentioned above, the white arrow pathway in Fig. 1 is a merged and abbreviated version of their six peroxisomal subforms (P1 to P6) model. Proteins in the ER are ostensibly directed through *gating* proteins to distinct ER subdomain(s) called the pre-peroxisomal template or peroxisomal ER (pER) Fig. 1, (Mullen et al. 1999). *Y. lipolytica* pre-peroxisomal ER vesicles are coated with at least two COPII components; this is in distinct contrast with results obtained with mammalian cells (see above). A similar overall vesicle fusion pathway has been proposed for biogenesis in the yeast *Pichia pastoris* (Faber et al. 1998; Subramani 1996).

A recent study with mouse dendritic cells provided a unique opportunity to examine the formation of peroxisomes using immunocytochemistry and three-dimensional (3-D) electron microscope tomography of cryosections (Geuze et al. 2003; Tabak et al. 2003). Compelling evidence was presented in support of peroxisome formation from a smooth-surfaced subdomain of the ER, called specialized ER. A modified version of their model is illustrated in Fig. 1 (white–black arrow pathway). The specialized ER is labeled as pER, consistent with the name of the subdomain as used by Titorenko and Rachubinski (2001a,b) and from Mullen et al. (1999) who coined the term for an ER subdomain in plant cells. Clear 3-D reconstructions detailed the membrane continuity between rough ER and pER in the mouse cells. As envisioned in the other ER-formation/assembly model (white arrows), early peroxins enter the rough ER post-translationally, but in this case, these proteins presumably stimulate formation of the pER subdomain. This subdomain segregates, possibly via dynamins and/or SNARE proteins, and becomes pre-peroxisomal lamellae, which acquire additional peroxins to form competent import machinery. Thereafter, lamellae give rise to spherical nascent peroxisomes, which may either be mature or acquire more components in a multistep elaboration/maturation process (Fig. 1).

The ER-lamellae peroxisome formation model certainly challenges the autonomy portion of the autonomous growth and division model as far as mammalian cells are concerned (dashed arrows, Fig. 1). Failure in other studies to demonstrate vesicle formation from the ER during peroxisome formation (e.g. through COPI and COPII inhibitors, variable BFA responses, cold treatments, *sec* mutations) might be explained by different dynamin or SNARE-mediated mechanism(s) involved in segregation of pER subdomains

(lamellae). This model provides an explanation for how the early-peroxin mutants can form new peroxisomes in the absence of peroxisomal ghosts (remnants) following transformation with competent peroxin genes. Thus, the nascent peroxins prime the rough ER to form template smooth-surfaced pER for subsequent lamellae formation and segregation. Overall, this model has a semi-autonomous character, whereby peroxin-induced ER priming generates membrane lamellae possessing peroxin PMPs that orchestrate subsequent autonomous elaboration/maturation events.

5
Evidence for the ER Semi-autonomous Growth and Division Pathway in Plants

5.1
Peroxisomal Growth is a Common Feature in Plant Cells

Microscopical studies document the enlargement (growth) of different kinds of peroxisomes in plant cells. Examples include glyoxysomes in oilseed cotyledons during postgerminative growth (Hayashi et al. 2000; Kunce et al. 1984; Wanner et al. 1982), leaf peroxisomes during greening of leaves (Gruber et al. 1973), and ureide-producing root peroxisomes in uninfected cells during development of root nodules (Kaneko and Newcomb 1987, 1990; Vaughn 1985; Webb and Newcomb 1987). Experiments with green fluorescent protein (GFP) chimeric proteins aimed at describing motility of peroxisomes in plant cells revealed changes in their size, shape, and number (e.g., Collings et al. 2002; Mano et al. 2002; Mathur et al. 2002). Higher-equilibrium-density cottonseed glyoxysomes apparently derived from lower-density pre-glyoxysomes were identified in sucrose gradients (Choinski and Trelease 1978), as were at least two buoyant-density populations of peroxisomes isolated from suspension cultures of anise (Kudielka et al. 1981) and *A. thaliana* (Lisenbee et al. 2003; Flynn et al. 2005). Other examples are given in Sect. 3.2, where regulated, induced proliferative events usually result in the subsequent growth of nascent plant peroxisomes.

5.2
Membrane-Bound Peroxins and Peroxisomal Enzymes Exist within the ER and Traffic Indirectly to Peroxisomes through the ER (Peroxisomal ER, pER)

5.2.1
APX Sorts Indirectly via a Targeting Signal to Peroxisomes through pER Subdomain(s)

Compelling evidence for post-translational insertion into plant ER came from extensive in vitro import experiments. Cottonseed peroxisomal membrane APX (GhAPX), a carboxyl tail-anchored type II ($N_{cytosol}$-C_{matrix}) integral membrane protein, was the first plant PMP found to sort indirectly through the ER to peroxisomes (Mullen et al. 1999). Radiolabeled GhAPX inserted into highly purified maize microsomal membranes in an SRP-independent, adenosine triphosphate (ATP)-dependent, chaperone-enhanced post-organelle addition manner. Indiscriminate binding/insertion of GhAPX into other organelles, including peroxisomes, did not to occur. Positive controls gave the expected results whereby ScPex15p and VAMP2 inserted only into ER microsomes, and CbPMP47 was incorporated only into peroxisomes. The co-operative action of ATP and three cytosolic chaperones (Hsp70, AtJ2, and AtE1) substantially enhanced the import of GhAPX into the ER microsomes.

In the same study (Mullen et al. 1999), transiently expressed, haemagglutinin (HA) epitope-tagged GhAPX trafficked in tobacco BY-2 cells to a reticular/circular network marked with the general ER stain $DiOC_6$ and then to pre-existing peroxisomes. Significantly, GhAPX co-localized with only a small portion of the total cellular ER marked with antibodies to three different reticuloplasmins. Dynamics of the GhAPX trafficking pathway was examined in BFA-treated cells where expressed CAT-APX (GhAPX fused at the N terminus of chloramphenicol acetyltransferase) accumulated in the reticular/circular network and then moved, presumably within ER-derived vesicles, to pre-existing BY-2 peroxisomes upon removal of BFA (during the recovery period). The conclusion was that GhAPX trafficked to peroxisomes indirectly through a distinct subdomain of the ER, which was named peroxisomal ER (pER). In accordance with this conclusion, Nito et al. (2001) found in a time-course study that pumpkin peroxisomal APX (CpAPX) was transported to pumpkin glyoxysomes/peroxisomes via a smooth-surfaced unidentified membrane structure, which they surmised was smooth ER or specialized subdomains of ER similar to pER described in BY-2 cells. One wonders now whether these structures are reminiscent of the ER-derived lamellae described in mouse dendritic cells (Geuze et al. 2003, and white–black arrow pathway Fig. 1). A modified version of the model of Mullen et al. (1999) is presented in Fig. 1 as the ER semi-autonomous growth and division pathway (dark arrows), excluding the constitutive daughter peroxisome formation because this was not addressed at the time.

In approximately 15% of BY-2 cells transiently transformed with HA-GhAPX, overexpression of HA-GhAPX led to altered morphology and/or distribution of peroxisomes and pER (immunofluorescence microscopy observations, Mullen et al. 1999). In another study (Mullen et al. 2001b), time-course observations revealed a shift from reticular to more circular pER as well as a more extensive aggregation of pre-existing peroxisomes. This phenomenon was particularly evident in cells transformed with CAT protein chimeras possessing *C*-terminal, tail-anchored proteins that could form dimers or trimers. The authors concluded that these images represented progressive association, or zippering, of organelles via oligomerization of folded polypeptides on the cytosolic side of the organellar membranes. Thus, this was an overexpression artefact for proteins sorted to ER (pER), but limited to those membrane proteins positioned mostly on the cytosolic surface and capable of oligomerizing (zippering) with like proteins extending from other like (or unlike) organelles.

Further characterization of the reticular/circular (immuno)fluorescence compartment led to redefining this putative pER image (Lisenbee et al. 2003). Chimeras were constructed with a peptide possessing the membrane targeting signal for GhAPX (Mullen and Trelease 2000) fused to GFP (GFP-APX). Transiently expressed GFP-APX sorted to BY-2 cell reticular/circular compartments and to peroxisomes with GFP on the cytosolic surface of the organelle membranes. Surprisingly, homotypic and heterotypic aggregates of peroxisomes, plastids, and/or mitochondria were formed. The circular portion of the reticular/circular compartment was comprised of mitochondria and plastids zippered together via cytosolic-facing GFP (capable of forming dimers). These and other results revealed that authentic pER is only the reticular compartment observed in (immuno)fluorescence images. Circular images were not formed when mutated GFP (deleted oligomerization residues) were used to generate GFP-APX constructs. These results establish beyond doubt that pER is an authentic compartment reliably and easily identified via (immuno)fluorescence microscopy in plant cells that are overexpressing (peroxisome) membrane proteins targeted to the ER en route to peroxisomes.

5.2.2 Overlapping Targeting Signals Direct GhAPX and AtPex16p through pER to Peroxisomes

Mullen and Trelease (2000) determined which part of the carboxyl tail-anchored type II integral membrane GhAPX served as the targeting signal(s) to pER and peroxisomes. They found that a patch of five basic residues (RKRMK) was necessary, but not sufficient, for sorting to peroxisomes via pER in vivo. However, the pentapeptide plus the immediately adjacent transmembrane domain (TMD) were sufficient for sorting CAT chimeric proteins

through the ER to peroxisomes. Further tests indicated that two overlapping molecular signals occurred within the TMD-pentapeptide signal described above. One, an ER (pER) portion of the signal was proposed for targeting GhAPX from the cytosol directly to a pER subdomain, or possibly to general ER from where it moved through gated privilege site(s) (Mullen et al. 1999) into the pER. The latter possibility is consistent with proposals of Titorenko and Rachubinski (2001a,b) and Tabak et al. (2003) for ER targeting in *Y. lipolytica* and mouse cells, respectively. The second overlapping signal was a membrane peroxisome targeting signal (mPTS) that functioned within pER to sort GhAPX to the boundary membrane of peroxisomes. The *C*-terminal ScPex15p (Elgersma et al. 1997) and *N*-terminal HpPex3p (Baerends et al. 2000) mPTSs are similar to the GhAPX signal (cluster of basic residues plus TMD). An overlapping targeting signal also was found for AtPex16p (SK Karnik, Ph.D. dissertation, Arizona State University, Dec. 2005).

Of interest, nearly all mPTS studies to date have focused on the elucidation of signals that target PMPs to mature peroxisomes, rather than to the ER or pre-peroxisomes. A surprising degree of complexity and variation has been reported within the common context of a TMD and a cluster of basic amino-acid residues (for details see Purdue and Lazarow 2001; Sparkes and Baker 2002; Trelease 2002; Eckert and Erdmann 2003; Murphy et al. 2003). Certainly this is an area where more data are needed so that we can better understand the integrated signaling phenomena involved in the uptake and sorting of PMPs into and out of pER subdomain(s), and subsequent trafficking (in vesicles) of these PMPs directly to peroxisomes, bypassing the Golgi apparatus.

5.2.3
Endogenous AtAPX Resides in ER and Peroxisomes

As is apparent from many of the discussions above, conclusions made from studies of overexpressed membrane proteins in transformed cells are subject to criticism. In view of this concern, studies of endogenous PMPs in nontransformed cells have been performed to substantiate/refute the role of the ER (or portions thereof) as an authentic pre-peroxisomal sorting compartment. Thus, Lisenbee et al. (2003) conducted a combined microscopy and cell fractionation study to examine endogenous AtAPX in nontransformed cultured Arabidopsis cells. AtAPX was consistently observed in vivo within peroxisomes, but detected only in small, subtle ER subcompartments. However, AtAPX and CpAPX (Nito et al. 2001) were reliably identified on immunoblots of ER and peroxisomal fractions recovered from isopycnic sucrose gradients. Mg^{+2}-induced shifts of AtAPX in sucrose density gradients provided convincing evidence for localization in rough ER vesicles. Immunogold electron microscopy verified the presence of AtAPX in the shifted rough ER vesicles. On the other hand, Nito et al. (2001) did not observe a Mg^{+2}-induced

shift for endogenous pumpkin CpAPX in microsomes loaded onto gradients, indicating a greater presence of CpAPX in a smooth ER (pER?) compartment. Topology experiments revealed that AtAPX was oriented mostly on the cytosolic face of both ER vesicles and peroxisomes. These results corroborated earlier interpretations for zippering of organelles together to form the artefactual (immuno)fluorescence circular structures (see above). Taken together, these results provide strong evidence that portions of rough ER (pER) serve as a constitutive sorting compartment for endogenous peroxisomal AtAPX and CpAPX in wild-type cells. This reenforces previous interpretations that pER is an authentic sorting compartment, rather than a transiently induced ER compartment formed as a consequence of overexpressed peroxisomal APXs.

5.2.4
Subcellular Localization(s) and Involvement of ER with "Early" Peroxin Homologs

Knowledge of the involvement of early peroxins (Pex3p, Pex16p, Pex19p and Pex10p) is not nearly as advanced for plants as for yeasts and mammals. Although Arabidopsis homologs have been identified for all four of these peroxins, only *Atpex16* and *Atpex10* null mutants have been examined (Lin et al. 1999, 2004; Schumann et al. 2003; Sparkes et al. 2003). Limited data were obtained with these mutants since the homozygous condition was lethal at the heart stage of seed embryogenesis. AtPex19p is a cytosolic protein in Arabidopsis cells (Trelease, unpubl.), which supports its predicted role in transporting/chaperoning membrane proteins to peroxisomes in plant cells as in other species (Baker and Sparkes 2005; Schliebs and Kunau 2004). AtPex3p (two forms) surprisingly was found to sort directly to pre-existing peroxisomes in transiently transformed Arabidopsis cells (Hunt and Trelease 2004), rather than indirectly through ER as expected from the location of Pex3p in ER of *H. polymorpha* (Salomons et al. 1997) and *Saccharomyces cerevisiae* (Hoepfner et al. 2005; Kragt et al. 2005; Tam et al. 2005).

5.2.5
AtPex10p

Schumann et al. (2003) described ultrastructural defects in heart-stage embryos of the At*pex10* lethal mutant. Extensive defects were apparent in the morphology of the rough ER and in the formation of protein and lipid bodies. Lipid-body membranes, derived from the ER, accumulated in the cytosol. Peroxisomes were not identified in any of the cells. This suggested that AtPex10p functioned through participation with ER in the formation of all three organelles: lipid bodies, protein bodies, and peroxisomes. Sparkes et al. (2003) also examined the At*pex10* mutant, and came to the more generalized conclusion that AtPex10p was essential for embryo development and viability,

but did not specify target organelles or possible involvement of ER. Evidence was obtained, however, for AtPex10p being a peroxin homolog in this and a subsequent paper (Sparkes et al. 2005). Autofluorescent chimeras AtPex10p-YFP and GFP-SKL (SKL is a *C*-terminal peroxisomal targeting signal), transiently expressed in epidermal cells of tobacco leaves, were co-localized in the tobacco peroxisomes.

A study with endogenous AtPex10p in Arabidopsis cells produced results that were both contrasting and supportive of those of other studies. Flynn et al. (2005) employed immunofluorescence microscopy and semiquantitative immunogold electron microscopy of Arabidopsis cells and sucrose-gradient fractions to elucidate the subcellular location(s) of AtPex10p. As was expected from the predictions of Schumann et al. (2003), but in contrast to negative evidence for trafficking to tobacco-cell ER (Sparkes et al. 2005), AtPex10p was observed in subdomains of ER. Despite numerous varied attempts and approaches, however, they were unable to demonstrate the occurrence of AtPex10p in Arabidopsis peroxisomes. The reason(s) for the apparent discrepancies between these results and those of Sparkes et al. (2003, 2005) are not known.

5.2.6 AtPex16p

Lin et al. (1999, 2004) suggested that AtPex16p, also called SSE1p from its shrunken seed mutant phenotype, was essential for ER-dependent protein and oil-body biosynthesis in developing Arabidopsis seeds. An implication for its involvement in peroxisomal biogenesis was the peroxisomal localizations of GFP-AtPex16p in root hairs and embryos of stably transformed Arabidopsis plants. Karnik and Trelease (2005) discovered a definite link between endogenous AtPex16p and ER in suspension-cultured Arabidopsis cells. AtPex16p coexisted at steady state within ER and peroxisomes. An example of these results is presented in Fig. 3. The immunofluorescence image in panel A shows AtPex16p throughout a reticular compartment in each cell, and within organelles exhibiting a punctate pattern. Panel B shows that the punctate organelles are catalase-containing peroxisomes. The punctate AtPex16p in panel A is co-localized with peroxisomes shown in panel B. Evidence that the reticular pattern (in panel A) is ER is convincingly demonstrated in the comparison of the confocal images presented in panels C and D. Virtually 100% of the reticular AtPex16p in panel C is co-localized with the reticular ER marker BiP in panel D. The extensive co-localization indicates that this peroxin homolog is distributed within most of the reticular ER in the cells. This ER localization is significantly different from the ER localization of APX, i.e., endogenous and transiently expressed APXs are observed only within subdomains of ER, which constitute a relatively small proportion of the total ER in each cell (Mullen et al. 1999).

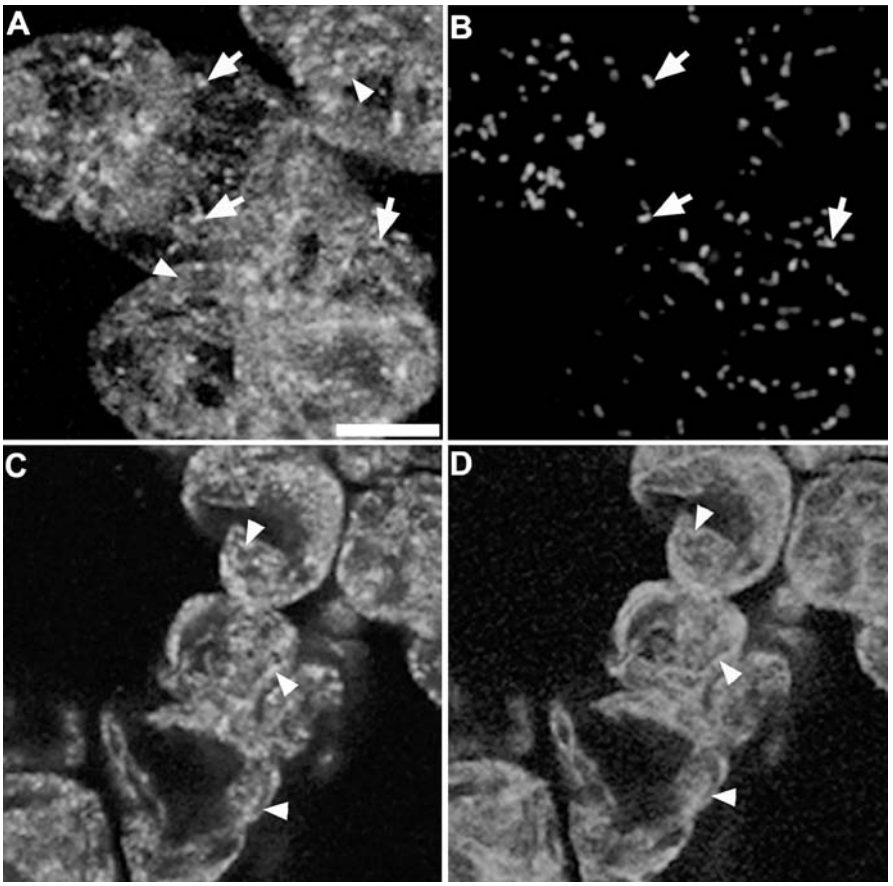

Fig. 3 Confocal immunofluorescence images reveal coexistence of endogenous AtPex16p in both ER and peroxisomes in wild-type suspension-cultured Arabidopsis cells. **A–D** Representative confocal section images of cells that were fixed in formaldehyde, treated with pectolyase and cellulase, permeabilized (membranes) with Triton X-100, and then primary and dye-conjugated secondary antibodies added to the cells adhered to glass microscope slides. **A** Cells exhibit Cy2 punctate (*arrows*) and reticular patterns (*arrowheads*) labeled with rabbit anti-AtPex16p IgGs (1 : 500, 1 h). **B** The same cells as those in A exhibit co-localized Cy5 peroxisomal catalase immunofluorescence due to mouse anti-catalase monoclonal IgGs (1 : 500, 1 h). **C** A different portion of the same population of cells exhibit a Cy2 punctate pattern and a reticular pattern (*arrowheads*) labeled with rabbit anti-AtPex16p IgGs (1 : 500, 1 h). **D** The same cells as those in **C** exhibit a co-localized Cy5 reticular pattern (*arrowheads*) attributable to the mouse monoclonal BiP antibodies (1 : 500, 1 h). *Bar* = 5 μm (from Karnik and Trelease 2005)

Results from cell-fractionation experiments confirmed the coexistence of AtPex16p in ER and peroxisomes (Karnik and Trelease 2005). Immunoblots revealed the presence of AtPex16p in purified peroxisome and ER fractions obtained from sucrose gradients. AtPex16p also exhibited a definite Mg^{+2}-

induced shift with ER vesicles. AtPex16 seems to be a peripheral, ionically bound membrane protein in both ER and peroxisomes. Topological orientation results were interesting and somewhat surprising in that AtPex16p was found mostly on the matrix side of the peroxisomal boundary membranes, whereas it was mostly on the cytosolic surface of ER membranes. The combined results indicate a bifunctional role for AtPex16p. That is, a portion of the ER-localized protein may function in constitutive oil- and protein-body biogenesis. Another portion of the ER-localized protein and that localized in ER vesicles and peroxisomes may function in the maturation of immature pre-existing peroxisomes, and/or constitutive/induced replication of mature pre-existing peroxisomes (semi-autonomous pathway, Fig. 1).

The trafficking pathway for nascent AtPex16p lends support to the latter function hypotheses (Karnik and Trelease 2006). Time course, cold treatment, and BFA incubation experiments with both wild-type and mycAtPex16p-transformed BY-2 and Arabidopsis cells resulted in unambiguous accumulations of mycAtPex16p in ER. During subsequent time and recovery periods, mycAtPex16p was detected in vesicles (likely derived from ER) en route to pre-existing peroxisomes. These results mirrored published results with the membrane enzyme APX. Interestingly however, the PMPs trafficked from ER in different vesicles en route to the same pre-existing peroxisomes.

6 Summary

Interesting and important features have emerged from studies on the membrane protein APX and various peroxin homologs in plant cells. There is a multitude of both indirect and direct evidence for participation of the ER in the biogenesis of different kinds of peroxisomes in plant cells. At this point, most evidence favors the ER semi-autonomous growth and division pathway presented in Fig. 1 (dark arrows). Closer examinations of selected cell systems with higher-resolution techniques such as electron tomography of quick-frozen hydrated/resin-embedded sections will likely provide more structural details of the pER subdomain(s) in addition to revealing more varied means of segregation from the rest of the ER, such as the lamellae described in the ER-lamellae peroxisome formation pathway (white–black arrows, Fig. 1). Essentially no evidence has been obtained in plants for the ER vesicle-fusion/maturation peroxisome formation/assembly pathway (white arrows). Events and mechanisms associated with the formation and distribution of daughter peroxisomes during cell division of plant cells is a major missing component of the pathways instigating the ER as the essential source of membranes (phospholipids). The prevalent membrane contact sites (MCS) in plant cells are possible sources of membrane components that likely complement events portrayed in the pathways shown in Fig. 1. Finally, certain peroxin ho-

mologs and other PMPs are sorted indirectly to peroxisomes through the ER via overlapping molecular targeting signals.

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The ER and Plant Hormones

Chris Helliwell

CSIRO Plant Industry, GPO Box 1600, 2601 Canberra,ACT, Australia
chris.helliwell@csiro.au

Abstract A large number of reactions in hormone biosynthesis and catabolism pathways are located in the endoplasmic reticulum (ER). These reactions are catalysed by cytochrome P450s, a large family of enzymes involved in many metabolic pathways in plants. As well as being involved in hormone synthesis and inactivation, many of the P450s involved in hormone biology are likely to be under regulation both to maintain hormone homeostasis and in response to environmental signals. This chapter describes the roles of the ER-located P450 enzymes in plant hormone biology.

1 Introduction

Plant hormones are involved in the control of a wide variety of developmental and environmental responses. The endoplasmic reticulum (ER) is the usual location of the cytochrome P450s, a major group of enzymes that are involved in the biosynthesis and catabolism of a number of plant hormones. This chapter will focus on the cytochrome P450s in hormone biosynthetic and catabolic pathways and their biological roles.

1.1 Plant Hormones

Plant hormones encompass a wide range of molecules, usually present at low concentrations. The classical plant hormones are indole-3-acetic acid (IAA, also known as auxin), cytokinins (CKs), abscisic acid (ABA), gibberellins (GAs) and ethylene. Other compounds have also been shown to act as hormones including brassinosteroids (BRs), jasmonic acid, salicylic acid and some small peptides. Physiological methods have defined many of the biochemical pathways leading to the synthesis and catabolism of many of the plant hormones. More recently the application of molecular genetics, particularly in the model species *Arabidopsis thaliana*, has led to the isolation of genes encoding many of the enzymes of hormone biosynthesis and catabolism pathways. As a result, there is now a good understanding of hormone biosynthetic and catabolic pathways and how they are regulated. Molecular genetics has also been the launching point for an increasing understanding of the mechanisms involved in the perception and signal transduction of hormone signals.

A key aspect of hormone regulation is the maintenance of hormone homeostasis, that is, the maintenance of a "default" concentration of hormone and a mechanism to restore the hormone concentration to the default concentration after a signal has been transduced. In the case of plant hormones, this is often achieved by regulation of expression of enzymes involved in both the biosynthetic steps leading to production of the biologically active hormone and also of catabolic enzymes which modify biologically active hormones to inactive forms. Typically the biologically active hormone acts via a receptor/signal transduction pathway to negatively feed back on expression of the biosynthetic enzymes (particularly those acting late in biosynthetic pathways) and positively feed forward on the expression of the catabolic enzymes. A well-characterised example of this is in regulation of GA homeostasis, where expression of soluble GA 20-oxidases and 3-oxidases in the biosynthetic pathway is down-regulated at the transcript level by increased GA concentrations and up-regulated in mutants that have reduced endogenous GAs. In contrast, expression of the catabolic GA 2-oxidases is increased when the GA concentration is high and reduced when the GA concentration is low. The feed back and feed forward controls do not operate in the dominant *gai* mutant, a dwarf that is unable to respond to GA, indicating that the regulation of biosynthesis and catabolism is under the control of the general GA perception and signal transduction pathway. Similar mechanisms control the homeostasis of other hormones via expression of ER-located cytochrome P450 enzymes, and will be discussed in this chapter.

1.2 Cytochrome P450 Enzymes

The cytochrome P450s are a large enzyme superfamily in plants. They are named after the 450-nm absorption maximum of the carbon monoxide-bound form of the protein. The P450 superfamily shows a great deal of amino acid sequence diversity, with some members having as little as 20% amino acid identity with each other. However, key amino acid residues and structures involved in binding the heme cofactor required for activity are conserved (Graham and Petersen 1999). Most plant P450s are likely to reside in the ER membrane, although examples of mitochondrial and plastidic P450s have also been found. A hydrophobic domain at the *N*-terminus of ER-localised P450s acts as the targeting sequence and also as a membrane anchor (Fig. 1). The membrane anchor is followed by a cluster of basic residues (a stop-transfer signal) and often a hinge region containing a cluster of prolines. The remainder of the protein, including the heme-binding motifs and substrate recognition sites, is termed the globular domain and is located on the cytosolic side of the ER membrane. The P450s catalyse a wide range of oxidation reactions in primary and secondary metabolic pathways.

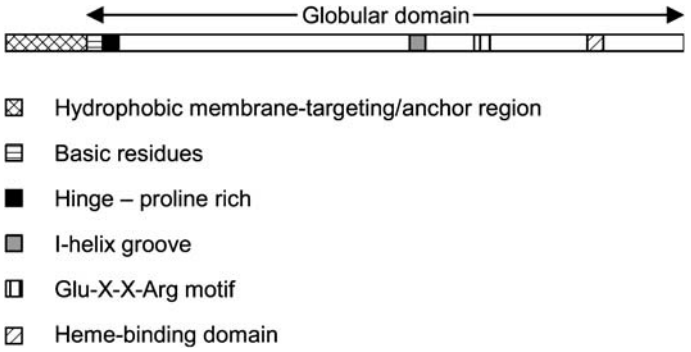

Fig. 1 Generalised structure of ER-bound cytochrome P450 enzymes. The major conserved regions involved in heme binding are shown, along with *N*-terminal membrane anchoring regions

In *Arabidopsis* there are approximately 272 cytochrome P450-encoding genes or pseudogenes and in rice there are 455 (Nelson et al. 2004). P450s are classified and named according to their amino acid sequence identity, gene structure and phylogeny (Nelson et al. 1996) into families (generally proteins with greater than 40% amino acid identity) and subfamilies (proteins with greater than 55% amino acid identity); for example, CYP701A3 is in family 701, subfamily A, gene number 3 in the subfamily. Plant P450s have been divided into two branches based on their amino acid sequences. The A type appears to be plant-specific, while the non-A types are more closely related to the P450s of other organisms. The P450s have diversified in plants to carry out a wide range of metabolic functions (Schuler and Werck-Reichhart 2003).

Cytochrome P450s require a number of other factors for activity. The protein itself binds a heme molecule, which is essential for activity. The P450s usually require one of a small family of ER-anchored NADPH-dependent P450 reductases for electron transfer from NADPH to the catalytic site of the P450. The requirement of these additional factors is one reason why many of the plant P450s have no assigned function. The most popular method of defining plant P450 function has been to express the cDNA for the P450 in yeast strains that also express one of two *Arabidopsis* cytochrome P450 reductases (Urban et al. 1994). This has been successful for a number of the P450s implicated in plant hormone biosynthesis (e.g. P450s in the GA biosynthetic and ABA catabolic pathways), but there are many cases where this approach has not been successful (e.g. a number of steps in the BR biosynthesis pathway). In these latter cases, determination of P450 function has relied on comparisons of measurements of biosynthetic intermediates in mutants against wild type or restoration of mutant phenotypes following the application of biosynthetic intermediates to the mutant plant.

2 Abscisic Acid

Abscisic acid (ABA) is a sesquiterpenoid hormone which has roles in many developmental processes and environmental responses. It is particularly important in dormancy, germination and stress responses. ABA is derived from carotenoid precursors, with the biosynthesis pathway split between early steps in the plastid and late cytosolic steps. As with other plant hormones, the catabolism of ABA is important in controlling cellular ABA concentration. Physiological analysis had suggested that a cytochrome P450 was responsible for the first step of ABA inactivation to give 8′-hydroxy ABA, an unstable intermediate that is rapidly converted to phaseic acid (PA) either enzymatically or spontaneously (Fig. 2).

The CYP707A P450 subfamily was identified as being up-regulated in response to ABA application in *Arabidopsis* using a microarray approach. All four members of the subfamily in *Arabidopsis* were shown to be ABA 8′-hydroxylases by functional expression in yeast (Kushiro et al. 2004), with PA being produced when the yeast was fed with ABA. Of the *Arabidopsis* CYP707A subfamily, CYP707A2 is most highly expressed in dry seed and its expression is rapidly induced following seed imbibition, suggesting that this enzyme may be important in reducing the ABA content of seeds to allow germination. Consistent with this, knockout mutants of CYP707A2 show increased seed dormancy and have increased seed ABA content. ABA levels also increase in plants exposed to dehydration stress; in parallel with the ABA increase there is a moderate induction of all four *Arabidopsis* CYP707A subfamily members. When plants are rehydrated, ABA levels are reduced at the same time as large increases in expression of the four CYP707A subfamily members occur. As all four CYP707As appear to be involved in the dehydration response they are likely to be functionally redundant, and consistent with this no phenotypes related to dehydration stress have been observed in knockout plants of the CYP707A subfamily. The evidence to date therefore suggests that ER-localised P450s play an important role in the control of ABA concentration, both in maintaining ABA homeostasis and in ABA-mediated developmental and environmental responses.

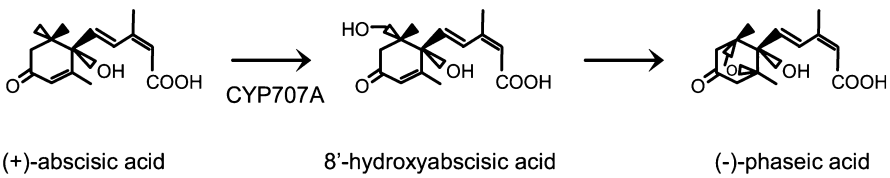

Fig. 2 The ABA 8-hydroxylation reaction catalysed by the CYP707A subfamily in *Arabidopsis* and the spontaneous conversion of 8′-hydroxyabscisic acid to (–)-phaseic acid

3 Auxin

The hormone auxin has been studied since the nineteenth century, and has been associated with a number of processes including apical dominance, shoot elongation, root initiation, cambial division and various tropisms. The main auxin in plants is indole-3-acetic acid (IAA). IAA can be synthesised from tryptophan and other precursors by a number of different pathways. All the individual pathways may not be present in one species and the relative contributions to the IAA pool from the different pathways can vary during the development of the plant. Members of two families of cytochrome P450 enzyme have been associated with IAA biosynthesis from tryptophan (Fig. 3): CYP79B2/B3 and CYP83B1. CYP79B2 and B3 were isolated as a result of a screen of yeast expressing *Arabidopsis* cDNAs for resistance to 5-fluoroindole, which is converted to the toxic 5-fluorotryptophan by yeast (Hull et al. 2000). Yeasts expressing CYP79B2 were resistant to 5-fluorotryptophan, presumably as they were able to metabolise the toxic 5-fluorotryptophan. Bacterially expressed protein was assayed in a reconstituted membrane system, and both CYP79B2 and the related CYP79B3 were shown to convert tryptophan to indole-3-acetaldoxime. Indole-3-acetaldoxime is a branch point between the IAA and glucosinolate biosynthesis pathways (Fig. 3).

CYP79B2 and B3 have a predicted chloroplast transit peptide at their *N*-termini; however, the functions of these predicted transit peptides have not been tested, so it is not clear if this enzyme is localised to the ER as would be

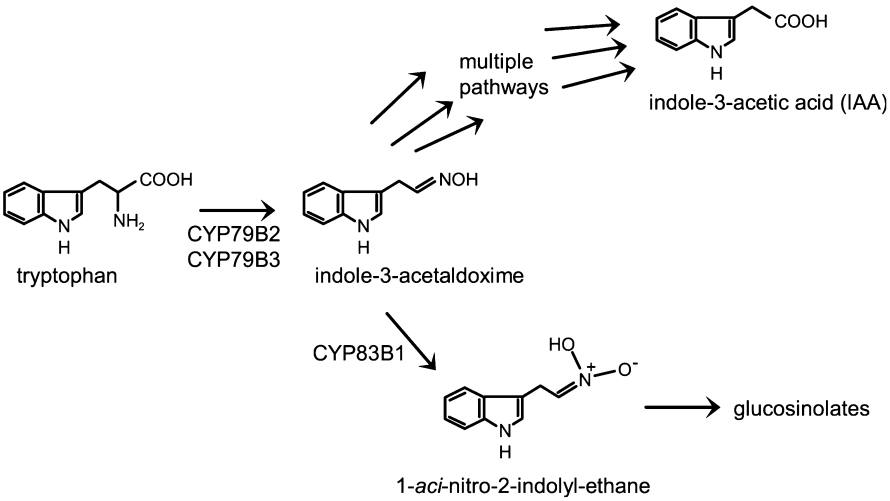

Fig. 3 Reactions in the IAA and glucosinolate pathways catalysed by CYP79B2/B3 and CYP83B1

expected or is one of the rare cases of chloroplast location. There is little information about the biological roles of CYP79B2 and B3. CYP79B2 is pathogen induced, which may indicate a defence role for this P450 which could be via auxin and/or glucosinolates (glucosinolates break down to toxic compounds upon herbivore attack). The second P450 with a role in auxin biosynthesis is CYP83B1. The P450 was identified from the *sur2* mutant, which has elevated IAA levels (Barlier et al. 2000). The enzymatic activity of CYP83B1 has been characterised by expression in yeast with a cytochrome P450 reductase, and was proposed to catalyse the first committed step of glucosamine synthesis from indole-3-acetaldoxime to 1-*aci*-nitro-2-indolyl-ethane (Bak et al. 2001; Fig. 3). The auxin-overproducing phenotype of *sur2* is therefore thought to be a result of increased IAA biosynthesis due to a block in glucosinolate biosynthesis. This branch point may well be a crucial point of control of IAA biosynthesis, as increased IAA leads to increased expression of CYP83B1 mRNA (Barlier et al. 2000). This could be a feedback mechanism acting to maintain IAA homeostasis by diverting indole-3-acetaldoxime from the IAA pathway to the glucosinolate pathway.

4
Brassinosteroids

4.1
Brassinosteroid Biosynthesis and Catabolism

The brassinosteroids (BRs) are a group of compounds closely related to the mammalian steroid hormones. While the existence of BRs had been known for some years, their functions in growth and development have only become clear following molecular genetics studies in *Arabidopsis*. Mutants in BR biosynthesis suggest that this hormone has important roles in cell elongation, xylem differentiation and photomorphogenesis.

The BR biosynthesis pathway is a continuation of the sterol biosynthesis pathway: the early committed steps to the sterol campesterol are catalysed by enzymes with similarity to mammalian steroid reductases. The pathway from campesterol to the active BRs, castasterone and brassinolide (Fig. 4), is best viewed as a network with the enzymes involved being capable of catalysing conversions of multiple substrates. All the biosynthetic enzymes which have been characterised at the molecular level in this part of the pathway to date are cytochrome P450s.

The P450 CYP90B1 from *Arabidopsis* was identified by the BR-responsive *dwf4* (*dwarf* 4) mutant (Choi et al. 1998). BR biosynthetic intermediates were fed to the mutant to define the block in the BR pathway. The mutant phenotype was rescued with the 22α-hydroxylated intermediates 6-deoxocathasterone and cathasterone (Fig. 4), as well as other 22α-hydroxyl-

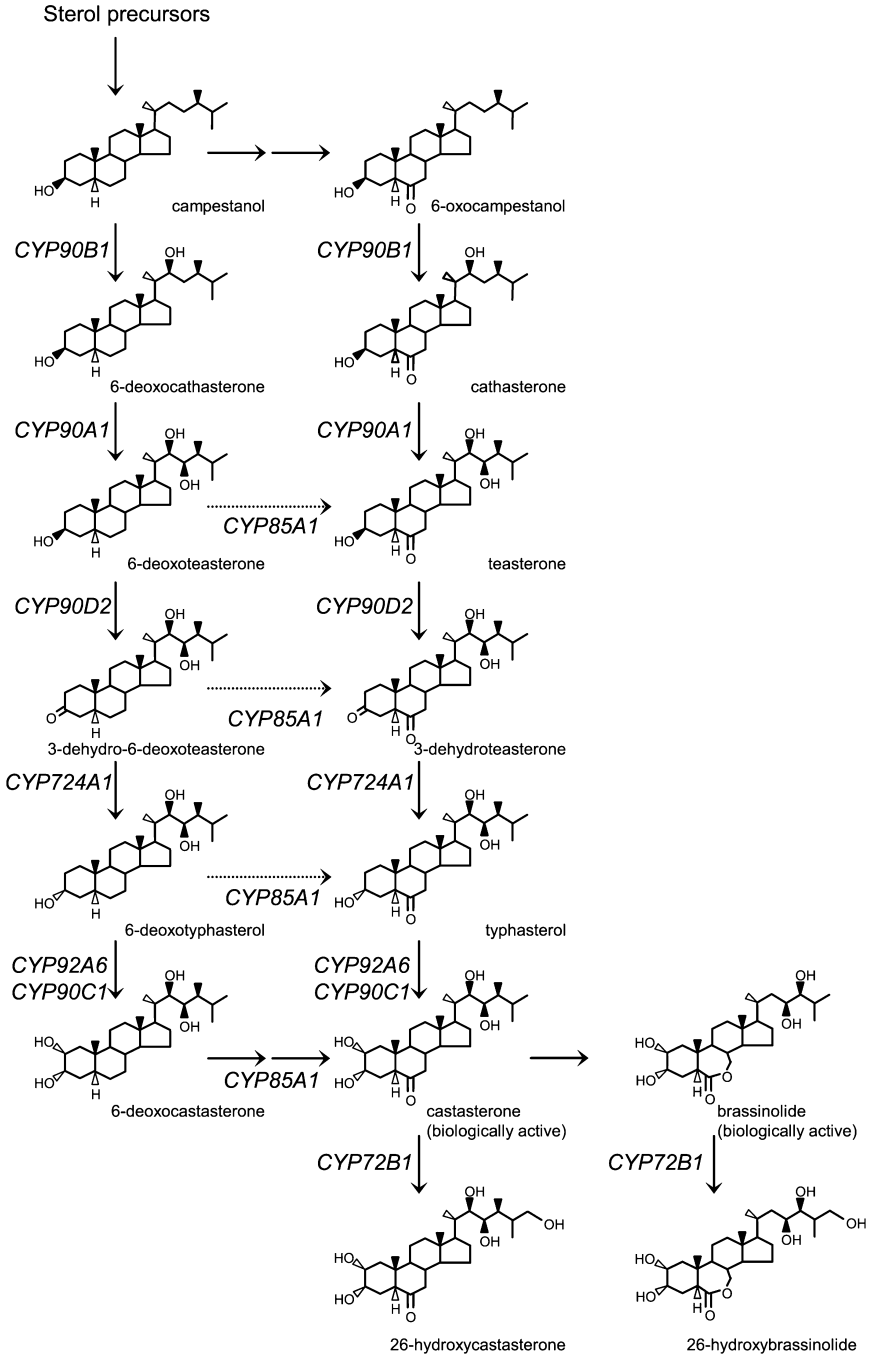

Fig. 4 The brassinosteroid biosynthesis and catabolic pathway with P450-mediated steps from various species indicated

ated intermediates (not shown in Fig. 4), and accumulated non-22α-hydroxylated intermediates (including campestanol and 6-oxocampestanol), strongly suggesting that CYP90B is capable of carrying out the 22α-hydroxylation of multiple substrates.

The 23α-hydroxylation steps in BR biosynthesis are catalysed by CYP90A1 in *Arabidopsis*, a P450 with 43% amino acid sequence identity to CYP90B1. This P450 was also identified by a BR biosynthesis mutant, *cpd* (constitutive photomorphogenesis and dwarfism; Szkeres et al. 1996). Feeding studies to rescue the mutant phenotype were used to define the likely reactions catalysed by this P450. Application of 6-deoxoteasterone or teasterone rescued the *cpd* mutant phenotype, whereas 6-deoxocathasterone and cathasterone did not, suggesting that CYP90A1 catalyses the 23α-hydroxylation steps in the BR biosynthesis pathway.

A rice dwarf mutant, *d2*, has a mutation in the gene encoding CYP90D2; this mutant accumulates 6-deoxocathasterone and the mutant phenotypes are rescued by 3-dehydro-6-deoxoteasterone and 3-dehydroteasterone but not by earlier intermediates (Hong et al. 2003), suggesting it is blocked between these intermediates. Another rice mutant in a P450 related to the CYP90 family of P450s, CYP724B1, has phenotypes including dwarfism that can be rescued by BR application. Feeding of intermediates suggests that the block in this mutant is in the steps of the pathway from 3-dehydro-6-deoxoteasterone and 3-dehydroteasterone to 6-deoxotyphasterol and typhasterol, respectively (Tanabe et al. 2005). So far there are no homologues of the CYP724B1 subfamily reported in other species.

CYP92A6 (*DDWF1*; dark-induced *DWF*-like protein 1) was isolated from pea in a yeast two-hybrid screen with the G-protein Pra2 (see below; Kang et al. 2001). This P450 has been shown by green fluorescent protein (GFP) fusion to be localised to the ER. Analysis of BR biosynthesis intermediates and feeding studies in plants carrying an antisense to CYP92A6 suggest that CYP92A6 catalyses the C-2 hydroxylations of 6-deoxotyphasterol and typhasterol to 6-deoxocastasterone and castasterone, respectively. This was confirmed by assaying bacterially expressed CYP92A6 mixed with yeast microsomes and demonstrating that the mix carried out the C-2 hydroxylation of typhasterol to castasterone. In *Arabidopsis* there is no CYP92A6 homologue, however evidence from quantifying intermediates in the BR biosynthesis pathway in a CYP90C1 mutant suggests that it catalyses the C-2 hydroxylations of 6-deoxotyphasterol and typhasterol, as both these intermediates accumulate in the mutant and the concentrations of the products 6-deoxocastasterone and castasterone are reduced (Kim et al. 2005). The CYP90C1 mutant could only be rescued by application of castasterone or brassinolide, which provides supporting evidence that it is a C2-hydroxylase. The CYP90C1 mutant (also known as *rotundifolia3*) does not show the classical BR-deficient dwarf phenotype but does have reduced petiole elongation and more rounded leaves. When combined with a mutant in the closely re-

lated CYP90D1, which has no phenotype as a single mutant in *Arabidopsis*, a plant with a strong BR-deficient phenotype is produced, suggesting there is some degree of functional redundancy between these two P450s. At present there is no evidence for the *Arabidopsis* CYP90D1 acting at the earlier steps in the pathway where the rice CYP90D2 is likely to act.

CYP85A1 was isolated using the tomato *dwarf* mutant and has been characterised by expression of the CYP85A1 cDNA in yeast together with a cytochrome P450 reductase (Bishop et al. 1999; Shimada et al. 2001). This analysis demonstrated that this P450 carries out the C-6 oxidation of multiple substrates (9-deoxoteasterone, 3-dehydro-6-deoxoteasterone, 6-deoxotyphasterol and 6-deoxocastasterone) in a reaction with two distinct steps. An activation tagging strategy in *Arabidopsis* identified CYP72B1 as a potential brassinosteroid catabolic enzyme. In the activation-tagged plants the CYP72B1 was overexpressed, the plants had low endogenous brassinolide and accumulated 26-hydroxybrassinolide (Neff et al. 1999). Subsequent expression of the CYP72B1 cDNA in yeast has shown that it catalyses the C-26 hydroxylation of both castasterone and brassinolide to 26-hydroxycastasterone and 26-hydroxybrassinolide, respectively (Turk et al. 2003). Castasterone and brassinolide both have biological activity, whereas 26-hydroxycastasterone and 26-hydroxybrassinolide are biologically inactive.

It is therefore likely that BR biosynthesis from campestanol and 6-oxocampestanol is exclusively catalysed by cytochrome P450s. The lactonisation of the B ring to form brassinolide from castasterone is the one step for which an enzyme has not been identified in any system, but this is also likely to be a P450-catalysed reaction. The P450s involved in BR biosynthesis are quite closely related, with the CYP90 family playing a prominent role. The evidence from some of the weaker mutant phenotypes is that there may be some functional redundancy, with multiple enzymes catalysing the same reaction. This could be resolved by heterologous expression of the enzymes; however, to date it has not been possible to assay the activities of any of the CYP90 P450 family by expression in yeast. The P450 enzymes of the BR biosynthesis pathway all appear capable of carrying out reactions at the same position on the BR skeleton on different substrate molecules, thus allowing BR biosynthesis to proceed as a network rather than a linear pathway. As the location of all the P450 enzymes in the BR pathway, the ER plays a central role in the biosynthesis and catabolism of this plant hormone.

4.2 Control of Brassinosteroid Biosynthesis and Metabolism

There is evidence for a feedback regulation pathway from active BR on the expression of the BR biosynthetic enzymes (Bancos et al. 2002). In *Arabidopsis*, expression of the mRNAs of CYP90B1, CYP90C1, CYP90D1, CYP85A1 and CYP95A2 is increased in the BR-deficient mutant *cpd* (blocked at the

CYP90A1-catalysed step of the BR biosynthesis pathway), and CYP90A1 expression is increased in the *cbb3* mutant (cabbage3, an EMS mutant in CYP90A which does not affect CYP90A mRNA expression). In all cases addition of BR dramatically reduces the expression of the mRNA of all six genes. Similarly, addition of BR to wild-type plants also reduces the expression of the mRNAs of these six genes. The same regulatory controls also seem to be acting on the expression of the rice CYP90D2 and CYP724A1 genes, as both are down-regulated following BR application. This feedback regulation presumably maintains BR homeostasis by reducing synthesis after BR concentrations have increased. Expression of the mRNA of the BR catabolising P450, CYP72B1, is up-regulated in response to applied BR (Goda et al. 2002) suggesting induction to catabolise increased BR and maintain homeostasis. The mRNAs of enzymes prior to the P450-mediated steps do not appear to be down-regulated by BR (Goda et al. 2002). The data to date suggest a concerted regulation of the expression of all the P450 enzymes involved in BR biosynthesis and catabolism rather than a single controlling step.

The feedback regulation is probably mediated via the BR perception and signal transduction mechanism. A plasma membrane leucine-rich repeat receptor protein kinase, BRI1, is an important part of the BR receptor. *Arabidopsis bri1* (brassinosteroid insensitive 1) mutants accumulate high levels of castasterone and brassinolide. The expression of BR biosynthetic P450 mRNAs are elevated in *bri1* mutants but not down-regulated by application of BR. The accumulation of BRs and the loss of feedback control in the *bri1* mutant provides strong evidence that perception of BR via BRI1 is crucial in the regulation of BR biosynthesis. The repression of CYP90A1 by BR application also requires protein synthesis (Mathur et al. 1998), perhaps of a signal transduction component.

One of the biological roles of BR is in the regulation of photomorphogenic pathways. Mutants deficient in BR biosynthesis often exhibit photomorphogenesis when grown in the dark, including opening of the apical hook, cotyledon expansion, suppression of hypocotyl elongation and leaf development. One of the suggested roles of BRs is in the dark-grown development (skotomorphogenic) and light-grown development (photomorphogenic) pathways. The evidence for this comes from BR-deficient mutants such as *det2* (deetiolated2, blocked in a sterol biosynthesis step) and *cpd* where constitutive photomorphogenesis is observed in dark-grown seedlings. BR appears to be needed for the elongation of the hypocotyl in both the light and the dark. CYP72B1 (the BR-inactivating P450) transcript is down-regulated compared to the dark expression level by white, blue and red light but not by far-red light, suggesting that this enzyme may be used to alter BR levels in response to light signals. The reduction of BR concentrations in response to light leading to de-etiolation is consistent with the phenotypes of the BR-deficient *det2* and *cpd* mutants, but BR is also required for photomorphogenesis as evidenced by the severe dwarf phenotypes of BR-deficient mutants. This re-

sponse is complex and probably involves changes in BR sensitivity as well as changes in BR concentrations (Turk et al. 2003).

Other evidence linking the regulation of the ER-located P450s in the BR pathway with light signalling comes from studies of the Pra2 protein in pea (Kang et al. 2001). Pra2 is a small G protein that is expressed in the dark and is down-regulated by light. A yeast two-hybrid screen with Pra2 as bait identified CYP92A6 as an interacting protein; both proteins localise to the ER as GFP or RFP fusion proteins in onion epidermal cells. Using activity assays of CYP92A6 with testosterone as substrate it was shown that Pra2 binding activates CYP92A6. This suggests a model where light signals regulate BR accumulation via the direct action of Pra2 on CYP92A6 in the ER. The P450s provide the major controls on the BR biosynthesis pathway, with a large body of evidence for elaborate transcriptional control emerging. The evidence from Pra2 also suggests that more direct controls are operating on the BR biosynthetic P450s in the ER in response to environmental signals.

5 Cytokinin

Cytokinins are a group of hormones derived from adenine usually with an isoprene-derived side chain. They are involved in a number of aspects of plant growth and development, notably cell division and leaf senescence. The physiologically active cytokinins are the free base forms such as isopentenyl adenine and *trans*-zeatin (Fig. 5). The synthesis of cytokinins in plants is thought to be largely via addition of dimethylallyl diphosphate to adenosine phosphates (inputs from the left side of Fig. 5) by adenosine phosphate-isopentenyl transferase (IPT), but may also be derived from mevalonic acid in the plastid (inputs from the right side of Fig. 5). The hydroxylation of the prenyl side chain of isopentenyl adenine-type cytokinins to form *trans*-zeatin-type cytokinins is a cytochrome P450-mediated reaction. An activity carrying out the hydroxylation reaction was detected in microsomal extracts of cauliflower and shown to be inhibited by metapyrone (a P450 inhibitor) and to be NADPH dependent (Chen and Leisner 1984). This enzyme hydroxylated isopentenyladenine riboside and isopentenyladenine to *trans*-zeatin riboside and *trans*-zeatin, respectively. This enzyme has not been further characterised and therefore the P450 enzyme involved is unknown.

More recently a screen for cytokinin hydroxylases was carried out by co-expression of IPT and a selection of *Arabidopsis* P450-encoding cDNAs in yeast (Takei et al. 2004). This identified CYP735A1 and CYP735A2, which are both capable of hydroxylating isopentenyladenine nucleotides but not isopentenyladenine riboside or isopentenyladenine (Fig. 5). It is therefore likely that this is a separate enzyme to the cytokinin hydroxylase activity previously detected in cauliflower microsomes. CYP735A1 and A2 show differential ex-

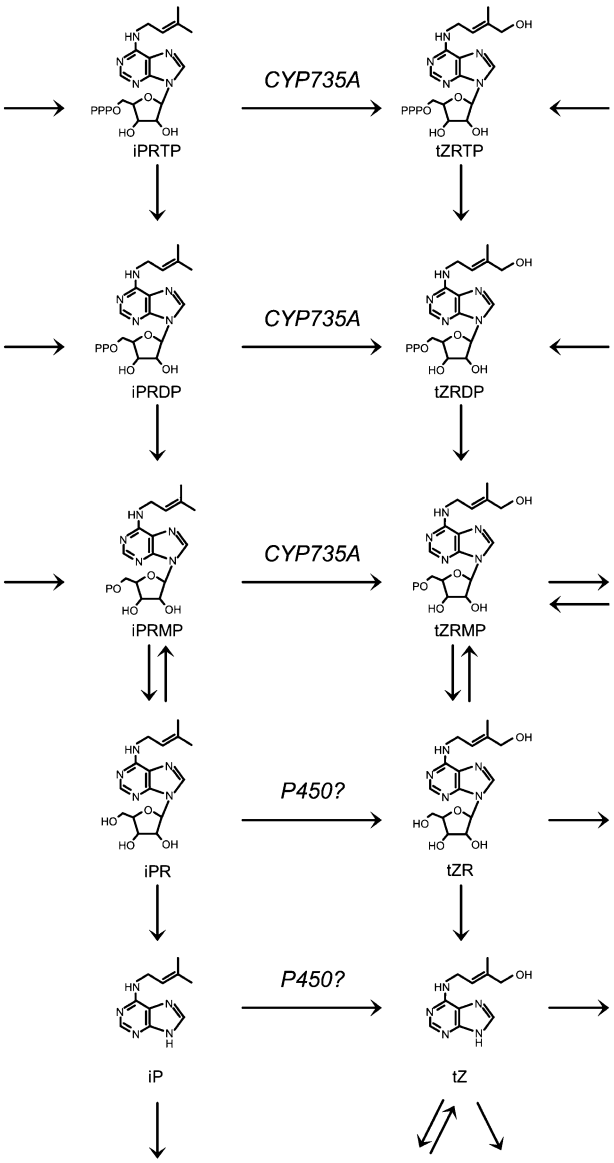

Fig. 5 Part of the cytokinin metabolic network showing reactions catalysed or putatively catalysed by cytochrome P450 enzymes. *Unattached arrows* (*left* and *right*) indicate entry and exit points of intermediates into the part of the network shown; *unmarked vertical arrows* are non-P450-mediated conversions. Key: iPRTP, isopentenyladenine riboside 5′-triphosphate; iPRDP, isopentenyladenine riboside 5′-diphosphate; iPRMP, isopentenyladenine riboside 5′-monophosphate; iPR, isopentenyladenine riboside; iP, isopentenyladenine; tZRTP, *trans*-zeatin riboside 5′-triphosphate; tZRDP, *trans*-zeatin riboside 5′-diphosphate; tZRMP, *trans*-zeatin riboside 5′-monophosphate; tZR, *trans*-zeatin riboside; tZ, *trans*-zeatin

pression patterns, with CYP735A expressed weakly in leaf, flower and root and CYP735A2 expressed strongly in root and stem. Expression of both P450s is increased with application of isopentenyladenine, which might reflect a role in maintaining the balance between non-hydroxylated and hydroxylated cytokinins; however, application of *trans*-zeatin also positively increases expression of both P450s. The significance of this is not clear at present; one hypothesis is that *trans*-zeatin is a substrate for a cytokinin catabolic step for which isopentenyladenine is not, so in the presence of high *trans*-zeatin the flux to hydroxylated cytokinins is increased to reduce physiologically active cytokinins via catabolism of *trans*-zeatin. Application of two other hormones, ABA and auxin, leads to down-regulation of CYP735A1 and A2 providing evidence that these enzymes may be important in mediating the cross-talk between these hormones and cytokinin. Our knowledge of the role of the ER in cytokinin biosynthesis is still in its infancy; however, the evidence to date suggests that cytochrome P450s may play an important role in regulating cytokinin activity in the cell.

6 Gibberellins

The gibberellins (GAs) are a large group of tetracyclic diterpenes that are present in plants and some fungi. The best-known biological function of GAs is in stem elongation; plants lacking GA due to mutation or the application of GA biosynthesis inhibitors are typically dwarf. GAs can also induce flowering, particularly in plants that require long days or prolonged cold to flower. Among other effects GA also induces starch mobilisation in germinating seeds.

The gibberellin biosynthesis pathway involves three classes of enzymes. Terpene cyclases catalyse the early steps of the pathway from geranylgeranyl diphosphate to *ent*-kaurene. These reactions are carried out in the plastid. Cytochrome P450s catalyse the oxidations from *ent*-kaurene to the first true GA, GA_{12}. The subsequent biosynthetic steps are catalysed by soluble 2-oxoglutarate-dependent dioxygenases.

Two families of cytochrome P450 have been shown to be involved in GA biosynthesis (Fig. 6). The first, *ent*-kaurene oxidase (P450s of the CYP701A subfamily), carries out a three-step oxidation of the C-19 methyl group to produce *ent*-kaurenoic acid via *ent*-kaurenol and *ent*-kaurenal (Helliwell et al. 1999). This activity has been demonstrated for *Arabidopsis*, pumpkin and pea CYP701A family members by assaying the activities of these P450s when expressed in yeast. Members of the CYP88A subfamily catalyse the oxidations and ring contraction reaction at the C-7 position that converts *ent*-kaurenoic acid to GA_{12} via the intermediates *ent*-7α-hydroxykaurenoic acid and GA_{12} aldehyde. This activity has been demonstrated for *Arabidopsis*, barley and pea

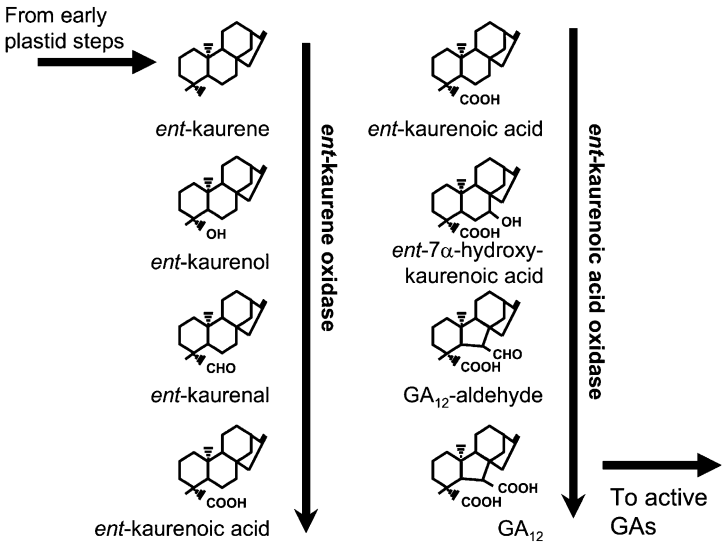

Fig. 6 The reactions of the GA biosynthesis pathway catalysed by cytochrome P450s. *ent*-Kaurene oxidases (CYP701A) catalyse the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid. *ent*-Kaurenoic acid oxidases (CYP88A) catalyse the three-step oxidation of *ent*-kaurenoic acid to GA_{12}

CYP88As by expression of the cDNAs in yeast and assaying products when fed *ent*-kaurenoic acid (Helliwell et al. 2001).

From studies carried out to date there is no clear regulation of either CYP701As or CYP88As in response to altered GA content or in response to environmental changes. CYP701A is probably present at limiting amounts in *Arabidopsis*, as *ent*-kaurene accumulates in plants overexpressing the terpene cyclases and the plants do not have elevated concentrations of active GAs or biosynthetic intermediates.

The localisation of the enzymes of the GA biosynthesis pathway has been experimentally demonstrated. The two *Arabidopsis* terpene cyclases have been shown to be located in the plastids by both GFP fusion and in vitro chloroplast import experiments. The results of GFP fusion and chloroplast import experiments with *Arabidopsis ent*-kaurene oxidase protein suggest that it is located on the outer envelope of the plastids with the possibility that some protein is also present in the ER (Helliwell et al. 2001). The two *Arabidopsis ent*-kaurenoic acid oxidases appear to be located only in the ER (Fig. 7). This suggests that *ent*-kaurene oxidase may act as a link between the plastid and ER parts of the GA biosynthesis pathway. The regulation of GA content appears to be largely at the later dioxygenase-catalysed biosynthesis pathway and catabolism steps of the pathway. Consistent with this there is no evidence for feedback regulation on expression of the genes encoding the P450 enzymes in the pathway or evidence for environmental regulation.

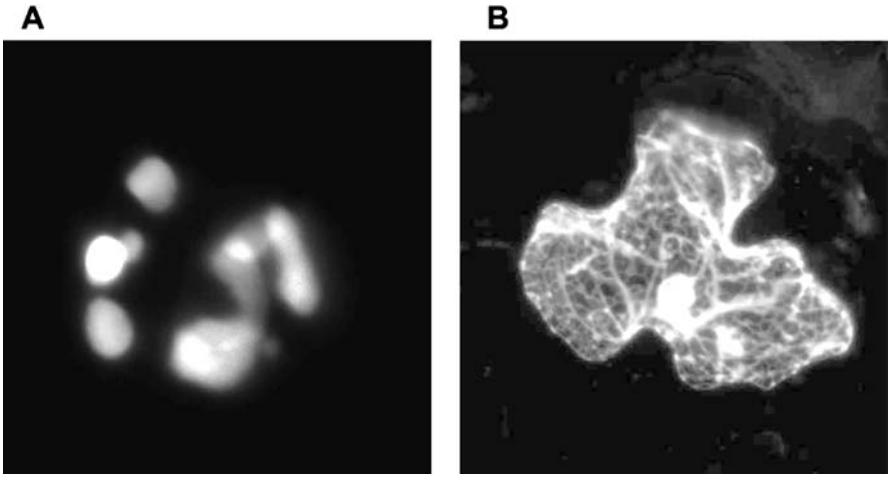

Fig. 7 GFP fluorescence images of **a** CYP701A3-GFP and **b** CYP88A3-GFP showing localisation to the plastids and ER, respectively, in transiently transformed tobacco leaf cells

7 Concluding Remarks

The ER plays an important role in both the biosynthesis and catabolism of plant hormones. In a number of cases there is evidence for changes in gene expression of the ER-located P450 enzymes in response to changes in hormone concentrations or environmental conditions. The activation of CYP92A6 by the dark-expressed Pra2 G protein in the ER suggests that direct regulation of hormone biosynthesis in the ER may occur in response to environmental signals, as well as the better characterised regulation of gene expression. Hormones are generally thought to act at plasma membrane receptors, which raises the question as to whether the hormone catabolic enzymes in the ER serve to regulate the intracellular pools of hormone or whether active hormones are also transported into the ER to be catabolised. Finally, there is little known about the role of the ER in hormone perception and signal transduction pathways. Understanding these presents a challenge for future research.

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The ER and Cell Calcium

Staffan Persson[1] (✉) · Jeff Harper[2]

[1]Department of Plant Biology, Carnegie Institution of Washington, Stanford, CA 94305, USA
shpersso@stanford.edu

[2]Department of Biochemistry, University of Nevada, Reno, Reno, NV 89557, USA

Abstract Calcium is one of the most versatile messengers in biological systems, translating developmental and environmental cues into cellular responses. The endoplasmic reticulum (ER) constitutes one of the most important calcium holding organelles in higher eukaryotes. Upon stimulation, calcium is released from the ER into the cytosol where it may trigger downstream effectors. However, the release of calcium may also affect internal ER functions, such as protein folding and secretion. It is therefore important not only to view cytosolic calcium signals as isolated events, but also in context to the organellar calcium status. In animals, the calcium levels of the ER can be sensed by other calcium resources, such as the plasma membrane, which may allow calcium uptake or release depending on the overall demand in the cell. In this chapter we have tried to convey the diverse aspects of calcium and its potential impact on different ER processes, and organellar communications, in plants.

1 Introduction

Plants are equipped with an intricate signalling network to interpret a constantly changing environment. This network constitutes a variety of components, ranging from proteins to ions, which trigger complex cellular responses. Calcium represents an important signalling molecule in all living systems (Sanders et al. 2002; Berridge et al. 2003). The plant cell sequesters calcium in different subcellular compartments, e.g. the vacuole, the cell wall, the chloroplasts, the mitochondria and the endoplasmic reticulum (ER). Upon stimulation, calcium may be released instantaneously from these compartments into the cytosol where it can coordinate downstream effectors. The calcium signalling network embraces so far approximately 700 proteins in the plant cell, creating a mosaic of potential interacting combinations (Reddy and Reddy 2004).

The resting level of free cytosolic calcium in a plant cell is approximately 100 nM, but may rapidly be increased to several mM during calcium influx (Sanders et al. 1999). Alterations in the spatiotemporal progression of the calcium influx may result in distinct Ca^{2+}-signalling signatures, i.e. variations in amplitude, duration and frequency of the signal (McAinsh et al. 1995; Allen

and Schroeder 2001). Although the calcium influx is necessary for the signal to progress, a sustained elevation of cytosolic calcium may pose a potential threat to the cell. High cytosolic calcium levels may cause energy-containing phosphate molecules, e.g. adenosine triphosphate (ATP), to precipitate, a process which may be lethal to the cell (Bush 1993). A tight coordination of the Ca^{2+}-signalling network is therefore of great importance to the cell.

A variety of abiotic stimuli, e.g. light (Johnson et al. 1995; Love et al. 2004), temperature variations (Catala et al. 2003; Kim et al. 2003), touch (Knight et al. 1991; Haley et al. 1995), gravity (Plieth and Trewavas 2002), osmotic and oxidative stress (Ng et al. 2001; Coelho et al. 2002), as well as biotic stimuli, e.g. hormones (Schroeder et al. 2001; Murata et al. 2001), fungal elicitors (Klusener et al. 2002), pathogens (Romeis et al. 2001) and nodulation factors (Levy et al. 2004), utilize calcium as a signalling factor. Although these disparate cues all trigger calcium releases, the cell's ultimate responses reflect the stimulus. Accordingly, the calcium paradox becomes apparent; How does the cell know which stimulus to react on, and how is a distinct response achieved? Several plausible and combinatorial theories have been presented: 1) Other signalling molecules may work in conjunction with calcium to trigger specific downstream effectors, 2) Depending on the primary messenger(s), different subcellular calcium-stores may be triggered, 3) The calcium signal may

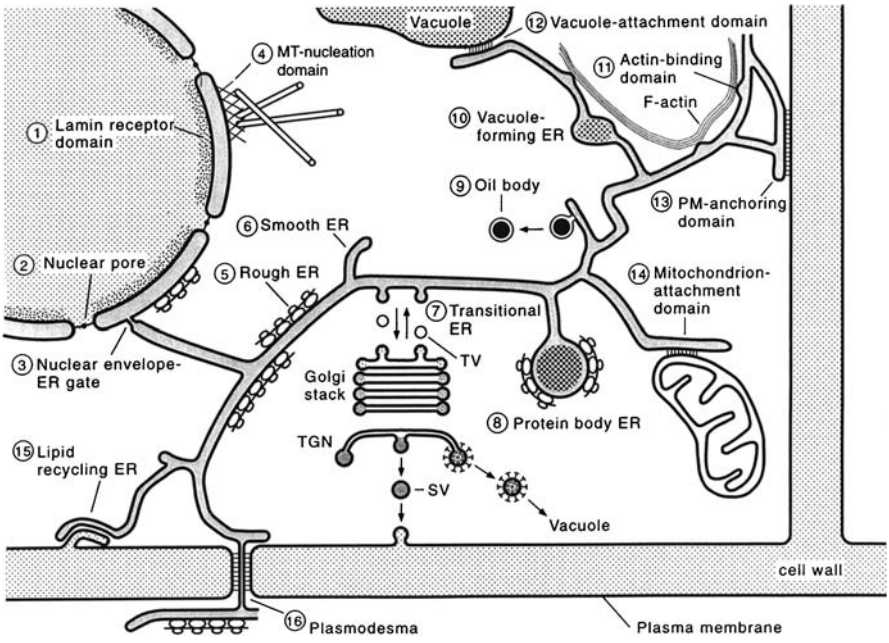

Fig. 1 Schematic model of different ER subcompartments in a plant cell. Reproduced from Staehelin (1997) with permission

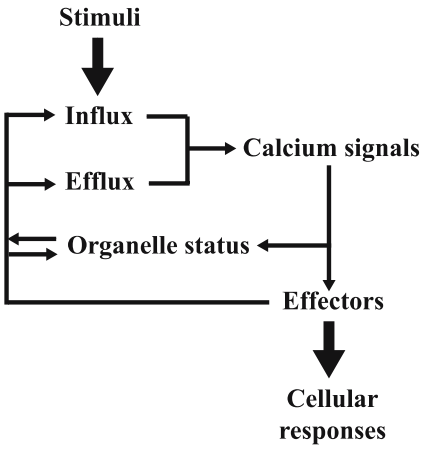

Fig. 2 Flow-chart of stimuli-induced Ca^{2+}-signalling network. Stimuli may activate calcium discharge from Ca^{2+}-holding compartments. The release of calcium affects downstream effectors and organellar processes. The effectors and the organellar status may in turn regulate the progression of the signalling pattern, i.e. the shape and size of the signal. The calcium signal is thus tightly monitored through a feedback network. Modified and extended from Sanders et al. (2002)

contain cues in itself, i.e. variations in amplitudes and frequencies of the signal, 4) The downstream effectors may differ depending on the developmental stage and type of cell that is triggered, 5) A sub-compartmentalization of calcium-release and uptake devices may generate calcium-denser areas in the cell. The latter provides an interesting aspect regarding the ER. At least 16 ER subcompartments have been described in plant cells (Fig. 1; Staehelin 1997). Several of these are in close proximity to other calcium-holding organelles and may therefore participate in calcium cross-talk between different stores.

Although calcium is well established as a cytosolic signalling source, the effects of calcium fluctuations on internal organellar processes are largely unexplored. Calcium fluxes over the ER membrane in animal cells may affect processes such as protein folding, secretion and glycosylation status. A dynamic view, incorporating cytosolic as well as organellar effects, of cellular calcium fluctuations may therefore be crucial to understand how calcium affects the physiology of the plant cell (Fig. 2).

2 Components

A reductionist approach to comprehend the ER calcium network requires the identification of its basic components. The ER in a typical plant cell contains Ca^{2+}-permeable channels, triggered by a variety of agonists, Ca^{2+}-pumps

Table 1 The major ER calcium network components in animals and plants

Components	Inhibitors	Activators	Physiological implications
Calcium permeable channels			
Animal:			
IP 3 -receptor (3)	High Ca^{2+}, CaM[1]	IP_3, Low Ca^{2+}, ATP, NADH, Phosphorylation[1]	Oozyte fertilization, Neuronal signalling[1]
Ryanodine- receptor (3)	High Ca^{2+}, Mg^{2+}, NO, CaM[2]	Ryanodine, cADPR, low Ca^{2+}, ATP, NO, CaM, Clq[2]	Mono- and divalent ion permeable[2]
Plant:			
IP_3-receptor (?)	—	IP_3[3]	—
Ryanodine-receptor (cADPR) (?)	Ruthenium red, 8-NH_2-cADPR[3]	cADPR[3]	—
NAADP- receptor (?)	Gd^{3+}, Ruthenium red[3]	NAADP[3]	—
LCC1- channel (?)	La^{3+}, Gd^{3+}, Erythrosin B[3]	Voltage-gated[3]	Ca^{2+}-, Ba^{2+}-, Sr^{2+}-permeable, Voltage-gated[3]
BCC1- channel (?)	Gd^{3+}, Cu^{2+}, Zn^{2+}, pH, H_2O_2[3]	Voltage-gated[3]	Ca^{2+}-, Ba^{2+}-, Sr^{2+}-permeable, Voltage-gated, Mechano-sensitive?[3]
Calcium efflux transporters			
Animal:			
Type IIA:			
SERCA1 (2)	Thapsigargin[4,5]		Brody's disease, Reduced Ca^{2+}-uptake[4,5]
SERCA2 (2)	Phospholamban, Sarcolipin, Thapsigargin[4,5]		Darier disease, Cardiac arrestment, Reduced Ca^{2+}-stores[4,5]
SERCA3 (5)	Thapsigargin[4,5]		Affect glucose homeostasis in pancreas, Altered Ca^{2+}-homeostsis in pancreas and aortic smooth muscle[4,5]

Table 1 continued

Components	Inhibitors	Activators	Physiological implications
Plant:			
Type IIA:			
ECA1	CPA, Vanadate, Erythrosin B[6]	—	Ca^{2+}- and Mn^{2+}-transport, Sensitivity to external Ca^{2+}-depletion, Cytoskeletal maintenance?[6]
Type IIB:			
ACA2	Vanadate, Erythrosin B, Phosphorylation[6]	CaM[6]	Ca^{2+}-transport[6]
Calcium binding proteins			
Animal:			
Calreticulin (2)	—	—	Chaperone, Ca^{2+}-homeostasis, Cardiac development, SERCA2B regulator, Cell adhesion, Immunology[7]
Calnexin/Calmegin (2)	—	—	Chaperone, Ca^{2+}-homeostasis, Fertility, SERCA2B regulator[8]
Calsequstrin (2)	Phosphorylation[9]	—	Ca^{2+}-homeostasis, Ryanodine-R regulator, Cardiac development, thiredoxin-related[9]
Plant:			
Calreticulin (3)	—	—	Chaperone, Ca^{2+}-storage, Sensitivity to external calcium depletion[10,11]
Calnexin (2)	—	—	Chaperone[10]

References: [1] Patterson et al., 2004; [2] Fill and Copello, 2002; [3] White, 2000; [4] Prasad et al., 2004; [5] Wuytack et al., 2002; [6] Sze et al., 2000; [7] Gelebart et al., 2005; [8] Michalak et al., 2002; [9] Beard et al., 2004; [10] Vitale and Denecke, (1999); [11] Persson et al., 2001 and specific references within these. Numbers in parenthesis indicate number of isoforms.

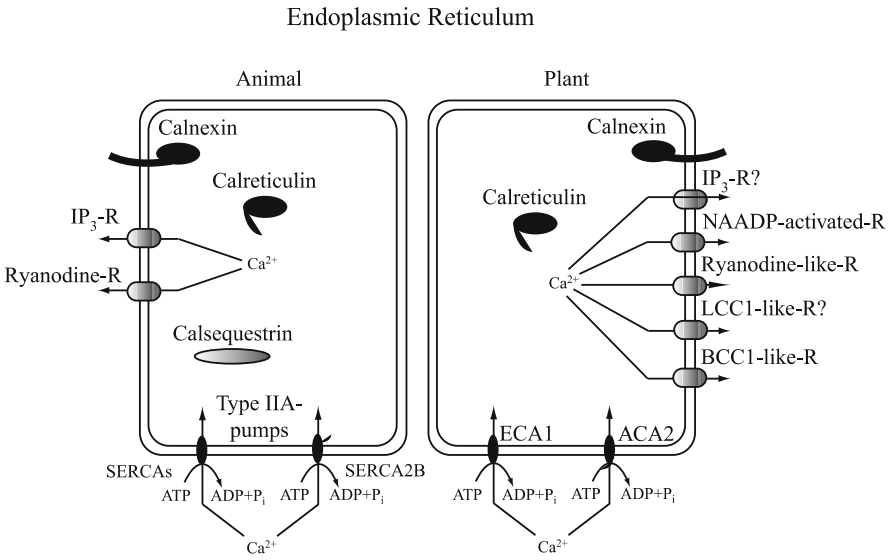

Fig. 3 Comparative representation of components of the ER calcium network in animals and plants. IP_3-R: Inositol 1,4,5-triphosphate-receptor, NAADP-R: Nicotinic acid adenine dinucleotide phosphate-receptor, BCC1: Bryonia calcium channel 1, LCC1: Lepidium calcium channel 1, ATP/ADP: adenosine triphosphate/diphosphate, SERCA: SR/ER calcium ATPase

and Ca^{2+}-binding proteins (Crofts and Denecke 1998; Sanders et al. 2002). These components were largely discovered and analyzed through biochemical efforts (for review see Bush 1993). Several of the components have subsequently been more thoroughly characterized, and placed into a cellular context, through multidisciplinary approaches (for review see Sanders et al. 2002). Data mining of the Arabidopsis and rice genomes have further increased the number of components connected to the ER calcium regulatory network in plants (Baxter et al. 2003; Persson et al. 2003). More recently, comparative analyses of calcium components in plants and animals have, not surprisingly, provided evidence for overlapping as well as diverging processes among the two kingdoms (Fig. 3, Table 1; Berridge et al. 2003; Reddy and Reddy 2004).

2.1 Calcium Permeable Channels

While the free cytosolic calcium level is kept at submicromolar levels, the total calcium concentration in the ER/Sarcoplasmic reticulum (SR) has been estimated to be 5–10 mM in animal cells (Meldolesi and Pozzan 1998). A similar level is also expected in typical plant ER (Sanders et al. 2002). The steep calcium gradient over the membrane may facilitate an instant discharge of

calcium from different organelles into the cytosol in response to agonists (Berridge et al. 2003). The calcium influx is mediated through ion channels, referred to as calcium permeable channels (Fig. 4; White et al. 2000). In plants, calcium channels have been detected in the ER (Klusener et al. 1995, 1999), the plasma membrane (PM), the tonoplast, the nucleus and the plastid envelope (for review see White 2000). The channels are classified according to the mode of activation, i.e. voltage-gated, ligand-activated, cytoskeletal connectivity and/or membrane stretch activation.

ER-localized calcium channels have only recently begun to get characterized in plants (Table 1; Klusener et al. 1995, 1997, 1999). The first ER channel to be identified was purified from touch-sensitive tendrils of the dicot plant *Bryonia dioica* and was referred to as BCC1 (Klusener et al. 1995, 1997). This voltage-gated channel was analyzed through lipid bilayer techniques and exhibited a high calcium conductance at low pH (Klusener et al. 1995, 1997). Inhibition studies further showed that BCC1 is sensitive to Gd^{3+}, H_2O_2, Cu^{2+} and Zn^{2+}.

Another putative ER calcium permeable channel (LCC1) was purified from garden cress (*Lepidium sativum* L.); Klusener et al. 1999). Characterization via lipid bilayer techniques suggested that the channel is voltage gated and strongly rectifying. The LCC1 channel further showed a simpler gating mechanism for calcium release than BCC1 (Klusener et al. 1999). LCC1 may be inhibited by both erythrosine B and the trivalent cations La^{3+} and Gd^{3+}. Although some similarities are evident between the two channels, the kinetic and pharmacological properties are distinctly different (Klusener et al. 1999). These observations suggest that the two channels are not simply orthologs from two different plant species. However, whereas the BCC1 appeared to

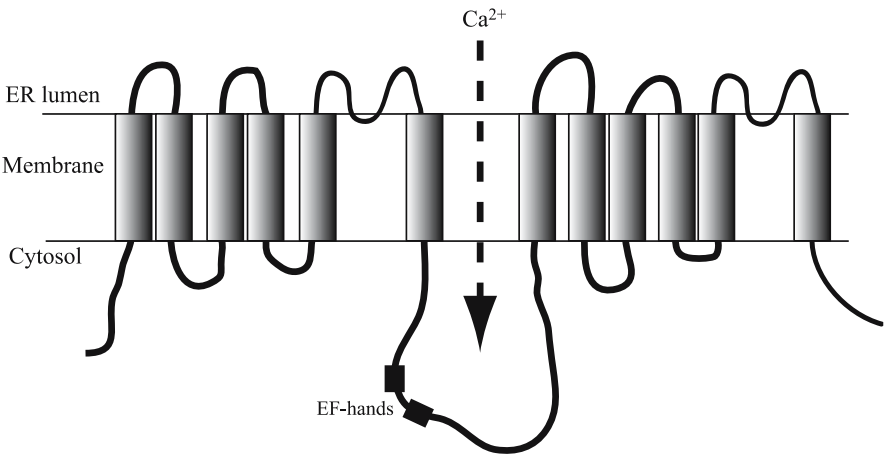

Fig. 4 Schematic topological model of a PM voltage-gated Ca^{2+}-permeable channel (TPC1). Putative EF-binding hands are indicated

be highly enriched in ER membrane fractions, an alternative location of the LCC1 is plausible (Klusener et al. 1999).

Additional calcium permeable channels have also been implicated for the plant ER (Table 1; Muir and Sanders 1997; Navazio et al. 2000, 2001). Radiolabelled calcium was used to show that influx of calcium could be mediated by applying either nicotinic acid adenine dinucleotide phosphate (NAADP), cyclic-ADP-ribose (cADPR) or inositol-1,4,5-triphosphate (IP_3), all potent calcium elicitors in animal cells. NAADP may trigger intracellular Ca^{2+} releases in animal cells (for review see Patel 2004). An NAADP-sensitive Ca^{2+}-store was detected in ER-enriched membrane fractions from cauliflower (Navazio et al. 2000). The channel was independent of the cytosolic level of free calcium, and thus not responsible for calcium induced calcium releases (Navazio et al. 2000). Surprisingly, the Ca^{2+}-release was insensitive to typical L-type channel antagonists, i.e. verapamil and diltiazem.

In contrast to the novelty of NAADP-sensitive calcium stores in plants, cADPR-sensitive stores have been detected in the tonoplasts (Allen et al. 1995). A potential non-vacuolar cADPR-releasing activity was, furthermore, detected in ER-enriched fractions from cauliflower (Navazio et al. 2001). Analogous to the vacuolar cADPR-activated Ca^{2+}-release, the ER-associated release was fully inhibited by ruthenium red and 8-NH_2-cADPR, a specific antagonist for cADPR-gated channels in animal cells (Navazio et al. 2001). IP_3 is the most prevalent Ca^{2+}-releasing agonist for the ER/SR in animal cells (Berridge et al. 2003), and IP_3-triggered Ca^{2+}-releases have been observed from tonoplasts in plants (Allen et al. 1995). However, using microsomal fractions from cauliflower, Muir and Sanders (1997) demonstrated a non-vacuolar IP_3-sensitive calcium store. IP_3 may furthermore bind ER-enriched membrane fractions from *Chenpodium rubrum*, suggesting a potential IP_3-triggered calcium pool in the ER (Martinec et al. 2000).

The diversity of ER Ca^{2+}-releasing agonists further emphasizes the importance of the organelle in different intracellular Ca^{2+}-signalling events. However, similar to the identification of LCC1, the localization of the cADPR-, IP_3- and NAADP-releasing sources, i.e. the ER, in plants were achieved by enriching membrane fractions through sucrose gradient separation (Muir and Sanders 1997; Navazio et al. 2000, 2001). The NAADP-releasing source in animal cells appears, however, to be clearly separated from the ER/SR compartment (for review see Patel 2004). Identification of the genes encoding ER localized calcium channels may therefore provide further evidences to the subcellular localization of the cADPR-, IP_3- and NAADP-sensitive calcium stores.

2.2 Calcium Efflux Transporters

The activity of a variety of Ca^{2+}-transporters are coordinated with the calcium channels to tune the frequencies and amplitudes of the progressing

calcium signal and are used to restore resting cytosolic calcium levels following the signalling event. Two major classes of Ca^{2+}-efflux transporters exist in plants: Ca^{2+}/H^{+}-antiporters and Ca^{2+}-pumps. The Ca^{2+}/H^{+}-antiporters are driven by a proton gradient (Hirschi 1999), whereas the Ca^{2+}-pumps (Ca^{2+}-ATPases) are directly coupled to ATP hydrolysis as an energy source (Sanders et al. 2002).

The Ca^{2+}-pumps and Ca^{2+}/H^{+}-antiporters have distinctly different kinetic properties. Whilst the antiporters have low affinity, but high transporting capacity for calcium (Pittman and Hirschi 2003), the pumps exhibit opposite properties (Sze et al. 2000). These characteristics suggest distinct complementary roles in the calcium efflux process, where the low affinity antiporters rapidly remove the majority of cytosolic calcium and the high affinity pumps fine-tune the basal cytosolic calcium levels. These complementary activities make sense in subcellular locations in which both efflux systems reside, such as the vacuole and the PM (Hirschi 1999; Kasai and Muto 1990; Sanders et al. 2002). Antiporters are, however, not expected to function in the ER, since the proton gradient over the ER membrane is believed to be too weak to drive proton-coupled antiporters. Thus, the dynamics of Ca^{2+} transport into the ER are controlled exclusively by Ca^{2+}-ATPases, providing a potentially important distinction from the multiple transporters systems operating at vacuoles or PM.

A compelling difference between calcium transport into the plant and animal ER is that plants utilize two types (IIA and IIB) of Ca^{2+}-ATPases instead of one (Table 1; Sze et al. 2000; Geisler et al. 2000). The type IIA pumps are found in the ER of both plants and animals. Due to their subcellular location, these pumps are referred to as ER-type calcium pumps. The type IIA pumps are not stimulated by calmodulin (CaM), but are inhibited by cyclopiazonic acid (CPA) and thapsigargin, two well-characterized inhibitors of animal ER/SR Ca^{2+}-ATPases. In contrast to animals, the plant ER is also equipped with a calmodulin-stimulated pump that belongs to the type IIB family. In animals, the type IIB pumps are instead exclusively localized to the PM. Because of their initial discovery in the PM of animal cells, they are referred to as PM-type Ca^{2+}-ATPases (PMCA). Since the type IIB type pumps are not restricted to a single membrane system in plants, they are referred to as ACAs (Autoinhibited Ca^{2+}-ATPases). In contrast to the type IIA (ER-type) pumps, the type IIB (ACA-type) pumps are stimulated by CaM, and are relatively insensitive to CPA and thapsigargin. Members of both type IIA and B pump families are clearly distinguished by sequence homologies and appear to have emerged before the separation of the plant and animal kingdoms (Sze et al. 2000; Baxter et al. 2003). The presence of two distinct types of Ca^{2+}-ATPases further illustrates the complexities in regulating calcium transport into the plant ER.

The Arabidopsis genome contains fourteen genes encoding Ca^{2+}-pumps (Sze et al. 2000; Baxter et al. 2003). Four genes belong to the type IIA (ER-type) family and are referred to as ECAs (ER-type Ca ATPases). There is

immunological and membrane fractionation evidence that ECA1 is most abundant in the ER, providing corroborating evidence that at least some members of this plant family are ER localized. The remaining 10 genes belong to the type IIB (ACA-type) family. Similar to ECA1, immunology and microsomal fractionation strongly indicates that ACA2 is most abundant in the ER. However, other members of the plant ACA family have been found to reside in the vacuole or PM. Thus, the CaM-regulated ACAs may be used in multiple subcellular locations. Of the 10 ACAs in Arabidopsis, a total of 3 isoforms are closely related to ACA2 and are expected to be ER localized (Baxter et al. 2003).

The mechanism of CaM activation of type IIB pumps has been examined for both plant (Sze et al. 2000) and animal isoforms (Clapham 1995). The animal type IIB pumps contain an autoinhibitor and CaM binding sequence located in the C-terminal domain. However, the plant ACAs have their autoinhibitor and CaM binding sequence at the opposite end of the protein, i.e. N-terminal domain (Fig. 5; Malmström et al. 1997; Harper et al. 1998; Baxter et al. 2003). The model for CaM-activation involves interaction of CaM to a site overlapping or juxtaposed to the autoinhibitor in the pumps. This binding event somehow disengages the autoinhibitor. A mutation that removes this N-terminal regulatory domain results in a constitutively active pump (Harper et al. 1998).

A useful tool for dissecting biochemical properties of both ACA and ECA Ca^{2+}-pumps has emerged through the use of heterologous expression in the K616 mutant strain of the yeast *Saccharomyes cerevisiae*. In the mutant, a deletion of two Ca^{2+}-pumps; PMR1 in the Golgi and PMC1 in the vacuolar membrane, result in a yeast strain that requires high levels (> 1 mM) of calcium for normal growth (Sze et al. 2000). The growth defect on calcium-depleted media appears to result from the inability to load the ER secretory system with sufficient amounts of calcium. The phenotype was complemented by the expression of the plant ECA1. Biochemical analyses of ECA1 expressed in the K616 yeast system furthermore confirmed that the activity of ECA1 was CPA sensitive, and that the pump was not stimulated by CaM (Liang et al. 1997; Liang and Sze 1998). In addition, the yeast system provided evidence that ECA1 has a dual capacity to transport both Ca^{2+} and Mn^{2+} (Wu et al. 2002). A biological function for Mn^{2+} transport by ECA1 is supported by the observation that a deletion of *ECA1* in Arabidopsis exhibits an increased sensitivity to Mn^{2+} toxicity.

In contrast to ECA1, ACA2 did not rescue the growth of yeast K616 on calcium-depleted media (Harper et al. 1998). However, the pump was able to complement the yeast strain when the N-terminal autoinhibitory domain was truncated. Using this observation as the foundation for a mutant screen, seven random intragenic mutations were identified that allowed ACA2 to complement the yeast strain (Curran et al. 2000). Three of these mutations were unexpectedly found in the stalk that connects the ATPase and trans-

membrane domains, a region outside the N-terminal autoinhibitory domain and not previously recognized as having regulatory functions. These new mutations implicate the stalk region as an important point of control for ATPase activity, possibly through a direct interaction with the autoinhibitor (Fig. 5). An analogous stalk mutation engineered into an animal PMCA-pump sug-

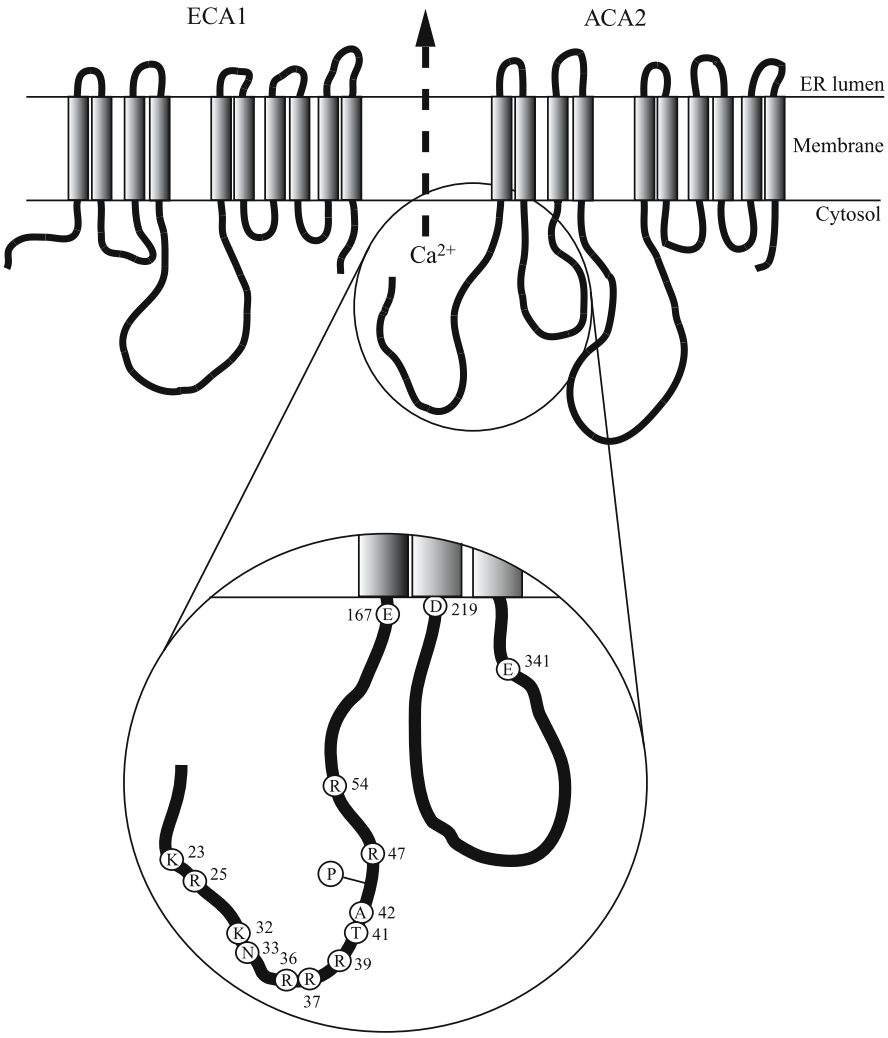

Fig. 5 Schematic topological models of the ER Ca^{2+}-ATPases, ECA1 and ACA2, in plants. The N-terminal region in ACA2 is enlarged to show amino acids involved in the autoinhibition of the ATPase (The corresponding numbers of the amino acids are indicated). The inhibitory phosphorylation site (Ser^{45}) on ACA2 is indicated with a **P**

gests that this region has general importance for regulation of all members of the type IIB pumps (Bredeston and Adamo 2004).

The distinct and redundant functions of the ECA and ACA pumps in the plant ER have yet to be delineated. However, the observation that ACA2 is CaM-activated identifies CaM as part of a feed-back regulatory pathway that controls the calcium dynamics associated with an ER calcium release. In addition, ACA2 may be inhibited by a separate calcium signalling pathway involving a plant-specific calcium-dependent protein kinase (CDPK; Hwang et al. 2000). Thus, the presence of both an ACA and ECA in the plant ER strongly suggests that plants carefully regulate the ER calcium efflux, and that this regulation is directly connected to feed back control by calcium signalling pathways.

2.3 Calcium-binding Proteins

The Ca^{2+}-holding capacity of different organelles is crucial for the calcium signalling system to perform efficiently. Maintenance of Ca^{2+}-buffering devices inside the organelles may therefore be essential to the cell (Crofts and Denecke 2001; Corbett and Michalak 2000). The ER/SR in animal cells contains a variety of calcium-binding proteins, e.g. calreticulin (Crt), calnexin (Cnx) and calsequestrin (Clq) (Corbett and Michalak 2000). These proteins may perturb both the calcium levels, as well as the influx/efflux efficiency, of the ER/SR (Camacho and Lechleiter 1995; Arnaudeau et al. 2002). Several homologs to these proteins have also been identified in higher plants (Fig. 6, Table 1; Vitale and Denecke 1999). In addition, other plant compartments, e.g. the vacuole and cell wall, also appear to hold Ca^{2+}-binding proteins (for review see Hirschi 2004).

The two major ER Ca^{2+}-binding proteins are Crt and Clq in animal cells (Table 1; Michalak et al. 1999). Whereas plant cells do contain Crt homologs (Vitale and Denecke 1999), no closely related Clq homologs have been identified. Crt is an ER luminal Ca^{2+}-binding chaperone, which holds three distinct domains; An N-terminal N domain with conserved histidine residues, a P do-

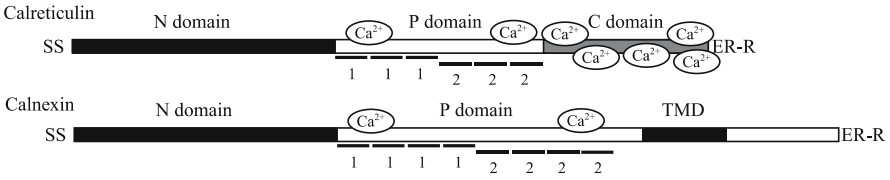

Fig. 6 Schematic representation of the two Ca^{2+}-binding proteins Crt and Cnx. Approximate regions for the N, P and C domains are indicated. Two repeat segments in the P domain are indicated with **1** and **2**, respectively. ER-R: ER retention signal, SS: Signal sequence, TMD: Transmembrane domain

main with low capacity but high affinity Ca^{2+}-binding properties and a C domain with high capacity but low affinity Ca^{2+}-binding properties (Fig. 6; Michalak et al. 1999). The protein can bind approximately 40 moles calcium per mol protein, mainly facilitated through the high capacity binding C domain (Baksh and Michalak 1991). Hence, the ER calcium content may depend on the Crt protein levels. Several studies in animal cells have also shown that overexpression of Crt leads to both an increase in the Ca^{2+}-signalling pool, and in free calcium levels, of the ER (Mery et al. 1996; Arnaudeau et al. 2002). Although no direct evidences for Crt as an effector of cytosolic calcium signalling exists in plants, Crt has been shown to be a potential effector of the ER calcium holding capacity and to protect against changes in external calcium levels (Persson et al. 2001; Wyatt et al. 2002; Åkesson et al. 2005).

Structural analyses of the central P domain of a rat Crt revealed an extended hairpin structure, comprised of three antiparallel β-sheets (Ellgaard et al. 2001). A similar structural feature is also evident in Crt's membrane bound homolog Cnx (Schrag et al. 2001). A putative calcium-binding site was indicated between Asp437, Asp118 and Ser75 in Cnx and was suggested as having a conformational role rather than directly affecting ligand-binding characteristics (Schrag et al. 2001). The tight coordination of the bound calcium to the P-domain of Crt/Cnx also implies constant maintenance of the bound calcium, even during prolonged depletion of the ER calcium (Schrag et al. 2001).

Whereas two Crt isoforms are present in animals (Persson et al. 2002), higher plants contain three Crt isoforms; Crt1a, Crt1b and Crt3 (Persson et al. 2003). While Crt1a and b show high sequence similarity (86% identity), Crt3 is distinctly different in several aspects. For example, both Crt1a and b contain higher levels of negatively charged amino acids in their C domains compared to Crt3 (approximately 35 and 25%, respectively). The high amount of negatively-charged amino acids in the C-domain is suggested to provide the main Ca^{2+}-binding characteristics for the protein (Baksh and Michalak 1991). However, no study has provided evidences for a correlation between the Ca^{2+}-holding potential of Crt and the amount of negatively-charged amino acids. A comparison between the three Crt isoforms in higher plants should therefore be relevant to resolve the Ca^{2+}-holding capacity of Crt.

Preliminary large-scale microarray data analyses have revealed that Crt1a and b are expressed in a very similar manner over approximately 1500 microarray datasets (Nottingham Arabidopsis Stock Center (NASC); Thelin and Persson, unpublished data). Several other ER chaperones, e.g. protein disulfide isomerase (PDI)-related homologs, also show a high degree of coexpression with Crt1a and b, indicating presence of an interactive chaperone matrix in the ER. Crt3, on the other hand, did not show coexpression with any known chaperone-like proteins, but instead exhibited coexpression with an iron-binding oxygenase family member and several pathogen-related proteins (Thelin and Persson, unpublished data). This further underscores a func-

tional dichotomy for the Crt family (Persson et al. 2003), and may provide insight to the physiological context of different Crts.

3 Orchestration of the Components

Identification and characterization of the components of the ER calcium network have provided important information to how these components may be regulated individually. A tight coordination of these components is, however, necessary to regulate both calcium fluxes, i.e. the amplitude and frequency of the cytosolic signal, as well as the internal ER calcium level. The challenge therefore lies in how these components act together, and affect each other, to achieve this regulation. While several aspects of how such coordination affects the calcium status of the ER/SR are becoming resolved in animal cells, analogous information is still largely lacking in plants.

The basic regulatory function is achieved through transcriptional coordination of the various components. This may be facilitated by the level of luminal ER calcium level, which works as a transcriptional switch for several components of the ER calcium network in animal cells (Waser et al. 1997). Both SERCA3 and Crt expressions are induced in response to depletion of ER calcium levels, e.g. by treatments with the ER Ca^{2+}-pump inhibitor thapsigargin or the calcium ionophore A23187, implying an attempt of the cell to increase the Ca^{2+}-holding potential of the ER (Waser et al. 1997; Liu et al. 2002).

The ER luminal proteins Cnx and Crt may coordinate the ER calcium dynamics in animal cells, i.e. influx, efflux and holding potential (Camacho and Lechleiter 1995; Michalak et al. 1999; Arnaudeau et al. 2002). Cnx and Crt can modulate the uptake of calcium by direct interactions with the Ca^{2+}-pump SERCA2B (Camacho and Lechleiter 1995; Roderick et al. 2000). In contrast to other SERCAs which consist of ten transmembrane segments, SERCA2B has eleven transmembrane regions (Bayle et al. 1995). Consequently, the carboxy terminus of the protein is located within the ER lumen. Crt may directly interact with the luminal carboxy terminus of SERCA2B, a characteristic possibly regulated by the glycosylation status of the pump (John et al. 1998). Coexpression of Crt and SERCA2B in *Xenopus* oocytes revealed that Crt effectively inhibits IP_3-mediated calcium oscillations, resulting in a sustained elevation of cytosolic calcium (John et al. 1998). In addition, Cnx may also affect SERCA2B activity, possibly through a direct interaction with the pump (Roderick et al. 2000). Analogous to Crt, coexpression of Cnx and SERCA2B in *Xenopus* oocytes caused sustained elevation of cytosolic calcium, generated through inhibition of SERCA2B. The inhibition was promoted by phosphorylation of a serine residue in the cytosolic carboxy-terminal of Cnx (Roderick et al. 2000). The two chaperones Cnx and Crt may thus both affect the ER

Ca^{2+}-uptake, coordinated through either internal ER processes or through cytosolic phosphorylation events.

Plants lack a SERCA2B homolog with a C-terminal luminal extension, and a direct effect on Ca^{2+}-fluxes by any luminal ER Ca^{2+}-binding proteins remains to be demonstrated. However, the activity of the Ca^{2+}-pump ACA2 may be regulated by phosphorylation (Hwang et al. 2000). The phosphorylation occurs in the cytosolic N-terminal region and inhibited the activity of the pump (Fig. 5). As described above, CaM may activate ACA2 by interacting with the autoinhibitory N-terminal domain of the pump. When CaM was bound to the pump, the inhibitory phosphorylation was prevented (Hwang et al. 2000). The "signalling" dynamics of the cytosol may thus orchestrate an activation or inhibition of ACA2. The phosphorylation may be mediated through a CDPK referred to as CPK1 (Hwang et al. 2000). So far only one CDPK, CDPK2, has been shown to localize to the ER (Lu and Hrabak 2002). Whether this CDPK phosphorylates ACA2 in vivo has not yet been tested.

Crt may also be post-translationally modified through phosphorylation and glycosylation (Baldan et al. 1996; Li et al. 2003; Persson et al. 2003). In vitro studies suggest that the phosphorylation is mediated through a casein kinase II/CK2 (Baldan et al. 1996). Whether this kinase can enter the ER lumen remains undetermined. Although the glycosylation status of animal Crts is suggested to mediate a cellular redistribution of the protein, no physiological functions for either glycosylation nor phosphorylation are so far apparent for plant Crts.

4 Cellular and Physiological Implications

The ability to coordinate the Ca^{2+}-signalling network, i.e. to achieve specific spatiotemporal characteristics of the signal, is of great importance for an agonist-stimulated signal to progress properly. Altering the abundance of different Ca^{2+}-signalling components may consequently skew the calcium signal and hence affect different sets of downstream targets. These targets may range from signal transmitters to signal effectors (Sanders et al. 2002). The signal transmitters, such as transcription factors and CaM-like proteins, may undergo conformational changes in response to Ca^{2+}-fluxes, which alter their binding ability to target molecules. In contrast, the signal effectors, such as various kinases and phosphatases, may directly trigger a covalent modification of their target molecules.

A major breakthrough in the visualization of calcium signals has been the development of calcium sensitive probes (Knight et al. 1991; Miyawaki et al. 1997). The most widely used probes are aequorins and cameleons. Whereas the aequorins are useful when measuring calcium changes in whole tissues

or a cell population, the cameleons can be used to measure Ca^{2+}-oscillations on a single-cell level. The cameleon construct consists of a recombinant protein based on a CaM flanked by two fluorescent proteins (Fig. 7A), which can be transformed into any organism and targeted to different organelles. When the calcium levels increase the CaM backbone undergoes a conformational change which brings the two fluorescent proteins in close proximity to each other (Fig. 7A; Miyawaki et al. 1997). By choosing fluorescent protein-parts with different excitation and emission wavelengths, the energy from one of the fluorescent protein-parts may be transferred to the other, referred to as fluorescent resonance energy transmission (FRET).

Combining cameleons with reverse genetic and molecular approaches has begun to increase our understanding for how different components affect the cellular calcium status in plants (Allen et al. 1999, 2000). This approach was used to dissect how a vacuolar H^+-ATPase affects Ca^{2+}-signalling in guard cells (Allen et al. 2000). By introducing cameleons into a line with a mutated vacuolar H^+-ATPase (*det3*), Allen et al. (2000) showed that the spatiotemporal calcium patterns generated by different stimuli changed in the mutant line compared to wild-type, and resulted in altered physiological responses of the guard cell (Fig. 7B,C; Allen et al. 2000). This report provided the first genetic evidence that distinct stimulus-specific calcium oscillations may facilitate specific physiological responses in plants.

The only component in the plant ER calcium network that has been investigated so far using genetic approaches is the Ca^{2+}-pump ECA1 (Wu et al. 2002). Disruption of the *ECA1* gene did not result in any visible phenotype when grown on standard Murashige Skoog growth medium (Wu et al. 2002). However, when grown on either low calcium (0.2 mM) or high manganese (0.5 mM) levels, seedlings grew very poorly (Fig. 8A). This may be due to an inhibition of cell expansion or cell division (Wu et al. 2002), similar to what has been reported for the yeast *pmr1* mutant, lacking a Golgi Ca^{2+}/Mn^{2+}-pump (Durr et al. 1998). In addition, the *pmr1* mutant showed defects in protein sorting and glycosylation processes, indicating a link between calcium and internal ER processes. Perturbing the cellular calcium homeostasis in plants by deletion of *ECA1* related Ca^{2+}-pumps may also affect intracellular organization (Adamikova et al. 2004). Disruption of an *ECA1* homolog in the fungus *Ustilago maydis* resulted in randomization of the microtubule network due to a sustained elevation of cytosolic calcium (Adamikova et al. 2004).

Overexpression of *CRT* also results in a conditional growth phenotype (Persson et al. 2001). Arabidopsis seedlings expressing a maize Crt1a/b homolog showed higher resistant to low levels of calcium in the growth medium (Fig. 8B). The increase in *CRT* expression further mediated a higher Ca^{2+}-holding potential of the ER in vitro (Persson et al. 2001). In addition, Arabidopsis plants expressing GFP fused to the high capacity Ca^{2+}-binding C domain of Crt showed an overall increase in cellular calcium (Wyatt et al. 2002).

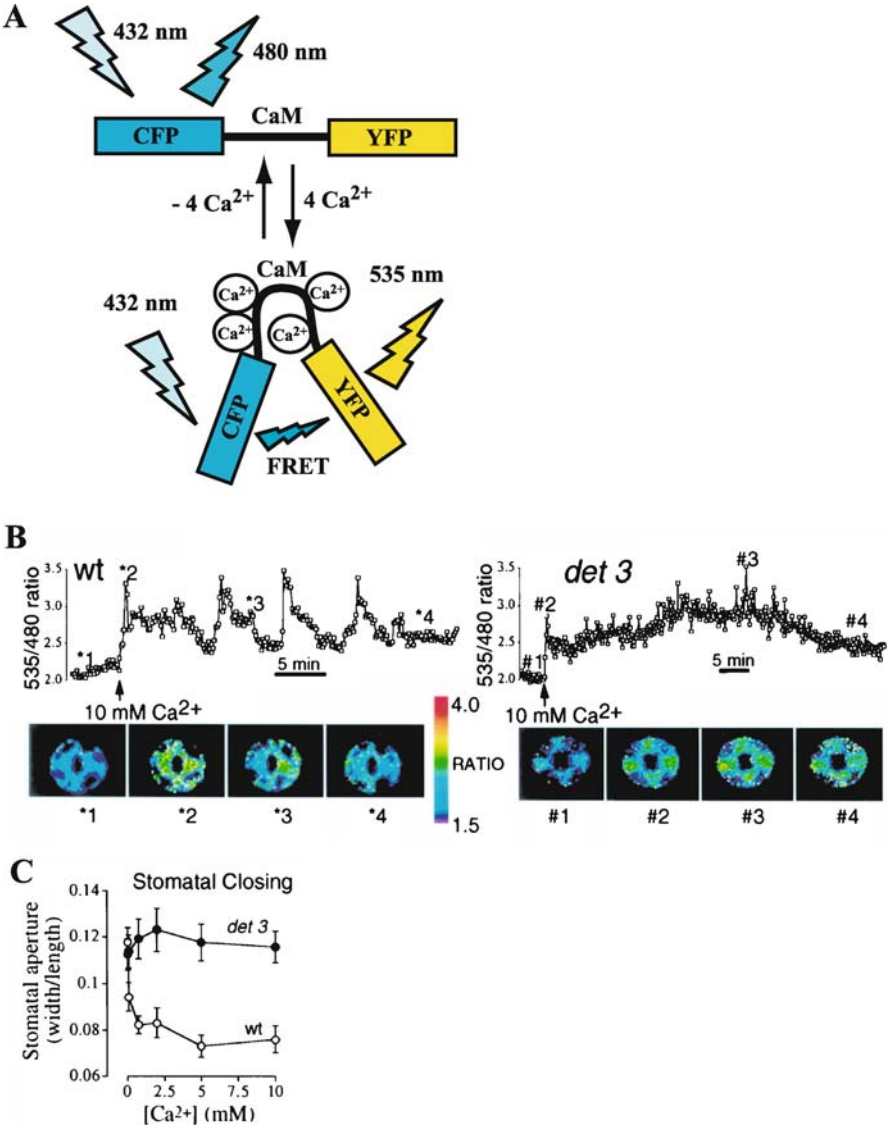

Fig. 7 Schematic model of the Ca^{2+}-indicator cameleon and its utilization in measuring defective Ca^{2+}-oscillations in the *det3* mutant. **A** The cameleon molecule, and its activation, consisting of a calmodulin (CaM) backbone fused to two fluorescent proteins. Modified from Miyawaki et al. (1997). **B** Application of external calcium evokes differences in cytosolic calcium responses in guard cells from wild-type and *det3* mutants. Intracellular Ca^{2+}-oscillations were induced applying 10 mM external calcium. Whereas the wild-type exhibited oscillatory Ca^{2+}-signals, Ca^{2+}-oscillations were abolished in the *det3* mutant. **C** The increase in external calcium caused stomatal closure in wild-type but not in the *det3* mutant. Reproduced from Allen et al. (2000) with permission. YFP: *Yellow* fluorescent protein, CFP: *Cyan* fluorescent protein, FRET: Fluorescent Resonance Energy Transfer

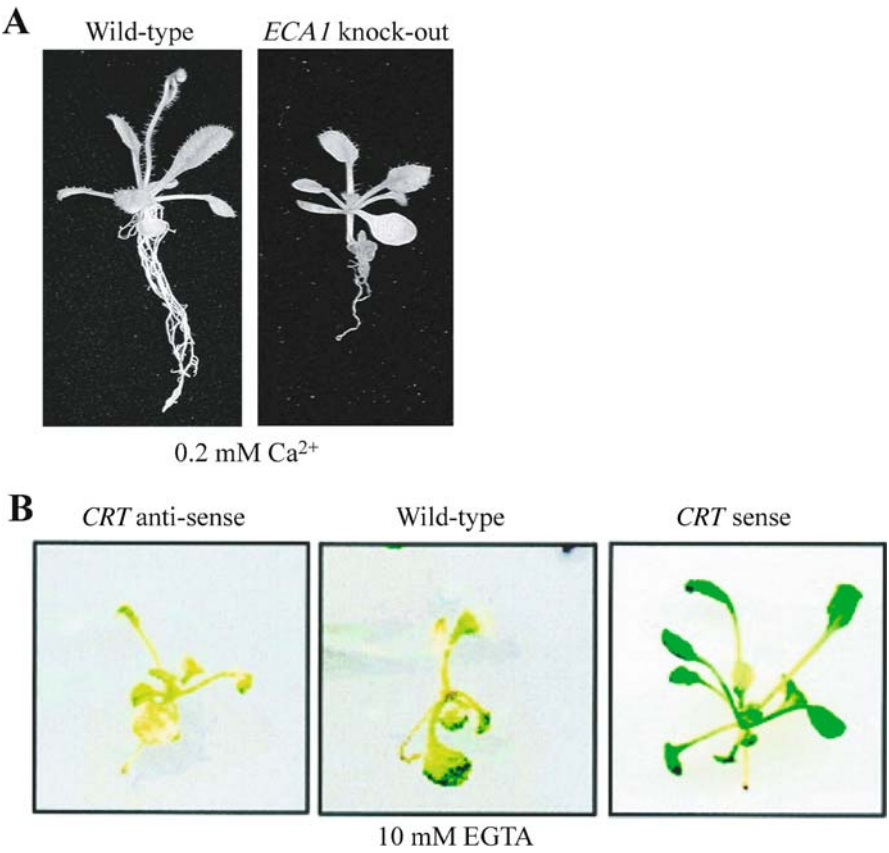

Fig. 8 Conditional growth phenotypes exhibited in *ECA1* knock-out plants and seedlings over expressing *CRT*. **A** Deletion of the *ECA1* gene results in delayed growth on medium containing reduced levels of calcium. Seedlings (5-day-old) transferred from MS medium to medium containing reduced calcium and grown for 10 days. **B** Correlation of *CRT* expression and seedlings ability to maintain growth on calcium-depleted medium. Seedlings (19 day-old) transferred from MS medium to medium containing reduced calcium (10 mM EGTA) and grown for 9 days. Reproduced from Wu et al. (2002) and Persson et al. (2001) with permission

The unique and redundant functions of different calcium stores in plant cells are not fully understood. However, calcium signals originating from the ER are expected to affect localized and global events, e.g. regulation of ER-generated calcium signatures and transcriptional events. The components decoding these calcium signals include CDPKs, CaM, as well as many of the other approximately 400 proteins in plants that have defined calcium binding EF-hands (Reddy and Reddy 2004). Of the 34 identified CDPKs in Arabidopsis (Harper and Harmon 2004), only AtCPK2 has been shown to localize to the cytosolic surface of the ER (Lu and Hrabak 2002). There is experimental

evidence that this association is dependent on myristoylation at the N terminus of AtCPK2. While the in vivo target substrates of CPK2 have yet to be determined, CDPKs may phosphorylate the ER calcium pump ACA2 (Hwang et al. 2000). In this case, phosphorylation is predicted to slow the rate of calcium efflux back into the ER, thereby possibly increasing the duration and magnitude of a calcium signal in the micro-environment around the ER. Since CDPKs are thought to be multifunctional kinases (Harper et al. 2004), many ER surface proteins are expected to be regulated by calcium signals decoded by isoform CPK2.

While investigations of how altered levels of Ca^{2+}-signalling components affect physiological responses have been initiated in plants, potential cross-talk between different Ca^{2+}-containing organelles are still largely unexplored. The ER calcium status in animal cells is tightly linked to Ca^{2+}-channels at the PM (Randriamampita and Tsien 1993; Fasolato et al. 1993; Putney et al. 1999). Consequently, depletion of the ER calcium stores activates the PM Ca^{2+}-channels and allows for refilling of the ER stores. This feedback process is referred to as capacitative calcium entry (Putney et al. 2001). The presently favoured model for capacitative calcium entry is a direct communication between PM Ca^{2+}-channels and the cADPR activated ryanodine receptors in the ER (Putney et al. 2001). The tight monitoring of the ER calcium status further strengthens the notion that the calcium ion is essential for proper maintenance of a variety of ER functions.

The ER calcium fluxes in animal cells also appear to influence the calcium status of the mitochondria (Rizzuto et al. 1993). This may be facilitated via the "hot-spot" hypothesis, in which IP_3-activated receptors in the ER are enriched at ER/mitochondria interfaces (Rizzuto et al. 2004). When calcium is released through the receptors, mitochondrial Ca^{2+}-uniporters facilitate uptake of calcium into the mitochondria. The increase in mitochondrial calcium may activate ATP production, but could also alter the organelle structure and trigger a release of apoptosis-activating substrates (Rizzuto et al. 2004).

Potential organellar cross-talk in the plant cell may, however, offer clues to phenotypic behaviours generated by genetic and molecular procedures. As discussed above, lower expression of either *CRT* or *ECA1* results in reduced growth on medium containing low levels of calcium (Fig. 8; Persson et al. 2001; Wu et al. 2002). These phenotypes were largely attributed to an overall decrease in ER calcium. However, overexpression of the vacuolar Ca^{2+}/H^+-antiport, *CAX1*, in tobacco displayed severe symptoms of calcium deficiency and contained a two-fold increase in cell calcium (Hirschi 1999). The phenotype could be reversed by adding exogenous calcium. Thus, a decrease in expression of at least two ER calcium network components, and an increase in expression of a vacuolar calcium network component, causes similar phenotypes. The explanation for this deceptive contradiction may lie in the distribution of calcium between the two organelles. If we assume that the protein level of a specific calcium handling protein is directly related to its

activity, then the decrease in both Crt and ECA1 should result in lower ER calcium levels. Similarly, increasing the *CAX1* expression may increase the vacuolar calcium efflux efficiency and therefore reduce the levels of accessible calcium for other organelles, e.g. the ER. Introducing cytosolic- and ER-targeted cameleons in crossed combinations of Crt, ECA1 and CAX1 plants should certainly present useful tools for visualization of potential cross-talk between the vacuole and the ER.

5
Calcium and Internal ER Regulation

It is well established that calcium works as an important cytosolic signal. In addition, the levels of calcium may be a potent switch for internal processes in various oganelles (Corbett and Michalak 2000; Rizzuto et al. 2004). The release and subsequent uptake of calcium during signalling events, creates continuous fluctuations of the available calcium in the organelle (Yu and Hinkle 2000). In animal cells, changes in the free ER calcium levels may control a variety of processes, e.g. protein synthesis and secretion (Corbett and Michalak 2000). Consequently in animal cells several ER chaperones, i.e. folding mediators, are regulated by calcium (Corbett et al. 2000; Frickel et al. 2002). Both Crt and Cnx interact with their substrates in a Ca^{2+}-dependent manner in animal cells. In vitro studies show that the lectin-like properties of these chaperones are significantly reduced when the calcium concentrations drop to levels observed in Ca^{2+}-depleted ER (Vassilakos et al. 1998). Furthermore, the latter unifies the dual functions of these proteins in the ER lumen; i.e. the chaperone and calcium-regulatory characteristics.

Expression of recombinant Crt proteins in a *CRT* knock-out mouse cell background, further emphasized the intimate link between the chaperone and calcium-regulatory aspects of Crt (Nakamura et al. 2001). Cells expressing *CRT* lacking its major Ca^{2+}-binding C-terminal domain exhibited a substantial decrease in the bradykinin-sensitive Ca^{2+}-signalling pool. The agonist bradykinin binds to a PM localized receptor protein and triggers an internal IP_3-induced ER calcium release. However, when measuring the binding of bradykinin to the receptor, Nakamura et al. (2001) discovered that the binding was severely impaired compared to control cells. The observed decrease in the ER calcium signalling may thus be explained by a misfolded bradykinin receptor.

Crt and several PDI-related proteins interact with each other in a Ca^{2+}-dependent manner in animal cells (Corbett et al. 1999). Interactions have been observed between PDI and Crt, which mainly seem facilitated by the high calcium capacity binding C domain in Crt and are reversibly increasing when the ER calcium levels are low. The interaction between Crt and PDI inhibits the isomerase activity, and thus the chaperone activity, of PDI

(Baksh et al. 1995). Crt can also interact with the PDI related protein ERp57. The initial interaction between Crt and ERp57 is independent of calcium concentration. However, the conformation of ERp57 may be indirectly altered through Crt at higher levels of calcium. This change presumably results in a tighter interaction between the two chaperones and the substrate. In addition, the substrate binding of Crt is reduced at low levels of calcium suggesting a lower chaperone efficiency in ER Ca^{2+}-depleted cells.

Additional chaperones and protein processors may also be affected by changes in the ER calcium levels. The binding protein BiP binds calcium and possesses a lower chaperone activity at low calcium levels in animal cells (Lievremont et al. 1997). Several other steps during protein synthesis, glycoprotein maturation and secretion are also impaired during lower calcium levels. The lower efficiency during these conditions may be reflected in a lower mode of protein–protein interactions.

Although very little is known about internal ER processes and calcium in plants, several of the affected components are present in plant cells. The model plant Arabidopsis contains two Cnx and three Crt isoforms (Persson et al. 2003). In addition, four PDI and two ERp57 homologs are also expressed in Arabidopsis (Persson et al. 2005). The interactive sites between ERp57 and Crt in animals are conserved in all of the plant homologs (Svensson and Persson, unpublished data), indicating that interactions may take place. Interestingly, whereas Crt and Cnx are conserved in most higher eukaryotes, the PDI protein family is extremely diverse (Persson et al. 2005). A comparative network scheme of interacting ER proteins from different species may therefore provide important evolutionary clues to the conservation and propagation of the ER chaperone matrix.

6 Perspectives

In plants, as expected by analogy to well studied animal systems, the ER is emerging as an important Ca^{2+}-source for intracellular signalling. Several important signalling components have been identified and characterized (Table 1; Sanders et al. 2002). However, approaches to reveal how these components are functionally orchestrated will be necessary to understand calcium related functions within the organelle, as well as cytosolic signalling events. Comparable studies in animal systems have revealed the ER, although a physical continuum, as a diverse and heterogenous organelle (Papp et al. 2003).

The construction and distribution of Arabidopsis insertion lines have provided the plant scientific community with a powerful tool to explore functional implications of genes of interest. The first gene involved in ER Ca^{2+}-signalling to be investigated using this approach was the ECA1 Ca^{2+}-

ATPase (Wu et al. 2002). Disruption of the *ECA1* gene resulted in reduced growth on medium with lower calcium contents. A similar growth defect was evident when an antisense construct of Crt was introduced into Arabidopsis plants (Persson et al. 2001). Combining molecular and genetic tools may therefore be a fruitful approach to assess the effects of altered ER calcium on plant growth. Crossing individual insertion lines may further reveal the extent of redundancy and functional overlap among different genes. Subsequent introduction of cameleons would then allow for a direct connection between the disruption/overexpression of genes and changes in calcium signatures.

Several additional approaches may be utilized to uncover the dynamics of the plant ER calcium network: 1) Comparable distribution of components using either fluorescently-labelled proteins or immuno-techniques, 2) Introduction of compatible fluorescently-labelled components for FRET analyses, 3) Large-scale microarray data mining to expose coregulatory networks of genes, 4) Assessment of protein–protein interactions through various proteomic efforts. These techniques should provide spatio-temporal information about how different components work in concert with each other under various conditions.

The emerging view in animal cells is that different organelles may sense and respond to the status of other organelles. This view has not been explored for Ca^{2+}-signalling and holding compartments in plants. The cross-talk in animal systems between, for example, the ER, PM and mitochondria has generated information to how changes in Ca^{2+}-homeostasis may affect the cellular status, e.g. apoptosis, protein synthesis and secretion. Given that the vacuole is the main Ca^{2+}-holding source in plant, a disruption of the calcium equilibrium between this organelle and the ER may provide insight into how the cellular distribution of calcium is facilitated.

Whilst most of the suggested approaches here refer to a macroscopic view of the ER and cellular Ca^{2+}-signalling, it is equally important to uncover structural information of the different enzymes. The structure of the P domain of Crt was solved using NMR and revealed a extended hairpin with three anti-parallel beta-sheets (Schrag et al. 2001). This information provided useful clues to how Crt interacts with other Ca^{2+}-regulated chaperones, such as ERp57 (Frickel et al. 2002). The structure of SERCA1a has similarly provided important information for functional aspects of Ca^{2+}-pumps (Toyoshima et al. 2004). These data may also reveal evolutionary relationships which may not be evident solely from sequence homology.

In this chapter we have tried to high-light the versatile aspects of calcium and its impact on a variety of ER processes. The tight coordination of cytosolic and organellar calcium contents emphasizes the importance of the ion in different compartments. The Ca^{2+}-fluxes should therefore simultaneously be viewed as a regulatory switch for cytosolic as well as internal organellar processes.

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The ER Within Plasmodesmata

Kathryn M. Wright (✉) · Karl J. Oparka

Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK
kwrigh@scri.sari.ac.uk, kopark@scri.sari.ac.uk

Abstract The endoplasmic reticulum (ER) is an essential component of plasmodesmata, the membrane-lined pores that interconnect plant cells. The desmotubule which traverses the centre of a plasmodesma is formed from, and continuous with, the cortical ER. Whilst the exact role of the ER is only now being characterised, it is recognised that the ER is intimately involved in the transfer of molecules to and through plasmodesmata, providing a number of pathways for movement between cells as well as being implicated in the mechanisms that control transport. It is believed that molecules may be transported by passive flow within the desmotubule lumen, by diffusion along the inner desmotubule membranes or by specific attachment to the cytoplasmic face of the desmotubule followed by facilitated transport through the cytoplasmic sleeve. The ER is also involved in the formation of plasmodesmata either during cell division or when formed de novo across non-division walls. This chapter focusses on the role of the ER in plasmodesmatal formation and function.

1 Introduction

Plasmodesmata (singular plasmodesma) are membrane-lined pores that interconnect plant cells to form a functional cytoplasmic continuum known as the symplasm. Although first described in the context of cell-to-cell communication by Eduard Tangl in 1879 (cited by Carr 1976), it was not until relatively recently that the importance of the endoplasmic reticulum (ER) within plasmodesmata became appreciated. Whilst the exact role of the ER is only now being characterised, it is recognised that the ER is an essential component of the structure and function of plasmodesmata.

Neighbouring cells within all multicellular eukaryotes must communicate to allow coordinated differentiation (Hashimoto and Inze 2003). From recent ultrastructural, physiological, biochemical and molecular studies it is now clear that plasmodesmata are not merely static channels that allow the movement of low molecular weight solutes, but rather function as flexible structures that are able selectively to control the cell-to-cell movement of proteins and RNAs (Lucas et al. 1993; Blackman and Overall 2001; Ehlers and Kollmann 2001; Roberts and Oparka 2003; Ding et al. 2003). As will be described, the ER forms an essential component of plasmodesmata, and can also determine where they are formed within the cell wall. The ER is inti-

mately involved in the transfer of molecules to and through plasmodesmata, providing a number of pathways for movement between cells, and has been implicated in the mechanisms that control transport. A number of reviews have appeared recently providing a comprehensive description of plasmodesmata and their function, and the reader is referred to these for more detailed discussions (Haywood et al. 2002; Roberts and Oparka 2003; Oparka 2004; Ruiz-Medrano et al. 2004; Heinlein and Epel 2004; Gillespie and Oparka 2005; Roberts 2005). In this chapter we will focus on the ER and its role in plasmodesmatal formation and function.

2
Structure

A simple plasmodesma comprises a single pore, lined by the plasma membrane and traversed in its centre by a structure known as the desmotubule. The desmotubule is formed by the cortical ER which remains continuous between cells via the desmotubule. The space between the plasma membrane and the desmotubule is called the cytoplasmic sleeve and is believed to be the principal route for the movement of small molecules between cells. Each plasmodesma is surrounded by an electron-lucent sleeve and at each end are neck regions, one or both of which may be constricted (Fig. 1 and Fig. 2a).

Structural models of plasmodesmata are based predominantly on data from transmission electron microscopy. On the basis of ultrastructure alone, plasmodesmata are classified into two basic types: simple and branched. The majority of models describe the structure of simple plasmodesmata, and interpretations have been influenced by the effects of the various fixation and staining procedures on plasmodesmata (Gunning and Robards 1976; Robards and Lucas 1990; Beebe and Turgeon 1991; Tilney et al. 1991; Ding et al. 1992b; Botha et al. 1993; Turner et al. 1994; White et al. 1994; Overall and Blackman 1996; Ding 1997, 1999; Waigmann et al. 1997; Radford et al. 1998; Overall 1999). Although there is consensus that all plasmodesmata have the same basic configuration, there is still much debate about their fine structure.

2.1
The ER Lumen

The desmotubule is now accepted to be a tightly furled membrane, running through the plasmodesma, in many cases appearing as an electron-dense rod (Lopez-Saez et al. 1966; Gunning and Hughes 1976; Overall et al. 1982; Olesen 1979; Gunning and Overall 1983; Robinson-Beers and Evert 1991; Ding et al. 1992b; Waigmann et al. 1997). There is a layer of proteinaceous material either integral to, or closely associated with, the desmotubule. Ding and co-workers (1992b) have shown that the wall of the desmotubule is com-

posed of up to ten electron-dense particles about 3 nm in diameter, embedded in the desmotubule membrane, possibly in a helical arrangement with a pitch of 20–30°. In contrast, Overall and Blackman (1996) suggest that the proteinaceous material appears as negatively stained, electron-lucent particles, these being interpreted by Ding and co-workers (1992b) as spaces between the electron-dense particles. In transverse views, at the centre of the desmotubule is an electron-dense rod of about 3 nm in diameter (Ding et al. 1992b) (Fig. 2a). Fine filamentous structures 1–1.5 nm in diameter may extend between this central rod and the desmotubule wall. In material treated without tannic acid or uranyl acetate/osmium tetroxide, most of the desmotubule particles, including the central rod particles, are absent (Ding et al. 1992b). These central rod particles, along with the proteins embedded in the outer membrane of the desmotubule cylinder, are believed to occlude the desmotubule lumen (Overall et al. 1982). However, this may be due to the fixation protocol employed, since other workers have estimated that the space between the central particles is between 2.5 and 3 nm (Botha et al. 1993).

The presence or absence of a desmotubule lumen may be tissue specific. In certain trichomes, instead of the desmotubule appearing as an electron-dense solid rod, the desmotubule may have an "open" electron-lucent lumen (Eleftheriou and Hall 1983; Waigmann et al. 1997).

2.2 The Cytoplasmic Sleeve

The plasma membrane within a plasmodesma is lined on its cytoplasmic face with electron-dense structures (Botha et al. 1993; Ding et al. 1992b; Tilney et al. 1991). These globular particles are connected by elongated spokes to the particles embedded in the wall of the desmotubule (Fig. 1) and may regulate transport by expanding or contracting the cytoplasmic sleeve (Ding et al. 1992b; Overall and Blackman 1996). Although the diameter of a plasmodesma channel is 20–50 nm and the desmotubule is about 15 nm in diameter (Ehlers and Kollmann 2001), the helical arrangement of the embedded particles produces spiralling channels that may reduce the functional diameter of the cytoplasmic sleeve (Zee 1969; Robards 1976; Olesen 1979; Overall et al. 1982; Wolf et al. 1989; Olesen and Robards 1990; Robards and Lucas 1990; Ding et al. 1991; Robinson-Beers and Evert 1991; Lucas and Wolf 1993; Lucas et al. 1993; Overall and Blackman 1996; Waigmann et al. 1997). Various estimates give the diameter of the resulting microchannels between 2.5 and 6 nm (Overall et al. 1982; Ding et al. 1992b; Botha et al. 1993).

Following the localisation of actin to plasmodesmata (White et al. 1994), Overall and Blackman (1996) proposed a model, in which a cable of actin runs through the centre of the pore, closely associated with the desmotubule. It has been suggested that the spokes may be composed of myosin and con-

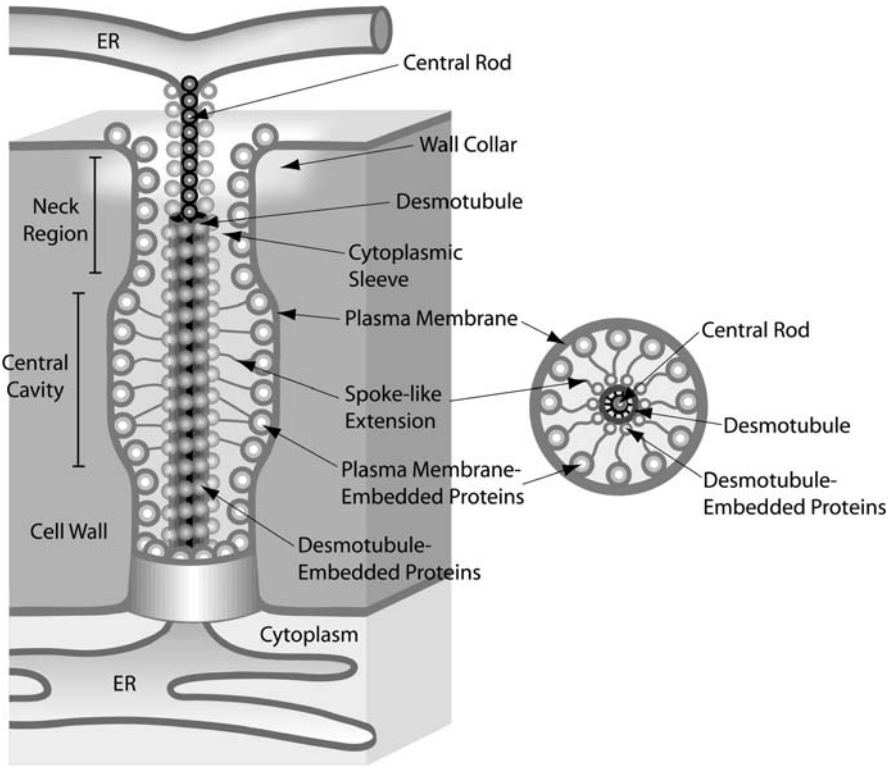

Fig. 1 Diagrammatic representation of the structure of a simple plasmodesma including all sub-structural components that have been confirmed to date. A longitudinal section through the pore is shown to the *left*, while a transverse section through the central cavity region is shown to the *right*. Based primarily on Ding (1992b) and reproduced with permission from Roberts (2005)

nect proteins embedded in the plasma membrane to the actin cable at the centre of the pore, forming an important regulatory structure that can alter the size exclusion limit of plasmodesmata (Oparka 2004). Additional support for this model is provided by the identification of myosin within higher-plant plasmodesmata (Radford and White 1998) and the green alga *Chara corallina* (Blackman and Overall 1998).

The neck region at each end of a plasmodesma may appear constricted, bringing the plasma membrane into close proximity with the desmotubule. This constriction may be the result of callose deposition since treatment with inhibitors of callose synthesis result in funnel-shaped rather than constricted plasmodesmata (Radford et al. 1998). Since callose deposition can be triggered as a response to wounding or chemical fixation, it remains to be determined whether the constricted state is an artefact of fixation or whether callose has a regulatory role in the control of plasmod-

esmal aperture (for discussions, see Overall 1999; Schulz 1999; Botha and Cross 2000).

2.3 Modifications to Plasmodesmatal Structure

In importing tobacco leaf tissue, plasmodesmata between most cell types are simple and lack a central cavity (Ding et al. 1992b). However, occasionally,

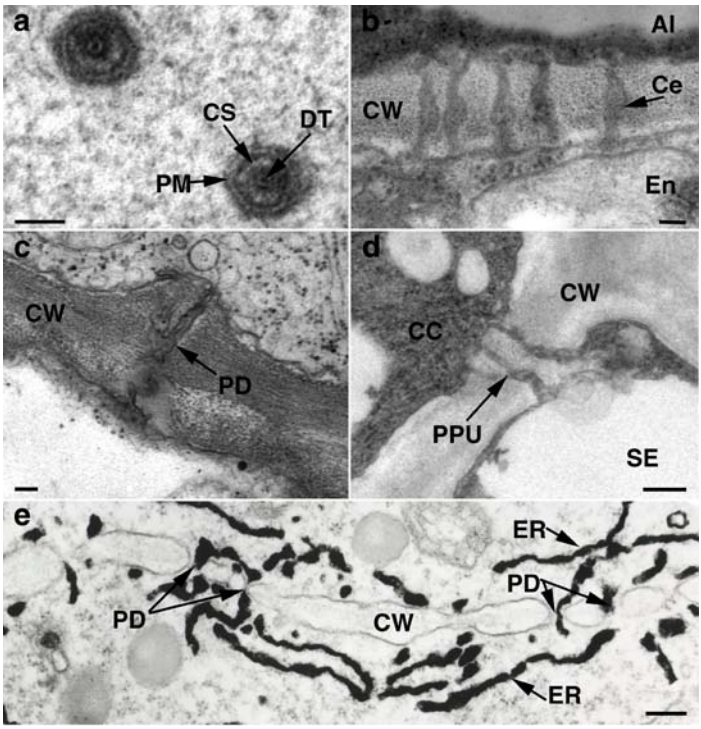

Fig. 2 **a** Transverse section through simple plasmodesmata connecting potato tuber parenchyma cells, showing the desmotubule (DT) with a central, electron-dense rod, the cytoplasmic sleeve (CS) and the plasma membrane (PM). Bar = 20 nm. **b** Longitudinal section through simple plasmodesmata between the aleurone layer (Al) and the starchy endosperm layer (En) in rice, showing the wide central cavity (Ce) within the cell wall (CW). Bar = 50 nm. **c** Longitudinal section through a branched plasmodesma (PD) connecting cells of *Narcissus* tuber. Bar = 100 nm. **d** Longitudinal section through a pore plasmodesma unit (PPU) connecting a companion cell (CC) and a sieve element (SE) in an *Arabidopsis* root. Bar = 25 nm. **e** Entrapment of the endoplasmic reticulum (ER) during cytokinesis in the root tip of *Zea mays*, resulting in the formation of primary plasmodesmata (PD) within the cell wall (CW). Bar = 50 nm. Image courtesy of C. Hawes, Oxford Brookes University. Images **a**–**d** courtesy of the Electron Microscopy Laboratory, SCRI

plasmodesmata between bundle-sheath cells and phloem-parenchyma cells contain extensive central cavities. The presence of a central cavity appears to be related to leaf development, since in non-importing tissue all plasmodesmata contain central cavities whilst retaining the neck constriction (Ding et al. 1992b). Simple plasmodesmata predominate in immature plant tissues (Oparka et al. 1999) and may be grouped together in primary pit fields. They are common in algae and mosses (Franceschi et al. 1994; Cook et al. 1997) and appear to represent a less evolutionarily advanced structure compared to branched plasmodesmata. Branched plasmodesmata comprise two or more channels on either side of the middle lamella, often joined by a central cavity (Fig. 2b–d), and are found extensively in mature tissues. Some simple plasmodesmata are gradually converted to branched plasmodesmata during the sink–source transition that occurs in leaves, by a mechanism that involves the formation of bridges between adjoining simple pores (Oparka et al. 1999; Roberts et al. 2001). The remaining simple plasmodesmata are lost during the phase of rapid leaf expansion, being ripped apart as intercellular air spaces form in the mesophyll (Roberts et al. 2001).

3
Role of ER in the Formation of Plasmodesmata

In order to allow the differential functioning of cell types and tissues, the plant symplasm behaves as a system of operational subunits termed symplasmic domains (Erwee and Goodwin 1985; for reviews see Robards and Lucas 1990; Lucas et al. 1993; McLean et al. 1997; Ding et al. 1999; Ehlers and van Bel 1999; Lucas 1999). It is now recognised that the amount of symplasmic connectivity at different cell interfaces, although not identical, is precisely controlled (Ehlers and van Bel 1999) with the degree of branching of plasmodesmata being genetically controlled, and specific for each cell type (Ehlers et al. 2004). Cells with common walls created during cell division may be connected by primary plasmodesmata that form during cytokinesis, whereas secondary plasmodesmata, that form de novo, are required to connect cells across non-division walls (Kollmann and Glockmann 1999; Ehlers and Kollmann 2001).

3.1
Primary Plasmodesmata

Based on where and how they are formed, plasmodesmata are categorised as either primary or secondary. Primary plasmodesmata form during cytokinesis at sites where ER tubules cross the phragmoplast of a dividing cell (Fig. 2e) (Hepler 1982; Staehelin and Hepler 1996). ER tubules, surrounded by cytoplasm, are trapped among the fusing Golgi vesicles that deliver wall

material to the developing cell plate (Fig. 3a). As the cytoplasmic bridges become increasingly constricted they develop into plasmodesmal strands that are lined with plasma membrane derived from the Golgi membrane (Fig. 3b). Although transformed into the desmotubule, the ER tubules remain connected with the cortical ER network of the adjacent cells. When formed these plasmodesmata are simple in structure, but during the course of growth and differentiation may be modified into branched forms (for reviews, see Ding and Lucas 1996; Kollmann and Glockmann 1999; Ehlers and Kollmann 2001).

One mechanism of plasmodesmal branching, observed in the division walls of protoplast-derived calluses (Ehlers and Kollmann 1996), is similar to the mode of formation of primary plasmodesmata. As the cell walls thicken the simple primary plasmodesmata have to elongate to maintain intercellular communication. Golgi-derived vesicles carrying wall material fuse with the plasma membrane and trap cytoplasmic strands, enclosing straight or branched cytoplasmic ER cisternae which are continuous with the desmotubule (Fig. 3c). The shape of the entrapped ER determines the shape of the resulting branched plasmodesmata (Ehlers and Kollmann 1996, 2001).

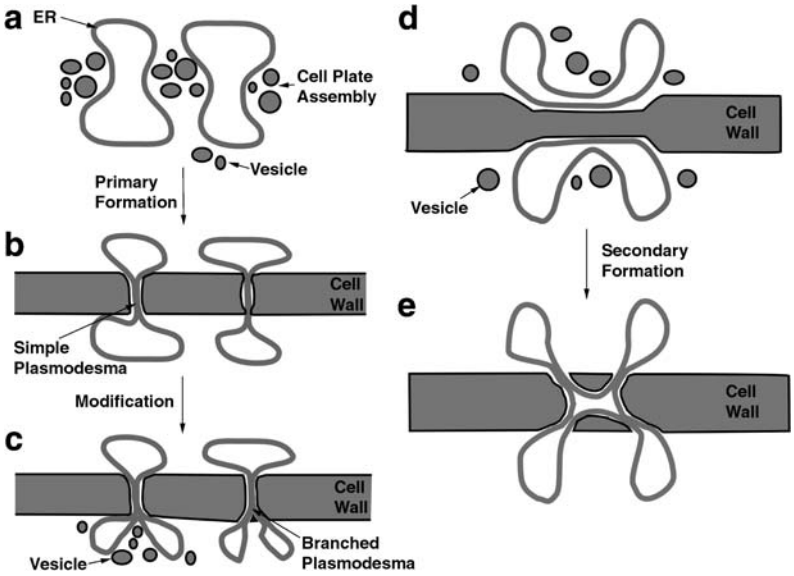

Fig. 3 Diagrammatic representation of plasmodesma biogenesis. Primary plasmodesmata (*left*) are formed during cytokinesis at sites where the ER tubules cross the phragmoplast (**a**). ER and cytoplasm are trapped among the fusing Golgi vesicles forming the desmotubule and cytoplasmic sleeve (**b**). These simple plasmodesmata may be modified into branched forms by the entrapment of additional ER strands during wall thickening (**c**). Secondary plasmodesmata (*right*) form in existing cell walls by the fusion of ER across thinned wall areas (**d**), establishing usually branched connections (**e**) (Modified from Kragler et al. 1998)

Similar entrapment of ER cisternae during cell wall deposition also results in the conversion of simple, primary plasmodesmata into complex plasmodesmatal morphotypes with median branching planes and central cavities. Primary plasmodesmata may become considerably extended in width during cell expansion growth and thinning of the cell wall. In this case, the dilated primary plasmodesmata form the median plasmodesmal parts in the middle lamella which later become the central cavities (for details, see Kollmann and Glockmann 1999; Ehlers and Kollmann 2001), with uneven wall expansion of the adjacent cells resulting in the formation of dilated central cavities (van der Schoot and Rinne 1999).

The lateral fusion of neighbouring simple primary plasmodesmata to form H-shaped plasmodesmata, which may possess dilated central cavities (Ding et al. 1992a, 1993; Glockmann and Kollmann 1996; Volk et al. 1996; Itaya et al. 1998; Kollmann and Glockmann 1999; Oparka et al. 1999), may involve local enzymatic digestion of existing cell wall to allow penetration of additional plasmodesmatal strands (Jones 1976; Ding et al. 1992a, 1993, 1999; Lucas et al. 1993; Lucas and Gilbertson 1994; Ding and Lucas 1996; Itaya et al. 1998), or the formation of further branches by ER entrapment (Ehlers and Kollmann 1996, 2001; Glockmann and Kollmann 1996; Kollmann and Glockmann 1999).

3.2
Secondary Plasmodesmata

By definition, secondary plasmodesmata are formed de novo across existing cell walls (Ehlers and Kollmann 2001). They cannot be distinguished from primary plasmodesmata on the basis solely of their structure, since they also may display simple or branched architectures (Ding et al. 1992b), and therefore can only be identified unambiguously by their location. At the time of their formation some secondary plasmodesmata, like primary plasmodesmata, have a simple structure that may subsequently be modified. However, other secondary plasmodesmata are branched when formed, and ultimately the majority of secondary plasmodesmata are modified to have a branched morphology (Lucas et al. 1993).

Although secondary plasmodesmata are formed during normal plant development (Jones 1976; Robards and Lucas 1990; Lucas et al. 1993; Ding et al. 1999; Kollmann and Glockmann 1999), detailed studies in a number of systems, including protoplast fusion, graft unions, plant chimeras and parasite–host interactions, have provided evidence for the de novo formation of plasmodesmata across cell types from different developmental origins. In all cases, the ER plays a central role in the formation of these secondary plasmodesmata (for reviews, see Jones 1976; Robards and Lucas 1990; Lucas et al. 1993; Kollmann and Glockmann 1999; Ehlers and Kollmann 2001).

As the outer cell walls of protoplast-derived cultured cells regenerate, half, branched plasmodesmata form by a passive entrapment mechanism resem-

bling primary plasmodesmata formation (Monzer 1990, 1991; Ehlers and Kollmann 1996). Cytoplasmic ER cisternae become closely associated with the plasma membrane and, along with the enclosing cytoplasmic strands, are trapped by Golgi-derived vesicles carrying cell wall material and fusing with the plasma membrane (Monzer 1990, 1991). The resulting half plasmodesmata, known as "outer-wall plasmodesmata" (Ehlers and Kollmann 1996), are scattered over the cell surface and when cultured cells come into intimate contact, opposite half plasmodesmata may fuse to form continuous, usually branched, plasmodesmata that connect the two cells (Monzer 1990, 1991; Ehlers and Kollmann 1996).

Heterografts of plants with species-specific subcellular markers have been used to identify the mechanism of formation of interspecific secondary plasmodesmata (Jeffree and Yeoman 1983; Kollmann and Glockmann 1985, 1991, 1999; Kollmann et al. 1985). This process involves the local thinning and loosening of the fusion walls between the cells of the graft partners followed by fusion of the plasma membranes which are associated with ER cisternae (Fig. 3d). Once again, during reconstruction of the modified wall parts, cytoplasm and ER cisternae become entrapped on either side of the graft interface (Fig. 3e). Although the resulting interspecific continuous secondary plasmodesmata may be simple strands, they are usually of a complex branched morphology with dilated central cavities in the median fusion plane. An exchange of information signals appears to be involved in this mechanism (Jeffree and Yeoman 1983; Kollmann and Glockmann 1991, 1999), and it has been shown that lack of cooperation between cell partners results in the formation of mismatching half plasmodesmata at graft interfaces of incompatible heterografts, between different cell types and between cells at different stages of differentiation (Kollmann et al. 1985).

Although not investigated in detail, it appears that the mechanism of formation of secondary plasmodesmata within chimeras and at host/parasite interfaces is essentially similar to that occurring at graft interfaces (for reviews, see Jones 1976; Robards and Lucas 1990; Lucas et al. 1993; Kollmann and Glockmann 1999; Ehlers and Kollmann 2001). Indeed, it is likely that a common mechanism is involved in the establishment and modification of primary plasmodesmata, as well as the de novo formation and modification of secondary plasmodesmata (Kollmann and Glockmann 1991, 1999; Kragler et al. 1998; Ehlers and Kollmann 1996, 2001).

4
Movement of Molecules Through Plasmodesmata

The architecture of a plasmodesma provides three possible routes for the movement of molecules from cell to cell: the ER, the cytoplasmic sleeve and the plasma membrane. Of these routes, movement within or along the ER

is the most contentious, while movement via the cytoplasmic sleeve is generally accepted for small solutes. It is a matter of debate as to whether all three of these pathways are available as transport routes within plasmodesmata, or whether the route via the desmotubule lumen is only available in plasmodesmata that have an "open" desmotubule configuration.

4.1 Transport Through Plasmodesmata via the Endoplasmic Reticulum

The ER–desmotubule–ER continuum provides three potential routes for cell-to-cell transport via plasmodesmata: (1) by passive flow within the desmotubule lumen; (2) by diffusion along the inner desmotubule membranes; or (3) by specific attachment of molecules to the cytoplasmic face of the desmotubule, followed by facilitated transport through the cytoplasmic sleeve. The last of these will be covered in Sect. 4.2.3, dealing with selective transport between sieve element and companion cell.

4.1.1 Transport Within the Desmotubule Lumen

In many cases plasmodesmata have been observed to be occluded by the proteins embedded in the desmotubule membrane, so it is envisaged that only a single water molecule could pass (Overall et al. 1982). However, other plasmodesmata have been observed to have an open desmotubule lumen. It appears that the open lumen in plasmodesmata of some trichomes may be a particular adaptation to their secretory role. In chickpea (*Cicer arietinum*) trichomes there appears to be a specialised system of vacuoles and tubules belonging to the ER system. Following the endocytic uptake of Lucifer Yellow carbohydrazide (LYCH) into this system the fluorescent probe was able to move, apparently within the ER network, through plasmodesmata (Lazzaro and Thomson 1996). Although not conclusively demonstrated, it has been proposed that the transport of pre-nectar in cotton papillae takes place through the open desmotubule lumen, followed by budding of pre-nectar filled vesicles from the ER and secretion of their contents via reverse pinocytosis (Eleftheriou and Hall 1983; Waigmann and Zambryski 2000). Movement of fluorescent probes through the desmotubule lumen has also been demonstrated. Fluorescently labelled dextrans with a molecular weight of 10 000 were able to move from cell to cell in *Torenia fournieri* stem explants following microinjection into the ER but not after injection into the cytoplasm, although it was not demonstrated whether the plasmodesmata involved had an open or closed lumen (Cantrill et al. 1999).

Although some fluorescent probes have been observed to move from cell to cell presumably via the ER lumen, ER-targeted green fluorescent protein (GFP) does not. When expressed under the AtSUC2 promoter, GFP targeted to the ER

lumen of companion cells was not able to move through the desmotubules into the neighbouring sieve elements (Martens et al. 2001; Wright et al. 2003).

4.1.2 Diffusion Within ER Membranes

A second route via the desmotubule potentially involves diffusion along the ER membrane. Hydrophilic fluorescent lipids partitioned within the ER membrane were demonstrated to be able to move from cell to cell following photobleaching, in contrast to lipids inserted within the plasma membrane (Grabski et al. 1993). Similarly, the fluorescent probe DiOC6 was observed to move from cell to cell, presumably via the membrane of the desmotubule, in pea root cortex tissue (Schulz 1999). It appears that the plasma membrane, but not the desmotubule, within plasmodesmata is modified in such a way that it forms a barrier to the diffusion of lipids across plasmodesmata (Grabski et al. 1993).

4.2 Transport Through the Cytoplasmic Sleeve

It is now generally accepted that the majority of molecular transport through plasmodesmata takes place via the cytoplasmic sleeve, through the helical channels between the plasma membrane and the desmotubule. This of course includes molecules moving in association with the ER. In many cases it is assumed that molecules are moving through the cytoplasmic sleeve without requiring any specific interaction with components of the pore, i.e. non-selectively (Schulz 1999; Zambryski and Crawford 2000; Oparka 2004).

The conductive properties of plasmodesmata have been established using a combination of techniques including monitoring of cell-to-cell transfer of radiolabelled substances (Arisz 1969), electrical coupling experiments (Overall and Gunning 1982) and dye coupling (Tucker 1982; Erwee and Goodwin 1983, 1984, 1985; Goodwin 1983; Palevitz and Hepler 1985; Tucker and Spanswick 1985; Madore et al. 1986; van der Schoot and van Bel 1989). Fluorescent probes in the form of fluorescein were first used to investigate short-distance transport through plasmodesmata in trichomes by Schumacher (1936), but it was not until 40 years later that fluorescent probes started to make a significant impact on the study of cell-to-cell communication.

4.2.1 Molecular (Size) Exclusion Limit

Open plasmodesmata allow the diffusive exchange of metabolites and small molecules (Tyree 1970; Tucker et al. 1989). The size of molecules able to pass through plasmodesmata determines the size exclusion limit (SEL) of the pore

but should more accurately be referred to as the molecular exclusion limit (MEL) when it is measured in terms of the mass, rather than the physical dimensions, of the molecules involved. On the basis of investigations using predominantly fluorescently labelled dextrans and other conjugates, the MEL for such movement through many plasmodesmata has been determined to be in the order of 850–900 Da. However, it is not the mass of the molecule that determines its ability to move through plasmodesmata but rather its physical size, which is usually measured in terms of its hydrodynamic radius or Stokes radius (R_s) (Terry and Robards 1987). Many open plasmodesmata allow the diffusion of molecules with an R_s of 0.75 nm. Predictions of the diameter of pores within plasmodesmata, determined using fluorescent dextrans or peptides, fall in the region of 3–4 nm (Terry and Robards 1987; Fisher 1999), which corresponds well with the measurements obtained from electron micrographs (2.5–6 nm) (Ding et al. 1992b; Overall and Blackman 1996; Overall et al. 1982). It should be noted that the relationship between molecular mass and size for dextrans differs greatly from that for proteins. For example, a 27-kDa dextran has the same R_s value (3.5 nm) as a 67-kDa protein (Jørgensen and Møller 1979; Le Maire et al. 1986; Fisher 2000). This becomes of particular relevance in considering more recent studies investigating the movement of the fluorescent protein GFP, which has a molecular mass of 27 kDa and an R_s of 2.82 nm (Terry et al. 1995).

As described previously, a plant is divided into a number of functional symplasmic domains (Erwee and Goodwin 1985; Robards and Lucas 1990; Lucas et al. 1993; McLean et al. 1997; Ding et al. 1999; Ehlers and van Bel 1999; Lucas 1999). In this context, it has been demonstrated that plasmodesmata do not all have the same SEL. Whilst leaf mesophyll cells are frequently quoted to have an MEL for dextrans in the region of 1 kDa, some cell types including epidermal cells have a lower MEL of 370 Da, as assessed by the movement of carboxyfluorescein (Erwee and Goodwin 1985; Duckett et al. 1994), while others have a higher $MEL_{(dextran)}$, including tobacco trichomes at 7 kDa (R_s 1.6 nm, estimated from Jørgensen and Møller 1979; Waigmann and Zambryski 1995).

Simple plasmodesmata have a much larger SEL than branched plasmodesmata (Oparka et al. 1999). In sink tobacco leaf epidermis, simple plasmodesmata predominate and allow the passage of GFP fusion proteins up to 47 kDa. However, after the sink–source transition, during which the simple plasmodesmata are replaced or modified to the branched morphotype, the SEL decreases so that GFP is able to move only from the bombarded cell to its immediate neighbours (Oparka et al. 1999).

4.2.2
Role of ER in the Regulation of SEL

The SEL of plasmodesmata is clearly highly regulated at both a tissue and developmental level, and a number of mechanisms have been proposed to

be involved. Plasmodesmata may be considered to have two potential functional states: closed or open (Lucas et al. 1993; Schulz 1999; Zambryski and Crawford 2000). Closed plasmodesmata are characterised by a lack of all intercellular exchange (Zambryski and Crawford 2000), but since this has often been determined using lower molecular weight fluorescent probes (340 Da) it has not been possible to prove that they are "watertight" (Schulz 1999). Closure of plasmodesmata may be temporary or more permanent, involving the breakdown and removal of plasmodesmata from the cell wall (Duckett et al. 1994; Oparka et al. 1995; Palevitz and Hepler 1985). The deposition of callose within the neck region of plasmodesmata has been implicated in both the transient closure and also in the fine regulation of SEL (Lucas et al. 1993; Roberts and Oparka 2003; Ruan et al. 2004).

Another possibility for regulation of plasmodesmata is that the central ER-derived desmotubule functions as an actin-clad scaffold, which is linked to the plasma membrane by myosin molecules that span the space between the desmotubule and the plasma membrane. In this model, myosin may provide a contractile mechanism for closing the pore aperture, in a similar way to which myosin VIII constricts microvilli in mammalian cells (Baluška et al. 2004; Oparka 2004). Myosin VIII has been detected within plasmodesmata, and it has been suggested that the spokes that radiate from the desmotubule to the plasma membrane may be myosin molecules (Overall and Blackman 1996). Myosin VIII has also been implicated as a structural support of the cortical ER elements tightly underlying the plasma membrane both outside and within plasmodesmata (Volkmann et al. 2003).

Calreticulin is a highly conserved calcium sequestering protein that resides in the ER lumen and is the first ER-resident protein to have been localised within plasmodesmata (Baluska et al. 1999, 2001). It may be involved in regulating plasmodesmatal transport in association with centrin, a calcium-binding contractile protein, localised to the neck region of plasmodesmata (Blackman et al. 1999). An increase in the concentration of cytoplasmic calcium causes a decrease in the phosphorylation of this protein, causing the centrin nanofilaments to contract (Martindale and Salisbury 1990; Blackman et al. 1999). In support of this model, increased levels of calcium have been shown to lead to plasmodesmal closure (Erwee and Goodwin 1983; Tucker 1990; Holdaway-Clarke et al. 2000), and two protein kinases, one calcium-dependent, have been localised to plant cell walls or plasmodesmata (Citovsky and Zambryski 1993; Yahalom et al. 1998).

4.2.3
The Specialised Case of Pore–Plasmodesma Units

Plasmodesmata connecting companion cells (CC) to sieve elements (SE) are specifically modified to form specialised structures known as pore–plasmodesma units (PPU) (van Bel 1996) (Fig. 2d). The ER within these PPU

is believed to play a vital role in the survival of SE and functioning of the phloem. The SE–CC complexes form the functional units of phloem and are specialised for the long-distance flow of solutes. Both cells originate from an unequal division of the SE–CC mother cell. Soon after division, the plasmodesmata on the CC side become highly branched, with up to 100 branches in some species (Evert 1990), coalescing in a central cavity that connects to a wider symplasmic pore at the SE side. It was previously reported that the ER was not continuous through the PPU, but it is now accepted that ER tubules traverse the PPU (Ding et al. 1993), although the ultrastructure of the central cavity has not as yet been resolved (van Bel and Kempers 1997). It has been suggested that branching of plasmodesmata occurs wherever the demand for symplasmic contact increases during development (Kollmann and Glockmann 1999; Ehlers and Kollmann 2001). If so, the asymmetric branching of the PPU implies the difference in permeability of the plasmodesmata on both sides of the cell wall (Schulz 2005).

During differentiation, selective autophagy results in the disintegration of most of the cellular components of the SE including the nucleus and vacuole. What remains at maturity is the plasma membrane, a thin layer of parietal cytoplasm composed of stacked ER or fenestrated ER (Thorsch and Esau 1981a,b; Sjolund and Shih 1983) and a few dilated mitochondria (Evert 1990). Phloem-specific plastids of two types, with proteinaceous (P-type plastids) or starch (S-type plastids) inclusions (Esau 1969; Behnke 1991a; van Bel and Knoblauch 2000; van Bel 2003), and SE phloem proteins (Eleftheriou 1990; Evert 1990; Cronshaw and Sabnis 1990; Behnke 1991b; Iqbal 1995) are also conspicuous in the SE. To prevent these organelles being dragged along in the turbulent mass flow within the SE, 7-nm-long macromolecular extensions anchor the ER, mitochondria and plastids to each other and to the plasma membrane (Ehlers et al. 2000). The resulting mature SE has been described as almost "clinically dead", and in many cases has to be maintained by the adjacent CC. The ER that is continuous from CC to SE is thought to provide a major "lifeline" for the trafficking of macromolecules that are essential to maintain the integrity of the SE (Oparka and Turgeon 1999; van Bel and Knoblauch 2000).

Compared to other organelles within the SE, the ER is well preserved in the form of nets or stacks (Thorsch and Esau 1981a,b; Sjolund and Shih 1983). The parietal fenestrated ER, which shares some traits with the cortical ER (Staehelin 1997), has been postulated to be a structural necessity for ATP-fuelled retrieval (Sjolund and Shih 1983), and may thus be essential for sucrose uptake and retrieval by the SE (van Bel 1996).

Attachment of macromolecules to the cytoplasmic face of the desmotubule has been proposed to allow the movement of proteins through the PPU connecting SE and CC (see Sect. 4.1 and Fig. 4). Due to the enucleate nature of SE and their lack of rough ER, it has been recognised that SE are dependent on CC for their functioning, in some cases for decades (Raven 1991). The flow

rate within sieve tubes has been estimated to exceed 40 cm h^{-1} (Fisher 1990). If proteins or mRNA entering the SE via the PPU were simply "dumped" into the translocation stream, they would be swept rapidly away to sinks. This might be the case for some macromolecules, but it is likely that others are in some way anchored, particularly those required for the maintenance of the SE. It has been suggested that proteins or mRNA trafficked across a PPU remain bound to the desmotubule and subsequently move in association with the ER (Fig. 4). Such a process may occur following the targeting of the movement protein (MP) of *Cucumber mosaic virus* (CMV), fused to GFP, to PPUs.

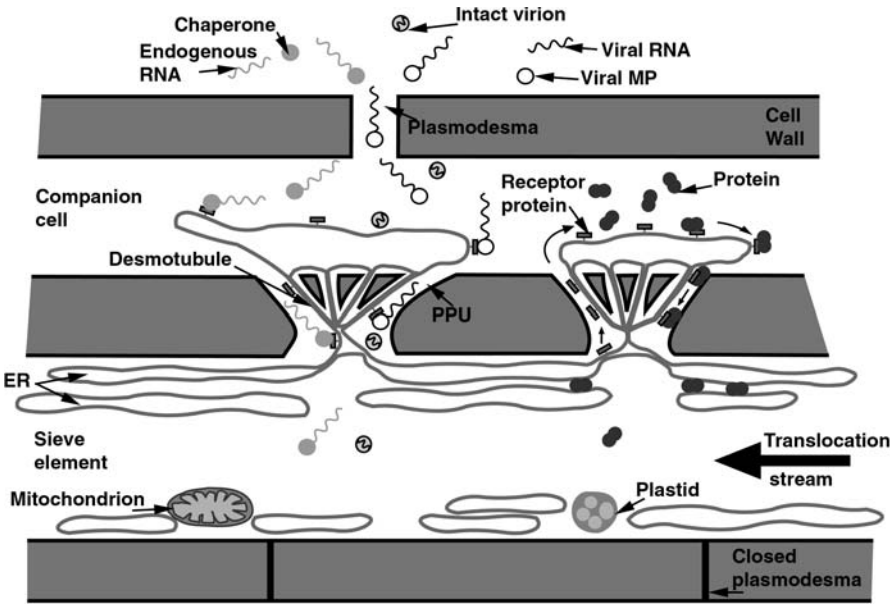

Fig. 4 Schematic model depicting molecular trafficking between the CC and the SE. *Left*: Endogenous RNA molecules originating in the mesophyll are trafficked by specific molecular chaperones into the CC. At the PPU, the chaperone interacts with a receptor protein located on the desmotubule. In the case of some systemic RNA viruses, the viral RNA is trafficked into the CC by a specific viral movement protein (MP). As in the case of endogenous RNA chaperones, the MP interacts with the desmotubule to facilitate RNA movement into the SE. Other systemic viruses may traffic across the PPU as intact virions, without the need for disassembly in the CC. *Right*: Selective protein trafficking between CC and SE. Proteins synthesized within the CC and destined for the SE parietal layer are trafficked across the PPU by an interaction with receptor proteins located on the desmotubule. Such receptors ensure that the proteins are delivered along the ER to their target sites without loss to the translocation stream. After delivery, the receptor proteins may be recycled back into the CC to collect further cargo (*arrows*). Other low molecular weight proteins may enter the SE from the CC by diffusion. Such proteins may not possess "retention signals" for the ER, and may be translocated and unloaded in sink regions of the plant. (Adapted from Oparka and Turgeon 1999)

Upon entry into the SE, the MP trafficked along a reticular structure within the SE parietal layer (Blackman et al. 1998).

It is interesting to note that the cortical ER of higher plants is a highly dynamic system of tubules and sheets, and that this ER flow is probably driven by a close association with an underlying actin –myosin network (Boevink et al. 1998; Brandizzi et al. 2002). This raises the possibility that macromolecules anchored on the cytoplasmic face of the cortical ER network might be delivered to the vicinity of plasmodesmata simply by random flow along ER membranes. Although the desmotubule is thought to be inserted into plasmodesmata from their inception, it is intriguing to speculate that in some circumstances the cortical ER might be able to physically move along actin filaments that traverse the plasmodesmal pore. In such a scenario, macromolecules (including viral ribonucleoprotein complexes) might be able to "hitch a ride" on the mobile cortical ER system (Oparka 2004).

5
Macromolecular Transport via Plasmodesmata

There is now a considerable body of evidence supporting the concept that plasmodesmata can mediate the cell-to-cell trafficking of numerous endogenous macromolecules including transcription factors, plant defence-related proteins and RNA (Mezitt and Lucas 1996; Ghoshroy et al. 1997; McLean et al. 1997; Haywood et al. 2002; Heinlein and Epel 2004). Intercellular trafficking through plasmodesmata is now thought to be an important means of regulating plant development, physiology and defence (Ding 1998). Two main mechanisms are now considered relevant to macromolecular transfer: non-selective transport and selective trafficking.

5.1
Non-selective Transport Through Plasmodesmata

It has been suggested that, given an adequate exclusion limit, non-cell autonomous proteins (NCAP) and other macromolecules diffuse from cell to cell via the cytoplasmic sleeve (Crawford and Zambryski 2000; Oparka 2004). According to this model, molecules are retained by cells by targeting to specific subcellular compartments, e.g. nucleus. This would explain the extensive cell-to-cell movement of GFP within epidermal cells of sink tissues (simple plasmodesmata and high exclusion limit) and the rarer movement in source tissues. In the case of the SE–CC complex, it appears that unless a protein has a retention signal for the CC or a targeting signal that directs it to the SE ER, it will be exported in the translocation stream (Oparka and Turgeon 1999). PPU connecting the CC and SE have been shown to have a large SEL for the non-selective movement of molecules. Using fluorescently tagged dex-

trans, the MEL has been demonstrated to be in excess of 10 kDa (Kempers and van Bel 1997) (R_s 2 nm, estimated from Jørgensen and Møller 1979), and proteins in excess of 67 kDa (R_s 3.3 nm, estimated from Jørgensen and Møller 1979) have recently been shown to move from the CC into the SE of *Arabidopsis* roots (Stadler et al. 2005). It is beyond the scope of this chapter to describe the wide range of macromolecules that pass through plasmodesmata, and the reader is referred to recent reviews on this topic (Haywood et al. 2002; Roberts and Oparka 2003; Oparka 2004; Ruiz-Medrano et al. 2004; Heinlein and Epel 2004; Gillespie and Oparka 2005).

5.2 Selective Trafficking Through Plasmodesmata

In contrast to non-selective movement, the selective or targeted movement of molecules requires conformational changes in the architecture of the plasmodesmal pore, and possibly conformational changes to the molecule being trafficked (Schulz 1999; Zambryski and Crawford 2000; Oparka 2004). Of the many proteins identified to date, many appear to show selective transport through plasmodesmata, and many also increase the exclusion limit of the pore.

5.2.1 Role of the ER in Macromolecular Trafficking from CC to SE

Due to the wealth of data provided by analysis of sieve tube exudates, much of the information regarding the mechanisms of macromolecular transport through plasmodesmata has come from studies of the PPU that connect CC and SE. A number of NCAPs have been shown to be synthesised in the CC before transfer to the SE. In cucurbits, the genes encoding the 25-kDa phloem lectin (phloem protein 2, PP2) and the 96-kDa phloem filament protein (phloem protein 1, PP1) are expressed in CC. Whilst the mRNA has been localised by in situ hybridisation to the CC, the proteins are the principal components of the P-protein aggregates in SE (Bostwick et al. 1992; Clark et al. 1997; Dannenhoffer et al. 1997). The phloem-specific thioredoxin h RPP13-1, a 13-kDa protein first identified in rice phloem sap (Ishiwatari et al. 1995) and subsequently shown to be present in vascular saps from a wide range of species (Schobert et al. 1998), has a similar localisation pattern with transcripts again accumulating specifically in CC (Ishiwatari et al. 1998).

In considering the major role of the phloem in sugar transport it is interesting to note that although some of the transporters involved in phloem loading, for example the SUC2 transporter (Stadler et al. 1995) and the proton pump (H^+-ATPase) (DeWitt and Sussman 1995), are located on the plasma membrane of the CC, the leaf sucrose transporter SUT1 is located only on the plasma membrane of the SE (Kühn et al. 1997; Lalonde et al. 1999). In this

case the mRNA has been localised to the SE as well as the CC, being preferentially associated with the PPU (Kühn et al. 1997). This raises the question as to why the *SUT1* mRNA is apparently trafficked into the SE. It has been proposed that *SUT1* transcript is translated in the CC followed by translocation of the SUT1 protein into the SE in association with the desmotubule and the ER of the SE, as described above (see Sect. 4.1) (Kühn et al. 1997). There has been some debate as to whether it is possible for *SUT1* mRNA to be translated in the SE (Thompson and Schulz 1999) but it has recently been postulated that the *SUT1* mRNA, which moves within the phloem translocation stream, is acting as a long-distance signalling molecule (Haywood et al. 2002).

5.2.2
Trafficking of mRNA from CC to SE

The localisation of *SUT1* mRNA within the SE provides some evidence in support of the trafficking of mRNA through PPU. In characterising other sieve tube exudate proteins, further support has been given to this process by the identification of a 16-kDa *Cucurbita maxima* (pumpkin) phloem protein (CmPP16). In common with viral movement proteins, CmPP16 binds to and mediates the cell-to-cell trafficking of non-sequence-specific RNA when microinjected into mesophyll cells. CmPP16 protein localises to the periphery of SE, possibly the plasma membrane. However, both *CmPP16* mRNA and CmPP16 protein are translocated in the phloem stream through a pumpkin-to-cucumber heterograft (Xoconostle-Cazares et al. 1999). This, therefore, appears to be a protein capable of mediating the transport of its own and possibly other mRNA into the phloem. In common with the association between viral MP and RNA, it is probable that endogenous RNA must also associate with a cellular protein to mediate its trafficking through plasmodesmata.

5.2.3
NCAP–ER Interactions

As yet it has not been possible to identify any common motifs in the NCAP characterised so far that are responsible for their interaction with plasmodesmata, and it is likely that at least some NCAP interact with other cellular proteins to mediate their intercellular passage (Oparka 2004). One such protein has recently been identified and indicates a role for the ER in this process. Using CmPP16 (Xoconostle-Cazares et al. 1999) as a bait for the affinity purification of interacting proteins present within a plasmodesmata-enriched cell wall fraction, a 40-kDa protein termed NCAPP1 (non-cell-autonomous pathway protein 1) was detected (Lee et al. 2003) and immunolocalised to the cortical ER. Silencing studies suggest that NCAPP1 has a role in the selective transport of key developmental proteins, possibly shuttling NCAP to the plasmodesmata pore.

5.2.4 Cell-to-Cell Movement of Small RNAs

As well as the trafficking of proteins and large mRNA molecules, the cell-to-cell movement of the as yet unidentified systemic silencing signal, believed to be a small RNA (Mlotshwa et al. 2002), is also thought to occur via plasmodesmata (Voinnet et al. 1998; Lucas et al. 2001; Mlotshwa et al. 2002; Himber et al. 2003). The silencing signal is not able to enter meristems or stomatal guard cells that have lost their plasmodesmatal connections (Voinnet et al. 1998). There has been speculation that if these signals are small RNAs they should be able to diffuse through plasmodesmata. However, microinjection of various fluorescently labelled single-stranded or double-stranded 25-nucleotide (nt) RNAs showed no movement from the injected cell unless in the presence of phloem-purified CmPSRP1 (*C. maxima* phloem SMALL RNA BINDING PROTEIN1) (Yoo et al. 2004). This protein is believed to facilitate the specific trafficking of small single-stranded RNA, but only into neighbouring cells as expected for trafficking of RNA from a CC into a SE. It remains to be shown whether the ER plays a specific role in this process.

6 Viruses, Plasmodesmata and the ER

Until relatively recently the study of virus movement provided the only evidence available relating to macromolecular transfer via plasmodesmata. Most viruses encode movement proteins (MP) that facilitate the movement of the viral genome either as virions or some form of ribonucleoprotein complex, through plasmodesmata (Carrington et al. 1996; Ding et al. 1999; Gilbertson and Lucas 1996; Lazarowitz and Beachy 1999; Nelson and van Bel 1998; Sakuth et al. 1993). A number of viral MP bind nucleic acid (Waigmann et al. 1994), target plasmodesmata (Blackman et al. 1998; Itaya et al. 1998; Oparka et al. 1997; Ryabov et al. 1998; Santa Cruz 1999; Tomenius et al. 1987) and gate plasmodesmata to a higher than normal SEL (Itaya et al. 1998; Oparka et al. 1997; Santa Cruz 1999; Wolf et al. 1989). It has been speculated that during co-evolution with host plants, viruses "hijacked" one or more host MP for trafficking molecules through plasmodesmata. As a result many models of macromolecular trafficking are based on these viral studies (Oparka 2004; Lucas et al. 2001; Haywood et al. 2002; Ruiz-Medrano et al. 2004).

Investigations into viral movement have implicated the ER in the MP-mediated transport of viral RNA to and through plasmodesmata. The ER is recognised to be the replication site for *Tobacco mosaic virus* (TMV) RNA (Heinlein et al. 1998; Mas and Beachy 1999; see also Chap. Ritzenthaler and Elamawi, this volume). The ER has also recently been shown to be involved, along with actin, in the movement of *Potato mop-top virus* (PMTV) granules

to and through plasmodesmata (Haupt et al. 2005). PMTV encodes three proteins associated with movement. The first triple gene block protein (TGB1) is a sequence non-specific RNA binding protein with helicase activity and is believed to produce a movement competent viral ribonucleoprotein complex, while TGB2 and TGB3 function in movement to and through plasmodesmata (Heinlein and Epel 2004; Haupt et al. 2005). Early in the infection, TGB2 and TGB3 associate with the ER and co-localise in motile granules. Both proteins are able to increase the SEL of plasmodesmata and TGB3 targets plasmodesmata in the absence of TGB2. TGB3 contains a putative Tyr-based sorting motif, which when mutated abolished the ER localisation and plasmodesmatal targeting. In this study it is suggested that the TGB2/3 complex uses the ER–actin network to facilitate movement to plasmodesmata (Haupt et al. 2005). A similar conclusion was reached for the movement of *Poa semilatent hordeivirus* (PSLV) TGB3 protein (Zamyatnin Jr. et al. 2002; Gorshkova et al. 2003).

In contrast to the TGB viruses, TMV employs only one virus-encoded MP. This 30-kDa protein from TMV has been shown to target plasmodesmata, increase the molecular exclusion limit and potentiate cell-to-cell movement as a complex with viral genomic RNA (for reviews, see Haywood et al. 2002; Roberts and Oparka 2003; Heinlein and Epel 2004). TMV-MP shows an early association with the ER and later with microtubules (Heinlein et al. 1998) and it has been proposed that the MP participates in the formation of ER-associated viral ribonucleoprotein complexes, and mediates their interaction with molecular microtubule motors for movement to plasmodesmata (Heinlein and Epel 2004). However, a more direct involvement of ER and actin in TMV movement to plasmodesmata has also been suggested based on the observation that disruption of microtubules does not prevent cell-to-cell movement of TMV, while treatment with inhibitors that disrupt ER or actin reduce the targeting of TMV-MP to plasmodesmata (Gillespie et al. 2002).

7 Conclusions

From the evidence gathered so far it is clear that the selective transport of proteins and RNAs through plasmodesmata is complex and highly regulated. Many current models of cell-to-cell transport through plasmodesmata implicate the cortical ER and desmotubule in trafficking NCAP through plasmodesmata. The greatest advances at present are emerging from new discoveries at the protein level using genomic and proteomic techniques (Cutler et al. 2000; Escobar et al. 2003; Tian et al. 2004; Oparka 2004). These should allow the identification of the receptor, docking and regulatory proteins at present hypothesised to be part of the pore and increase our understanding of the role of the ER within plasmodesmata.

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The ER in Replication of Positive-Strand RNA Viruses

Christophe Ritzenthaler (✉) · Rabab Elamawi

Institut de Biologie Moléculaire des Plantes, 12, rue du Général Zimmer,
67084 Strasbourg cedex, France
christophe.ritzenthaler@ibmp-ulp.u-strasbg.fr

Abstract All eukaryotic positive-sense single-strand RNA viruses, (+)ssRNA, replicate their genome in association with membranes of host cells. The presence of a replicating virus frequently induces proliferation and rearrangement of the host membranes into various cytopathic structures, including invaginations, vesicles, spherules or membranous webs. Such structures are considered to be virus-induced organelles specialized in replication functions. Virtually all membranes are able to be rearranged to support replication. Thus, membranes from peroxisomes, endosomes, lysosomes, vacuoles, mitochondria, and chloroplasts are used for (+)ssRNA virus replication, but the endoplasmic reticulum (ER) is by far the preferred organelle. The specific type of membrane system utilized in assembling the viral replication complex is strictly dependent on individual viruses and is likely to be genetically determined. The various molecular interactions that govern ER targeting of plant viruses highlight how viruses can exploit the diversity of interactions that occurs between proteins and membrane or lipid structures.

1 Introduction

Positive-sense single-stranded RNA [(+)ssRNA] viruses represent the largest class of viruses. They infect both animal and plants and encompass 23 families, which represent over one third of all virus genera (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). (+)ssRNA viruses replicate their genomes through negative-strand RNA intermediates and encapsidate messenger-sense genomic RNAs. Numerous well-known human and animal pathogens belong to this class, including *Hepatitis C virus* (HCV), *Yellow fever virus*, *Poliovirus*, *Foot-and-mouth disease virus*, and *Coronavirus*. The latter becoming well known recently as being responsible for severe acute respiratory syndrome (SARS). Remarkably, the replication of all known eukaryotic (+)ssRNA viruses occurs in association with host cell membranes. These viruses modify the intracellular membranes to create a compartment, with defined components and functions, often considered as a virus-specific organelle dedicated to RNA replication.

Although the strict requirement for membranes in viral RNA synthesis is not well understood, multiple properties have been attributed to membranes. Thus, membranes may provide a physical support on which the RNA

replication complex can anchor (Lyle et al. 2002). Membranes may also offer the possibility to locate and concentrate cellular and viral replication factors (Ahola et al. 1999; Schwartz et al. 2002). In addition, the flexibility of membranes might allow the replication complex to be compartmentalized into a specific virus-induced organelle, thereby sequestering RNA replication factors and genomic RNAs from competing RNA templates and competing processes such as translation (Schwartz et al. 2002). This "caging" mechanism could also offer adequate protection against host antiviral defence mechanisms such as RNA interference, or interferon-induced responses (Baulcombe 2004; Pelham 1996). Finally, evidence also exists for the involvement of membranes in tethering viral RNA during unwinding (Egger et al. 2002).

Depending on the virus, replication may occur on altered membranes derived from very diverse organelles. Thus, *Turnip yellow mosaic virus* (TYMV) is probably the best studied virus whose replication occurs in association with chloroplasts. TYMV infection is accompanied by the induction of characteristic membranous vesicles at the periphery of the chloroplast envelope. Cytological abnormalities include the swelling and clumping of the chloroplasts and the appearance of peripheral structures consisting of membrane vesicles, 50–100 nm in diameter, that are likely to result from the invagination of the chloroplast envelope into the organelle (Hatta et al. 1973; Prod'homme et al. 2001, 2003). Membranes from mitochondria support the replication of the tombusvirus *Carnation Italian ringspot virus* (CIRV). Studies of CIRV infections have identified vesiculated structures (multivesicular bodies [MVBs]), made up of a main body surrounded by many spherical to ovoid vesicles 80–150 nm in diameter, resulting from proliferation of the limiting mitochondrial membrane (Rubino and Russo 1998; Rubino et al. 2001; Weber-Lotfi et al. 2002). Similar MVBs are also observed upon infection with the closely related tombusvirus *Cymbidium ringspot virus* (CymRSV). However, here membranes from peroxisomes rather than mitochondria were shown to support the biogenesis of the MVBs (Bleve-Zacheo et al. 1997; Burgyan et al. 1996; Navarro et al. 2004; Rubino and Russo 1998). With *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV), it is the vacuolar membrane or tonoplast that appears to participate in the replication process (Cillo et al. 2002; Van Der Heijden et al. 2001).

The endoplasmic reticulum (ER) can represent up to 50% of the intracellular membrane surface. It is the most versatile, adaptable, and largest organelle in eukaryotic cells (Staehelin 1997). It is also the most common organelle for the replication of viruses infecting animals and plants. Thus, the membranous web observed during HCV infection of human cells is found in close association with rough ER (for review see Moradpour et al. 2003; Moriishi and Matsuura 2003). For *Poliovirus*, it is believed that the translation of viral proteins and replication takes place at dispersed sites on the ER, resulting in membrane protrusions and the budding of vesicles from the ER (Egger et al. 2000; Egger and Bienz 2005). The emerging individual vesicles grow

rapidly into ER-associated small clusters dispersed throughout the cytoplasm and are engaged in RNA synthesis (Bolten et al. 1998; Egger and Bienz 2005). Although consensus is emerging indicating that ER membranes contribute to the origin of the *Poliovirus*-induced vesicles, the exact mechanism underlying their generation remains controversial. The data available indicates that different pathways, controlled by host cellular processes and enhanced by the virus, may be involved. Thus, it was initially suggested from the sensitivity of *Poliovirus* to Brefeldin A, that the viral-induced vesicles might be derived from the COPI pathway (Belov et al. 2005; Cuconati et al. 1998). However, Rust et al. (2001) provided evidence that vesicles could form at the ER by subversion of the cellular COPII budding machinery. More recently, Jackson et al. (2005b) highlighted the role of autophagy in *Poliovirus* replication. In particular, they showed that autophagy marker proteins colocalize in PV-infected cells, a phenomenon that otherwise occurs only upon induction of autophagy. In addition, they established that stimulation of autophagy by tamoxifen or rapamycin, increased *Poliovirus* yield, whereas inhibition of the autophagosomal pathway decreased *Poliovirus* yield (Jackson et al. 2005).

In plants, more than 90% of all known viruses possess a (+)ssRNA genome. Based on their genome organization, they have been classified into three main superfamilies: alphavirus-like, picornavirus-like, and flavivirus-like (Koonin and Dolja 1993). All contain members whose replication occurs in association with the ER (for a recent review see Salonen et al. 2005). We will present in some detail relevant studies on plant viruses belonging to the alphavirus-like and picornavirus-like superfamilies that replicate on ER membranes.

2
Plant Viruses Belonging to the Alphavirus-like Superfamily

Plant viruses belonging to the alphavirus-like superfamily can target different types of membranes for their replication as exemplified above with TYMV and AMV. *Brome mosaic virus* (BMV) and *Tobacco mosaic virus* (TMV), probably two of the best-studied members of the alphavirus-like superfamily, exploit ER membranes for their replication.

2.1
Brome Mosaic Virus

BMV is the type-member of the genus *Bromovirus* within the family *Bromoviridea*. Its replication cycle has been carefully reviewed in recent years (Noueiry and Ahlquist 2003). Its genome consists of three positive-sense RNAs designated RNA 1, RNA 2, and RNA 3. All RNAs possess a "cap" structure at their 5′ extremities and contain a conserved 200-nucleotide tRNA-like

structure that can be acylated by tyrosine at their 3′ends (Ahlquist 1992; Ahola and Ahlquist 1999). The monocistronic RNAs 1 and 2 encode the nonstructural proteins 1a and 2a, respectively, which are essential for viral RNA replication. The 109-kDa 1a protein contains a *N*-proximal domain with m7G methyltransferase and covalent GTP-binding activities required for viral RNA capping in vivo, and a *C*-proximal RNA helicase homology domain (Ahola and Ahlquist 1999; Ahola et al. 2000; Kong et al. 1999). The 94 kDa 2a protein contains a large central RNA-dependent RNA-polymerase-like domain (Sivakumaran et al. 2000). RNA 3 is dicistronic and dispensable for replication. It encodes the 3a cell-to-cell movement protein and the coat protein that is required for systemic movement during virus infection (Mise and Ahlquist 1995). The coat protein is translated from the subgenomic RNA 4 synthesized from the negative-strand RNA 3 during replication.

Remarkably, proteins 1a and 2a can direct replication of all BMV RNAs not only in plant cells, but also in the yeast *S. cerevisiae*, indicating that the essential host features required for BMV replication are widely conserved (Janda and Ahlquist 1993). RNA replication, transcription, and persistence of BMV RNA replicons in *S. cerevisiae* have been reported (Ishikawa et al. 1997). In both organisms, RNA replication occurs on 50- to 70-nm spherular vesicles or spherules made from the invagination of the outer nuclear envelope or perinuclear ER membrane. The interior of these spherules remains connected to the cytoplasm via a narrow membranous neck contiguous with the ER membrane (Ahola and Ahlquist 1999; Restrepo-Hartwig and Ahlquist 1999; Schwartz et al. 2002). Such compartmentalization of BMV replication into ER-derived spherules was shown to protect viral RNA from nucleases (Schwartz et al. 2002) and is thought to preserve double-stranded viral replication intermediates from dsRNA-induced host defence responses such as RNA interference (Ahlquist 2002).

A combination of genetic, biochemical, and cell biology approaches has revealed that 1a is a multifunctional protein and the primary viral determinant for the subcellular localization of the BMV RNA replication complex. Thus, 1a localizes to the cytoplasmic face of the perinuclear ER independently of other viral factors and is sufficient for spherule formation (Schwartz et al. 2002). 1a expression increases total membrane lipids but not composition in yeast cells (Lee and Ahlquist 2003). 1a self-interacts and its binding domain has been mapped to the *N* terminus of the protein (O'Reilly et al. 1998). This self-interaction property is most likely responsible for spherule biogenesis, into which up to several hundred molecules of protein 1a can accumulate (Schwartz et al. 2002). In the absence of the polymerase, protein 1a also interacts with the genomic RNA resulting in a dramatic increase of stability, but reduced translatability (Janda and Ahlquist 1998). BMV RNA replication protein 1a dramatically increases in vivo stability but not translation of viral genomic RNA 3 (Sullivan and Ahlquist 1999). Finally, 1a recruits the RNA-dependent RNA polymerase 2a into the spherules (Schwartz et al. 2002). This

recruitment of 2a protein was shown to be driven by direct interaction between the *C* terminus of 1a and the *N* terminus of 2a (Kao and Ahlquist 1992; O'Reilly et al. 1995, 1997; Schwartz et al. 2002). In a recent study, the group of Ahlquist established by modulating the relative levels and interactions of BMV replication factors 1a and 2a, that spherules are dispensable for RNA replication. Thus, double ER membranes (termed karmellae) induced upon expression of 1a plus higher than normal levels of 2a supported RNA replication and protected viral RNA as efficiently as spherules (Schwartz et al. 2004). The mechanism that triggers the change from ER-derived spherules to karmellae is still not well understood, but involves protein–protein and protein–membrane interactions.

The capacity of yeast cells to duplicate all known features of BMV replication in plant cells has been exploited to identify cellular factors and functions required for BMV RNA replication. Thus, classical yeast genetics has been used to identify host genes involved in (i) BMV translation, (ii) selection of RNA templates, (iii) activation of the replication complex, and (iv) association of replication complexes with membranes (for a review see Noueiry and Ahlquist 2003). More recently, a high-throughput approach performed on 4500 yeast deletion strains, representing approximately 80% of all yeast genes, revealed nearly 100 genes whose absence inhibited or significantly stimulated BMV RNA replication (Kushner et al. 2003).

Not surprisingly, a number of the identified genes encode proteins whose function is related to host cell membranes (Table 1). Thus, BMV RNA replication was increased in *DRS2, RCY1, PBS2, NEM1, SPO7* and *GITI* knock-out yeast strains, whose functions involve phospholipid translocation, membrane recycling and MAP kinase activities. Conversely, mutations in *OLE1, ACB1, SEL1* and *SCS2*, whose involvement in membrane functions are also very diverse, resulted in mild to severe inhibition of BMV replication (Table 1). The mechanisms by which these genes contribute to BMV replication are still unknown except maybe for *OLE1*. This gene encode a delta9 fatty acid desaturase Ole1 involved in the conversion of saturated fatty acids (SFA) into unsaturated fatty acids (UFA) in yeast. UFAs are incorporated into membrane lipids and are major determinants of membrane fluidity and plasticity. Interestingly, Ole1 is an integral ER membrane protein and thus is present at sites of BMV RNA replication. However, (Lee et al. 2001) established that the Ole1 protein was not required for BMV RNA replication, but inhibition was rather linked to membrane composition. Thus, a 12% decrease in UFA levels resulted in a 95% or more reduction in BMV RNA replication. Interestingly, none of the above-described 1a properties (1a-induced membrane synthesis, 1a localization to the ER, recruitment of 2a polymerase, spherule formation) were altered in *OLE1* mutant yeast. However, membrane staining of spherules revealed that they are locally depleted in UFAs (Lee et al. 2001). This implies that the membrane, rather than Ole1 protein itself, is an essential functional component of the RNA replication complex. At this stage, the precise nature of the replica-

Table 1 Genes encoding membrane proteins involved in BMV replication (Adapted from Kushner et al. 2003, and Lee et al. 2001)

Genes	Protein name	Function/phenotype	References	Localization	Effect on BMV replication
OLE1	9 Fatty acid desaturase (Oleic acid linoleic acid)	Synthesis of unsaturated fatty acid	(Lee et al. 2001)	ER	– 50 X
ACB1	Acyl-CoA-binding protein	– synthesis of long-chain acyl-CoA esters – Intracellular transporter of acyl-CoA – Vesicles trafficking	(Rasmussen et al. 1993) (Rasmussen et al. 1994) (Faergeman et al. 2004)	Mitochondria + microsomes	– 11.1 X
SEL1	S-uppressor and/or enhancer of *lin-12*	– Secretion regulation – Regulate ER stress response – Protein-protein interaction	(Grant and Greenwald 1997) (Sundaram and Greenwald 1993)	Cytoplasm – intracellular vesicles	– 4.8x
SCS2	Suppressor of Ca^{2+} sensitivity VAP homolog SCS2 (vesicle-associated-membrane protein-associated protein)	– Myoinositol metabolism – Regulate phospholipid metabolism (serine palmitoyltransferase activity)	(Zhao et al. 1994) (Loewen and Levine 2005)	ER	– 3.6x
DRS2	Deficient in ribosomal subunits	Phospholipid-translocating ATPase	(Natarajan et al. 2004) (Ripmaster et al. 1993)	*trans*-Golgi network (TGN)	+ 4x
NEM1	Nemaline myopathy	Regulate nuclear membrane/ ER morphology	(Santos-Rosa et al. 2005) (Siniossoglou et al. 1998)	nuclear/ ER membrane	+ 4.1x
RCY1	Recycling1 (F-box protein)	Mediate endosome-to-Golgi transport	(Chen et al. 2005) (Wiederkehr et al. 2000)	Golgi and endosomes	+ 3.7x
GITI	Gastrointestinal tract injuries	Glycerophosphoinositol uptake	(Hughes 1999)		+ 3.5x
SPO7	Sporulation-defective	Regulate nuclear membrane/ ER morphology	(Santos-Rosa et al. 2005) (Siniossoglou et al. 1998) (Klapholz and Esposito 1980)	nuclear/ ER membrane	+ 3.2x
PBS2	Polymyxin B sensitivity	– Kinase activity – Osmoregulation – Scaffold protein	(Boguslawski 1992) (Bansal et al. 2001)		+ 3x

tion block remains to be determined. It is suggested that membrane fluidity or plasticity may be needed for the 1a or 2a polymerase to function correctly or to interact properly with each other or with host factors (Lee et al. 2001). Interestingly, *OLE1* gene is well conserved throughout kingdoms and the Ole1 protein from yeast OLE1 shares 31% identity with its *Arabidopsis thaliana* ortholog (accession number AK119146.1). Whether plant Ole1 protein acts on BMV replication similarly to it yeast counterpart needs to be determined.

2.2 *Tobacco Mosaic Virus*

The 6.3-kb (+)ssRNA genome of TMV encodes at least four proteins, of which the 5′ proximal open reading frame (ORF) encodes a 126 kDa protein terminated by an amber stop codon that when suppressed yields a read-through 183 kDa protein (Goelet et al. 1982; Pelham 1978). Both the 126 kDa and the 183 proteins represent essential replicase components, containing methyltransferase and helicase domains as well as a polymerase domain on the read-through portion of the 183 kDa ORF (Buck 1999; Koonin and Dolja 1993). The two additional proteins, the 30 kDa movement protein (MP) and the 17.5 kDa capsid protein are dispensable for replication but essential for cell–cell spread of infection and RNA encapsidation, respectively (for review, Heinlein 2002).

For TMV, the virus replication occurs on amorphous proliferations of membranes, previously termed viroplasms or X-bodies, which were initially observed in infected plant cells by Ivanowski (1903). Electron microscopy revealed that these inclusion bodies contained ribosomes, viral RNA, tubules, and 126/183 kDa replication proteins (Esau and Cronshaw 1967; Hills et al. 1987; Martelli and Russo 1977; Saito et al. 1987). Additionally, membrane fractions from TMV-infected cells also contained active replicase complexes capable of synthesizing both plus- and minus-strand viral RNAs in a template-dependent fashion (Osman and Buck 1996).

The use of GFP later revealed that TMV replication complexes localized into large, irregularly shaped, ER-derived structures (especially the cortical ER) containing viral RNA and also the MP (Heinlein et al. 1998; Mas and Beachy 1999; Reichel and Beachy 1998). Mas et al. (1999) further established that the MP was not required for association of the viral RNA with the ER, but was required for the formation of the large irregular bodies. Structural studies recently confirmed the membrane-binding properties of the MP (Brill et al. 2000, 2004). It has been suggested that two highly hydrophobic regions conserved among tobamovirus MPs, the domains I (residues 56–96) and II (residues 125–164) are responsible for the behavior of the MP as an integral membrane protein (Moore et al. 1992; Reichel and Beachy 1998), including its association with cortical, cytoplasmic, and perinuclear ER (Heinlein et al. 1998; Mas and Beachy 1999).

As was the case for the MP, homology searches of the 126 kDa protein failed to identify any potential ER retention motifs or obvious membrane-spanning domains (dos Reis Figueira et al. 2002). However, the TMV 126/183 kDa replicase proteins encode a predicted bipartite nuclear localization signal (NLS) at amino acids 29 to 47 that is conserved within the genus *Tobamovirus* as well as a potential 21 amino acid amphipathic helix between residues 708–728 (dos Reis Figueira et al. 2002). Interestingly, results from transient expression studies have demonstrated that:

1. The NLS present within the 126/183 kDa protein could function to nuclear localize large polypeptides
2. The NLS domain is necessary for TMV replication
3. The NLS is required but not sufficient for the formation of ER-associated inclusion bodies
4. The full-length 126 kDa protein associates with the ER in the absence of other viral proteins and components (dos Reis Figueira et al. 2002)

Whether the 21 amino acid amphipathic helix is involved in the retention of the 126 kDa protein to the ER is still not known. It has been proposed that a phenylalanine at one end of the helix could function in membrane attachment in a fashion similar to the alphavirus *Semliki forest virus* nsP1 protein (Lampio et al. 2000). Alternatively, the membrane retention of the 126 kDa could be conferred via a membrane-bound host protein.

In view of the retention of the TMV replication complex by host proteins, Ishikawa and colleagues have identified three Arabidopsis genes, *TOM1, TOM2A* and *TOM3*, that are required for efficient tobamovirus replication (Tsujimoto et al. 2003; Yamanaka et al. 2000, 2002). *AtTOM1* and *AtTOM3A* share a high degree of similarity (56% identical). They are predicted to be seven-pass transmembrane proteins and are likely to share a parallel and essential function in tobamovirus multiplication (Yamanaka et al. 2002). *AtTOM2A* is predicted to be a four-pass transmembrane protein. Remarkably, neither of these *TOM* proteins possesses well-known sorting signals to specific organelles (Tsujimoto et al. 2003; Yamanaka et al. 2000). *AtTOM1* and *AtTOM3* have been shown to interact with *AtTOM2A* and with the helicase domain of the 126/183 kDa proteins (Tsujimoto et al. 2003; Yamanaka et al. 2000), suggesting that *TOM* proteins are constituents of the replication complex of tobamoviruses and play important roles in the formation of the complex on ER membranes where they colocalize. In support of this hypothesis, subcellular fractionation patterns revealed that the viral RdRp activity coincided well with that of both the membrane-bound 126/183 kDa proteins and *TOM1–TOM2A* proteins (Hagiwara et al. 2003). However, confocal microscopic analyses of the GFP-tagged *TOM* proteins showed that *TOM1* and *TOM2A* were predominantly targeted to the vacuolar membranes and not to the ER (Hagiwara et al. 2003). Therefore, whether TMV replication complex is

associated with the tonoplast, as strongly suggested by the latter findings, or with the ER still needs to be determined.

2.3 Plant Viruses Belonging to the Picornavirus-like Superfamily

Viruses belonging to this superfamilly bear strong resemblance to animal picornaviruses both in gene organization and in the amino acid sequence of replication proteins. In plants, the genome of picorna-like viruses can be monopartite such as in *Potyviridae* or distributed over two RNAs as in members of the *Comoviridae* family. The RNAs of these viruses are characterized by a covalently genome-linked protein (VPg) at the 5′ end, a unique open reading frame encoding a single polyprotein and a poly (A) tail at the 3′-terminus. Within the *Potyvirida*, the *Tobacco etch virus* 6-kDa protein has been shown to localize specifically to ER membranes when expressed as a fusion with GFP or glucuronidase and was proposed to cause the ER modifications that are observed during *Tobacco etch virus* infection (Schaad et al. 1997). Here, the analysis has been restricted to some of the best-studied *Comoviridae* members such as *Cowpea mosaic virus* (CPMV), the type member of the genus *Comovirus*, and two nepoviruses, the *Tomato ringspot virus* (ToRSV) and the *Grapevine fanleaf virus* (GFLV).

2.3.1 *Cowpea Mosaic Virus*

CPMV RNA1 and RNA2 express large polyproteins, which are proteolytically cleaved into different proteins by the RNA1-encoded viral 24-kDa proteinase (24 K). The proteins encoded by RNA1 are necessary and sufficient for virus replication. The function of RNA1-encoded proteins functions has been attributed as follows. The 32-kDa protein (32 K) is a hydrophobic protein which does not contain a motif common to other positive-strand RNA viruses outside the *Comoviridae*. It is involved in the regulation of processing of RNA1-encoded polyprotein and is required as a cofactor in the cleavage of the RNA2 polyprotein (Peters et al. 1992). The 58 K is able to bind ATP via a conserved nucleotide-binding motif and has been proposed to be a viral helicase (Peters et al. 1994). The 28 amino acid VPg is linked to the 5′ end of the RNA via a serine residue and is likely involved in initiation of viral RNA synthesis (Carette et al. 2001). The 24 K protease is structurally similar to the trypsin-like family of serine proteases with residues His40, Glu76, and Cys166 representing the catalytic triad of the active site (Dessens and Lomonossoff 1991). The 87 K has a domain specific to RNA-dependent RNA polymerases; however, the 110 K (87 K plus 24 K) is the only viral protein present in highly purified "replication complexes" capable of elongating nascent viral RNA chains, suggesting that fusion to 24 K is required for replicase activity (Eggen et al. 1988).

Upon infection of cowpea plants with CPMV, a typical cytopathic structure is formed, often adjacent to the nucleus and consisting of an amorphous matrix of electron-dense material that is traversed by rays of small membranous vesicles (De Zoeten et al. 1974). Both subcellular fractionation experiments of CPMV-infected leaves and autoradiography, in conjunction with electron microscopy, on sections of CPMV-infected leaves treated with [^{3}H] uridine revealed that the membranous vesicles are closely associated with CPMV RNA replication (De Zoeten et al. 1974; Eggen et al. 1988; Zabel et al. 1974). Curiously, more detailed analysis later showed that the bulk of the replication proteins in CPMV-infected cells immunolocalized not to the vesicles, but to the adjacent electron-dense structures, suggesting that only a small part of the RNA1-encoded proteins contributes to the active replication complexes (Wellink et al. 1988). The accumulation of replication proteins into the electron structure is likely to depend on interactions between the 32 K and the 60 K precursor (VPg + 58 K) since deletion of the 32 K coding region, single amino acid substitution within the VPg, or the NTP binding of the 58 K protein all abolished formation of the dense bodies (Carette et al. 2001; Peters et al. 1994; van Bokhoven et al. 1993). The significance of these electron-dense structures for viral replication remains unclear as a mutation in the VPg was identified that prevented the formation of the cytopathic structures, without abolishing viral replication (Carette et al. 2001). They may simply represent deposition of inactive nonstructural protein or sites where overproduced proteins accumulate (Carette et al. 2001).

In an attempt to identify the membrane that contributes to CPMV replication, it was demonstrated through the use of transgenic *Nicotiana benthamiana* plants expressing ER- or Golgi-targeted GFP that CPMV infection causes a strong proliferation of ER membranes without affecting the structure of the Golgi stacks (Carette et al. 2000). ER modifications start at the cortical ER network and culminate with the apparition of a large region of densely packed ER membranes, often near the nucleus (Carette et al. 2000). Treatment with cytoskeleton inhibitor further revealed that intracellular trafficking of replication complexes to the large juxtanuclear structure occurs via association with the actin cytoskeleton and not the microtubular network (Carette et al. 2002a). The combined use of fluorescent in situ hybridization (FISH) and ER-GFP marker also showed that during the course of an infection, CPMV RNA colocalizes with the 110 K viral polymerase and other replication proteins and is always found in close association with proliferated ER membranes, supporting the view that ER membranes act as a source for the small membranous vesicles (Carette et al. 2002a). Not surprisingly, changes in ER morphology could be attributed to RNA1 alone. Thus, expression of RNA1-encoded proteins in insect cells, by using a baculovirus expression system, showed that 60 K, but not 110 K, is able to induce and associate with small membranous vesicles in the cytoplasm in these cells (van Bokhoven et al. 1992). More significantly, expression of individual viral proteins in *N. benthamiana* epi-

dermal cells using a viral vector revealed that both 32 K and 60 K, when fused to GFP, associate to membranes and induce rearrangement of the ER (Carette et al. 2002b). The alterations of the ER morphology resembled the proliferations that occur in CPMV-infected cells, although some differences could be observed. In particular, the GFP-32 K was present mainly in the cortical ER, whereas GFP-60 K was found mainly in the nuclear envelope, the plastidial membrane, and aggregates presumably derived from the ER. Other RNA1-encoded proteins, the 110 K polymerase and the *N*-terminal cleavage product 24 K proteinase, behaved as freely soluble proteins when expressed in isolation (Carette et al. 2002b). The localization signals that target the 32 K and 60 K, and probably also the replication complexes to ER membranes remain to be identified. It has been suggested that the three stretches of hydrophobic amino acids at the *C*-terminal end of the 32 K contribute to the membrane attachment (Carette et al. 2002b). Similarly, the 60 K contains two hydrophobic domains that are conserved among the comoviruses: an amphipathic helix at the *N* terminus (amino acids 45 to 61) and a 22-amino-acid stretch of hydrophobic residues (amino acids 544 to 565) at the *C* terminus immediately upstream of VPg (Carette et al. 2002b; Peters et al. 1992).

How ER membranes contribute to the formation the CPMV-induced vesicles is still a matter of debate. Immunogold labelling experiments on CPMV-infected tobacco leaf cells showed that they contained a relatively low amount of the luminal GFP-ER marker protein, indicating that luminal ER proteins are excluded (Carette et al. 2000) during generation of these vesicles. Additionally, it seems that the vesicle formation in CPMV-infected cells involves de novo membrane synthesis rather than a modification of preexisting membranes, as it was found that cerulenin, a fungal antibiotic which prevents de novo phospholipid biosynthesis and exerts its action by specifically inhibiting document β-ketoacyl-acyl carrier protein synthase (Moche et al. 1999), proved to be a strong inhibitor of CPMV RNA replication (Carette et al. 2000). Carette et al. (2002a) suggested that the vesicles may form as a result of the unfolded-protein response (UPR). This response can occur after various biochemical and physiological stimuli that affect ER homeostasis and impose stress to the ER, subsequently leading to accumulation of unfolded or misfolded proteins in the ER lumen (Shen et al. 2004). However, attempts to demonstrate the up-regulation of BiP that serves as a hallmark in the UPR remained unsuccessful (Carette et al. 2002a). Interestingly, a yeast two-hybrid search of an Arabidopsis cDNA library showed that the *C*-terminal domain of 60 K protein interacts with two vesicle-associated proteins (termed VAP27-1 and VAP27-2) that belong to the VAP33 family of SNARE-like proteins (Carette et al. 2002c). These host proteins also localized with the 60 K in CPMV-infected protoplasts. Carette et al. (2002c) suggested that VAP27-1 and VAP27-2 could play an important role in vesicular transport to or from the ER and may also act as a membrane anchor for the virus replication complex. Alternatively, interference of 60 K with VAP function could contribute

to the ER vesiculation process seen during infection. However, experimental evidence demonstrating the involvement of VAP27-1 and VAP27-2 in CPMV replication is still missing.

2.3.2
Grapevine Fanleaf Virus and *Tomato Ringspot Virus*

Grapevine fanleaf virus (GFLV) and *Tomato ringspot virus* (ToRSV) are very closely related nepoviruses belonging to the subgroups a and c, respectively. Their bipartite genome encodes two polyproteins P1 and P2 that are cleaved into final maturation products by the RNA1-encoded proteinases (Hans and Sanfaçon 1995; Margis et al. 1994). Similarly to CPMV, the polyprotein P1 contains the domains for proteins likely to be involved in replication, including a putative helicase (Hel), the VPg, the proteinase (Pro), and the RNA-dependent RNA polymerase (Pol) (Ritzenthaler et al. 1991; Rott et al. 1995). For GFLV, five final products referred to as 1A, $1B^{Hel}$, $1C^{VPg}$, $1D^{Pro}$ and $1E^{Pol}$ are generated by P1 processing, whereas ToRSV P1 is maturated into six proteins in vitro due to the presence of an additional cleavage site within protein 1A (Andret-Link et al. 2004; Wang et al. 1999; Wang and Sanfaçon 2000). Similarly, the processing of polyprotein P2 of GFLV generates three proteins termed 2A, 2B, 2C, whereas an additional *N*-terminally located protein is produced from the maturation of polyprotein P2 from ToRSV (Carrier et al. 2001; Margis et al. 1993). Protein 2B and 2C are involved in virus movement, transmission by nematode vectors and encapsidation (Andret-Link et al. 2004; Laporte et al. 2003; Ritzenthaler et al. 1995; Wang and Sanfaçon 2000).

Initial studies on the cytopathology of nepovirus-infected cells have revealed the presence of diffuse inclusions, often near the nuclei, which consist of complex membranous structures, some of which form vesicles (Francki et al. 1985). Further studies performed in our laboratory using infectious full-length transcripts of GFLV showed that RNA1 is able to self-replicate in protoplasts and therefore encodes all the functions needed for its own replication (Viry et al. 1993). However, analysis of a set of deletion mutants in the P2-coding sequence demonstrated that protein 2A is necessary but not sufficient for RNA2 replication (Gaire et al. 1999). Remarkably, 2A protein, when fused to GFP, distributed to punctate structures throughout the cytoplasm when expressed alone, and accumulated in a juxtanuclear area together with other RNA1-encoded proteins upon infection. This perinuclear area (Fig. 1a), also termed the viral compartment and initially described by Franski et al. (1985), could be defined as the site of viral RNA replication where newly synthesized RNA molecules, double-stranded replicative intermediates, and RNA1-encoded protein accumulate (Gaire et al. 1999; Ritzenthaler et al. 2002). It was hypothesised from these results that protein 2A enables RNA2 replication through its association with the replication complex assembled from RNA1-encoded proteins (Gaire et al. 1999).

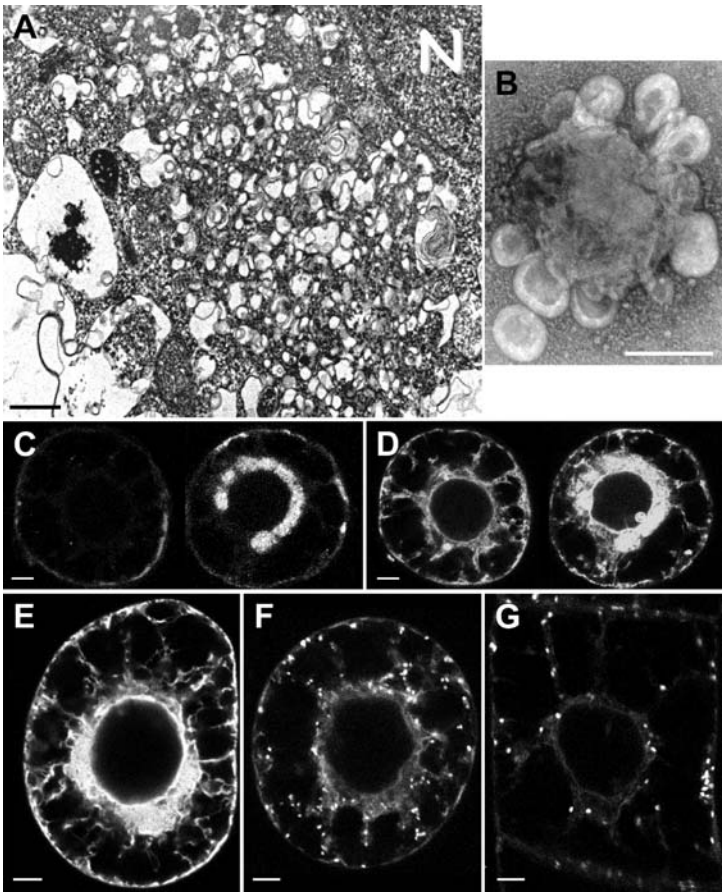

Fig. 1 Effects of GFLV replication on the endomembrane system of tobacco BY-2 cells. **a** Ultrastructural analysis of the viral compartment made of numerous vesicular structures and proliferated membranes. **b** GFLV "rosette" isolated from infected BY-2 cells after anti-VPg affinity-purification and negative-staining. **c** Detection of VPg in GFLV-infected protoplasts (*right cell* infected, *left cell* healthy). The protein specifically accumulates in the viral compartment where replication occurs. **d** The ER is visualised in the same cell as in **c**. Note that the ER is highly condensed in the viral compartment. **e** Another example of modified ER in GFLV-infected protoplast. Golgi distribution in **f** an infected and **g** a healthy cell. Note the regularly-shaped Golgi in healthy cells compared to the modified ones observed in infected cells. *Bars* = 5 μm except in **a** (1 μm) and **b** (0.5 μm)

How does the replication complex assemble, what type of intracellular membranes are used, and which are the viral and host proteins involved in the formation of the viral compartment, are questions that have been recently addressed for nepoviruses. By similarity with other picorna-like viruses, it was suggested that the $1B^{Hel}$ could act to anchor the replication

complex to membranes. In view of this hypothesis, stretches of hydrophobic residues were identified within the $1B^{Hel}$ from both GFLV and ToRSV (Rott et al. 1995; Ritzenthaler, unpublished results). Thus, the $1B^{Hel}$ from ToRSV, also named nucleoside triphosphate-binding (NTB) protein, contains a hydrophobic region at its *C* terminus consisting of two adjacent stretches of hydrophobic amino acids separated by a few amino acids. Sanfaçon and colleagues recently showed that $1B^{Hel}$VPg associates with canine microsomal membranes in the absence of other viral proteins in vitro and with ER membranes in planta (Wang et al. 2004). Analysis performed on truncated proteins fused to GFP confirmed the presence of a functional transmembrane domain within the 60 amino acids at the *C* terminus of protein 1B and also revealed the presence of a putative amphipathic helix within the *N*-terminal 80 amino acids of 1B (Zhang et al. 2005). In agreement with the replication of ToRSV on ER-derived membranes (Han and Sanfaçon 2003), both domains of the fusion proteins were sufficient to promote partial association with ER membranes, supporting the hypothesis that the HEL–VPg polyprotein acts as a membrane anchor for the replication complex of ToRSV (Zhang et al. 2005).

Similary to ToRSV, in silico analyses have revealed the presence of four stretches of 21 hydrophobic residues, predicted to correspond to transmembrane domains (TMD), situated near the *N* terminus (amino acids 518–538 and 547–567) and almost at the *C* terminus of protein $1B^{Hel}$ of GFLV 1B (amino acids 1168–1188 and 1191–1211). Transient expression of individual RNA1-encoded proteins as GFP fusions in vivo confirmed that only protein 1B was targeted to the ER and often led to the apparition of a highly modified ER network (R. Elamawi & C. Ritzenthaler, unpublished results). These $1B^{Hel}$-induced modifications are very reminiscent of those observed in vivo upon infection with GFLV (Fig. 1c–e) (Gaire et al. 1999; Ritzenthaler et al. 2002). Whether the $1B^{Hel}$ alone will also allow the formation of rosette-like structures as shown during viral infection (Fig. 1b) still needs to be determined. Nevertheless, they are perfectly in agreement with the replication of the virus on ER membranes (Ritzenthaler et al. 2002). Remarkably, transient expression of protein $1B^{Hel}$ in tobacco BY-2 cells also prevented the export of the resident Golgi protein α-1,2 mannosidase I (R. Elamawi & C. Ritzenthaler, unpublished results). Together with the facts that GFLV replication (i) is inhibited by brefeldin A (Ritzenthaler et al. 2002) and (ii) may affect the number and structure of Golgi stacks within infected cells (compare the distribution of Golgi stacks in Figs. 1f and 1g), it is suggested that the virus could exploit the cellular COPII transport pathway for the assembly of the replication complex and the biogenesis of the perinuclear viral compartment. Current research in our laboratory is aimed at testing this hypothesis.

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