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REVIEW PAPER

The endoplasmic reticulum is a hub to sort proteins toward unconventional traffic pathways and endosymbiotic organelles

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Abstract

The discovery that much of the extracellular proteome in eukaryotic cells consists of proteins lacking a signal peptide, which cannot therefore enter the secretory pathway, has led to the identification of alternative protein secretion routes bypassing the Golgi apparatus. However, proteins harboring a signal peptide for translocation into the endoplasmic reticulum can also be transported along these alternative routes, which are still far from being well elucidated in terms of the molecular machineries and subcellular/intermediate compartments involved. In this review, we first try to provide a definition of all the unconventional protein secretion pathways in eukaryotic cells, as those pathways followed by proteins directed to an 'external space' bypassing the Golgi, where 'external space' refers to the extracellular space plus the lumen of the secretory route compartments and the inner space of mitochondria and plastids. Then, we discuss the role of the endoplasmic reticulum in sorting proteins toward unconventional traffic pathways in plants. In this regard, various unconventional pathways exporting proteins from the endoplasmic reticulum to the vacuole, plasma membrane, apoplast, mitochondria, and plastids are described, including the short routes followed by the proteins resident in the endoplasmic reticulum.

Keywords: Apoplast, extracellular space, mitochondria, plasma membrane, plastids, signal peptide, unconventional protein secretion, vacuole.

Introduction

The endoplasmic reticulum (ER) is a compartment with many functions in eukariotes (Sparkes *et al.*, 2009), whose central role is the synthesis, folding, and quality control of secretory proteins (reviewed in Vitale and Denecke, 1999). It is also important for adaptation to abiotic and biotic stress (Nakano *et al.*, 2014; Ruberti and Brandizzi, 2014). Secretory proteins released in the ER lumen by an N-terminally located signal peptide may have two different destinies: they can be retained in this compartment or traffic along the secretory pathway. In the first case, secretory proteins can either be involved in specific ER functions or be accumulated in ER-derived protein bodies as storage proteins (Herman, 2008; Pedrazzini *et al.*, 2016*a*). In the second case, secretory proteins are transported to the extracellular space, or to vacuoles in plants and yeast as well as lysosomes in animals, following a conventional ER–Golgi secretory route. This pathway requires sequential budding and fusion of vesicular carriers and involves several endosomes as intermediate sorting stations (Bonifacino and

Glick, 2004). Soluble and membrane proteins, for example integral vacuolar or plasma membrane proteins, are delivered to their steady-state location through this secretory pathway (Viotti, 2016). Thus, the ER has been considered as the gateway of the conventional secretory pathway, which regulates many biological functions, such as cell homeostasis or defense, and is responsible for biogenesis and proper intracellular localization of proteins as well as complex carbohydrates and lipids (Brandizzi and Barlowe, 2013).

The classical view of the ER as the starting point of the secretory pathway has recently been expanded by several studies showing that some plant proteins harboring a signal peptide can traffic from this compartment to their destination, bypassing the Golgi apparatus (reviewed in De Marchis *et al.*, 2013*a*). Therefore, the ER can be considered as a hub to sort proteins toward unconventional traffic pathways, whereas other proteins follow the conventional secretory route and will be segregated by the Golgi apparatus to their final localization (Fig. 1A) (Banfield, 2011). Initially, the definition of unconventional protein secretion (UPS) pathways was applied to routes used by signal peptide-lacking proteins (also called leaderless secretory proteins) to reach the extracellular space bypassing intermediate compartments involved in secretion such as the Golgi apparatus (Fig. 1C) (Nickel and Rabouille, 2009). Examples of these proteins include the exocytosis of interleukin-1β (IL-1β)-containing secretory lysosomes (Andrei *et al.*, 2004; Piccioli and Rubartelli, 2013), the fusion of IL-1β-containing multivesicular bodies with the plasma membrane and release of their content outside the cell (Qu *et al.*, 2007), direct translocation of fibroblast growth factor 2 (FGF)2 across the plasma membrane by lipidic membrane pores (Zacherl *et al.*, 2015), members of the Annexin family (Valapala *et al.*, 2014), and others. In spite of the increasing number of leaderless secretory proteins ascribed to the secretome in eukaryotic cells (Bendtsen *et al.*, 2004; Agrawal *et al.*, 2010; Albenne *et al.*, 2013), few of them have been experimentally demonstrated to localize to the apoplast in plants (Pinedo *et al.*, 2012; Davis *et al.*, 2016; Robinson *et al.*, 2016). In addition, there is a growing number of polypeptides, discovered in different organisms, which, despite harboring a signal peptide, reach their steady-state location

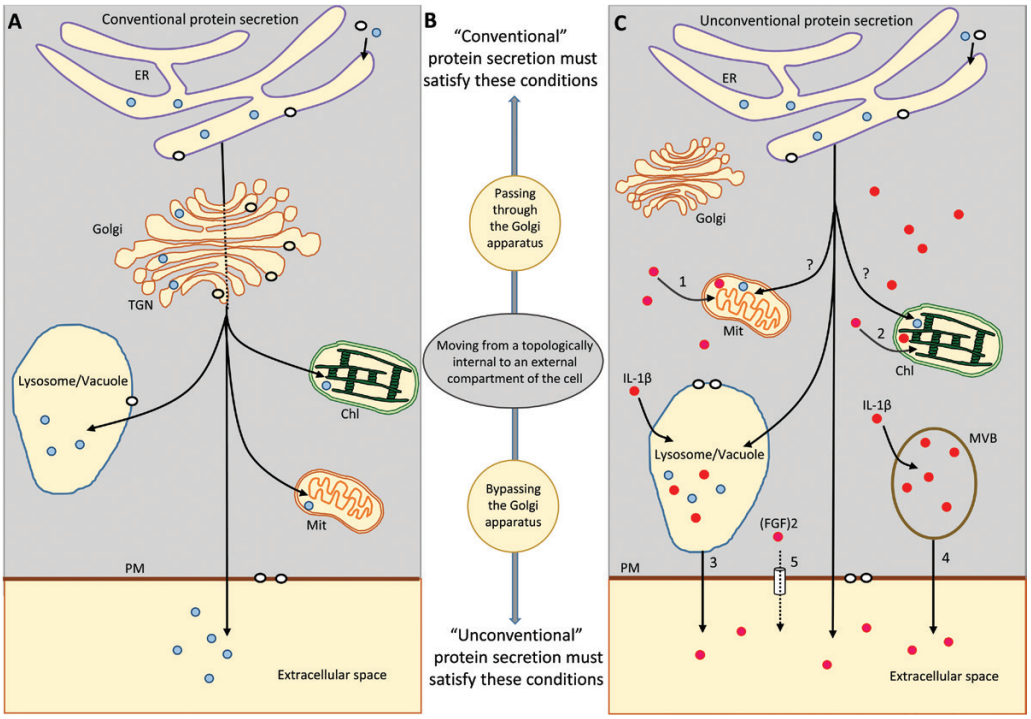

Fig. 1. Schematic definition of conventional and unconventional protein secretion routes in eukaryotic cells. (A) Secretory proteins with ER-targeting signals are transported following a conventional ER–Golgi secretory pathway to inner compartments such as vacuoles in plants and yeast or lysosomes in animals, and organelles (mitochondria and plastids) or to the cell exterior. Soluble proteins are indicated with pale blue circles, while membrane proteins are indicated with white circles. TGN, *trans*-Golgi network; Chl, chloroplast; Mit, mitochondrion; PM, plasma membrane. (B) The definition of unconventional protein secretion is summarized in two essential protein characteristics: first, polypeptides must be transported from a topologically internal (the cytoplasm) to an external compartment of the cell, which can be represented by the lumen of the ER, vacuole/lysosome, mitochondria, plastids, and the space external to the plasma membrane; secondly, proteins trafficking along UPS pathways must circumvent the Golgi apparatus. The definition of the conventional secretory pathway is also provided. (C) Secretory proteins are transported following UPS pathways from the ER to the same compartments and organelles indicated in (A), with the only difference that these proteins bypass the Golgi apparatus. Symbols are the same as in (A). Question marks indicate routes not yet described. In addition, examples of UPS routes of leaderless proteins (red circles) moving from the cytoplasm to other cell compartments and organelles are shown: (1) proteins with a cleavable N-terminal pre-sequence imported into mitochondria, (2) proteins with a cleavable N-terminal transit peptide imported into chloroplasts, (3) exocytosis of interleukin-1β (IL-1β)-containing secretory lysosomes (Andrei *et al.*, 2004; Piccioli and Rubartelli 2013), (4) fusion of IL-1β-containing multivesicular bodies (MVB) with the plasma membrane and release of their content outside the cell (Qu *et al.*, 2007), (5) direct translocation of fibroblast growth factor 2 (FGF2) across the plasma membrane by lipidic membrane pores (Zacherl *et al.*, 2015).

by UPS routes (Grieve and Rabouille, 2011). Since proteins with or without ER-targeting signals can both traffic along UPS pathways in eukaryotic cells, we suggest wider criteria to classify proteins transported by UPS routes. The concept of 'protein secretion' derives from studies published in the mid-1970s (Morré and Mollenhauer, 1974; Palade, 1975) and usually refers to proteins transported to the extracellular space. Even if the lumen of the secretory route compartments (ER, Golgi apparatus, *trans*-Golgi network, vacuole, etc.) is clearly different from the extracellular space, when a protein is translocated across the ER membrane to traffic along the secretory route, it leaves the cytoplasm and enters a type of 'external space'. In addition, the inner space of organelles of symbiotic origin, mitochondria and plastids, can also be defined as an 'external space' since most of the organellar proteins are post-translationally imported from the cytoplasm or other cell compartments. Therefore, UPS pathways can be defined as those routes followed by proteins directed to an 'external space' circumventing the Golgi apparatus, where 'external space' refers to the extracellular space plus the lumen of the secretory route compartments and the inner space of mitochondria and plastids (Fig. 1B, C). Described as a degradation mechanism, autophagy mediates cellular recycling by a trafficking pathway that carries cell material to the vacuole for recycling or to the plasma membrane for secretion (Kellner *et al.*, 2017). Involvement of the autophagy machinery in UPS has been recently reported (Zhang *et al.*, 2015). However, here we will not cover the relationship between autophagy and UPS pathways because it has already been extensively reviewed (Bhattacharya *et al.*, 2014; Michaeli *et al.*, 2014; Ng and Tang, 2016).

In this review, we highlight the role of the ER in regulating the UPS pathways that start from this compartment toward various 'external' locations in plants. Our idea of the ER as a decision-making center in protein sorting is also supported by recent publications regarding proteins delivered to the vacuole along the conventional secretory pathway. Results concerning vacuolar transport of some enzymes demonstrate that vacuolar sorting receptor–ligand interactions already occur in the ER lumen instead of in the *trans*-Golgi network as expected (Watanabe *et al.*, 2004; Künzl *et al.*, 2016). Moreover, Pompa *et al.* (2010) suggested that vacuolar delivery of the storage protein phaseolin is promoted by homotypic interactions at a very early stage of the sorting process within the ER. Based on these data, we can hypothesize that some protein sorting decisions already take place at the ER level (Niemes *et al.*, 2010; Robinson and Neuhaus 2016).

How can the ER sort proteins to UPS pathways and distinguish these proteins from those directed to the conventional secretory pathway? The mechanisms involved at the molecular level remain to be discovered, but the answer may reside in biochemical studies that must also take into consideration the existence of unknown ER domains. Indeed, the plant ER seems to have several functional domains, ranging from areas that accumulate specific products to connections with individual cell compartments or organelles (Staehelin, 1997). One of these domains, for example, is the ER export sites (ERES) to the Golgi apparatus where lipids and cargo are packed into coat protein complex (COP) II-coated vesicles (Sparkes *et al.*, 2009; Viotti, 2016). Another example is the enrichment of ER beneath the apex of tip-growing cells (Rounds and Bezanilla, 2013), described as a subapical ER scaffold (Griffing *et al.*, 2017). This condensed network of ER tubules is transiently connected with the plasma membrane in the cell apex and seems to be required for tip growth, as shown in pollen tubes (Lovy-Wheeler *et al.*, 2007) and in rhizoids of the green alga *Chara* (Limbach *et al.*, 2008). This functional organization is possible because the ER is a single extensive compartment composed of an interconnecting network of cisternal sheets and dynamic tubules, whose movement is driven by the cytoskeleton. Therefore, characterization of ER morphology is crucial for understanding its roles in the basic biology of cells, and many recent studies try to link ER morphology to its functions (Griffing *et al.*, 2017). In mammalian cells, components of the peripheral ER, which contacts many other intracellular compartments, do not comprise tubules and flat sheets, but almost exclusively tubules at varying densities. Nixon-Abell *et al.* (2016) found in live and fixed cells densely packed tubular arrays, termed ER matrices, which rapidly interconvert from tight to loose arrays. This rapid interconversion between loose and tight arrays of tubules may help the ER in reconfiguring its spatial footprint in response to intracellular structural rearrangements or cell shape changes. Moreover, dense ER matrices may also be necessary to enable ER interactions with other organelles by providing a tubular membrane reservoir (Nixon-Abell *et al.*, 2016). In addition, Summerville *et al.* (2016) demonstrate a specific mechanistic link between ER morphology and neuronal function. Thus, being organized in discrete functional domains, this compartment can accomplish its functions, for example exchange materials with other compartments by membrane contact sites at which two heterologous membranes are closely apposed without fusing, or by vesicle-mediated trafficking. All these observations lead us to believe that the ER indeed has the required characteristics to represent a sorting compartment of protein cell trafficking.

ER-resident proteins traffic along a minimal UPS pathway

In eukaryotic cells, newly synthesized secretory membrane proteins are inserted into the ER membrane, while nascent secretory soluble proteins are translocated in the ER lumen. A co-translational mechanism of protein translocation mediated by the cytosolic signal recognition particle (SRP), the ER-localized SRP receptor, and the ER-localized translocon formed by the Sec61 complex occurs for both soluble and membrane proteins (Osborne *et al.*, 2005; Shan and Walter, 2005). SRP interacts with the N-terminal signal sequence or the transmembrane domain (TMD) of a nascent polypeptide chain immediately after it emerges from the ribosomal exit tunnel, then the SRP receptor and the Sec61 complex are involved in the translocation across the ER membrane (Viotti, 2016). Alternative post-translational mechanisms

have been shown for specific proteins. For example, proteins with a single C-terminal TMD take a post-translational pathway to be inserted into the ER membrane, because they have been released from the ribosomes before the TMD emerges from the ribosomal tunnel, precluding the SRP-mediated co-translational targeting (Yamamoto and Sakisaka, 2015). After reaching their correct conformation in the ER through the action of molecular chaperones and enzymes, secretory proteins can be retained in the ER (ER-resident proteins) or be transported from the ER to the Golgi (Vitale and Denecke, 1999). It is widely accepted that in all eukaryotes, the most prominent anterograde protein transport out of the ER is the COPII-mediated transport. COPII-coated vesicles bud off from ERES which are dynamic entities (Robinson *et al.*, 2015). Incorporation of soluble proteins into COPII vesicles can occur by bulk flow (Phillipson *et al.*, 2001), whereas selection of membrane proteins and receptors for inclusion in these vesicles may depend on the specific interaction of vesicle coat subunits, or adaptors, with diacidic or dihydrophobic motifs present in the protein cytosolic domains (Schekman and Orci, 1996; Hanton *et al.*, 2005; Barlowe and Miller, 2013).

According to our definition of unconventional secretion, the ER-resident proteins could be considered as polypeptides trafficking along UPS pathways because they are retained in the ER by various mechanisms, which prevent their progression through the different Golgi subcompartments (Pagny *et al.*, 2000). One of these mechanisms is protein oligomerization. In cereal seeds, due to interchain disulfide bonds or hydrophobic interactions, the prolamin storage proteins accumulate within the ER as largely insoluble protein bodies. When permanently unable to traffic along the secretory pathway, these proteins can be considered as those following a UPS route (Pedrazzini *et al.*, 2016*b*). Another retention mechanism, suggested for ER membrane-localized proteins, relies on the properties of the TMD: length and physical–chemical features, and the TMD interaction with membrane microdomains of different lipid composition (Szczesna-Skorupa and Kemper, 2000; Ronchi *et al.*, 2008). Due to a different composition in terms of sterols and sphingolipids, the thickness of the lipid bilayer along the secretory pathway is increased (Lippincott-Schwartz and Phair, 2010). As a result, the plasma and Golgi membranes are usually thicker than the ER membranes, thus single transmembrane proteins with longer TMDs would be selected for the transport out of the ER, whereas those with shorter TMDs would be retained in the ER (Brandizzi *et al.*, 2002). Therefore, TMD length and sequence could determine whether a protein is included or not in the transport vesicles (Szczesna-Skorupa and Kemper, 2000; Gao *et al.*, 2014).

In the absence of the above-mentioned direct ER retention mechanisms, ER-resident proteins may escape through passive incorporation into COPII vesicles. In this case, COPI vesicles mediate retrograde transport of these proteins from the *cis*-Golgi back to the ER (Barlowe and Miller, 2013; Gao *et al.*, 2014). We consider the ER proteins that reach the *cis*-Golgi and then return to the ER as proteins trafficking along a UPS pathway (Fig. 2). In fact, these proteins do not transit through the Golgi stacks where they could be modified by the Golgi enzymes. For example, many reticuloplasmins, the soluble enzymes and molecular chaperones responsible for the ER protein quality control functions (Anelli and Sitia, 2008; Ellgaard *et al.*, 2016), utilize a recycling mechanism to maintain their ER residency based on a C-terminal H/KDEL

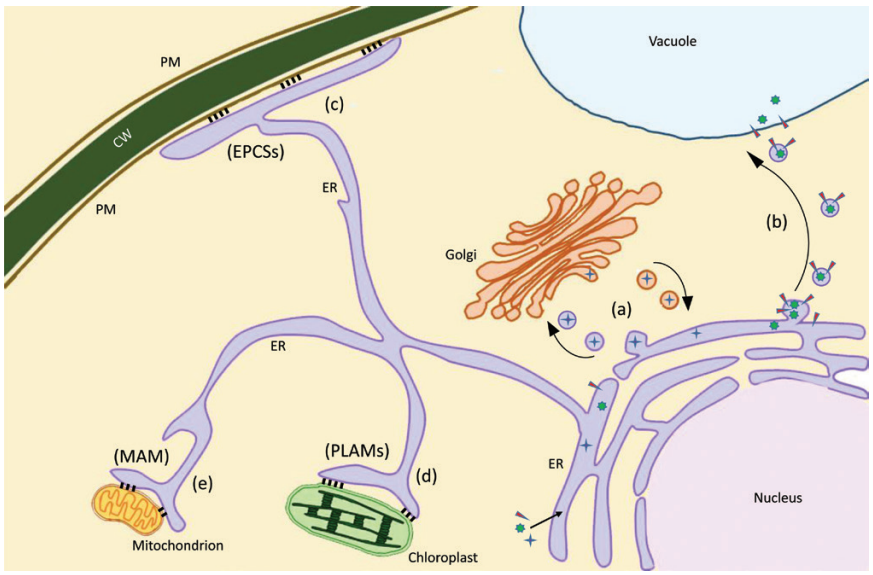

Fig. 2. Unconventional protein secretion pathways of polypeptides with ER-targeting signals in plant cells. The ER is represented as a sorting point for proteins that start their traffic from this compartment versus different external locations. (a) An example of a 'minimal UPS pathway' followed by H/KDEL-tailed ER-resident proteins (indicated with four point stars) which are bound by *cis*-Golgi-localized receptors and retrieved back to the ER. (b) Direct delivery of membrane (indicated with triangles) and soluble (indicated with seven point stars) proteins from the ER to the vacuole by ER-derived bodies. (c) ER–plasma membrane contact sites (EPCSs) that can favor protein translocation from the ER to the plasma membrane or to the apoplast; unknown UPS pathways between the ER and the plant organelle of endosymbiotic origin involving membrane contact sites. (d) Plastid-associated membrane (PLAM) sites. (e) The mitochondria-associated membrane (MAM). PM, plasma membrane; CW, cell wall.

motif. This tetrapeptide is recognized by *cis*-Golgi-localized receptors, Erd2 in yeast and plants (Semenza *et al.*, 1990; Lee *et al.*, 1993) and KDELRs in mammals (Lewis and Pelham, 1992, Pulvirenti *et al.*, 2008), which bind the tetrapeptide-containing protein and retrieve it back to the ER by COPI vesicles.

We must underline that some ER-resident proteins can pass through the Golgi complex and reach the vacuole, but this is not a secretory route. Disposal of structurally defective secretory proteins usually takes place through retrotranslocation from the ER into the cytosol, ubiquitination, and proteasomal degradation, so-called ER associated degradation (ERAD). Alternatively, ER proteins might also be transported to the vacuole for degradation and the Golgi complex may be involved in these vacuolar disposal routes. For example, the ER chaperone BiP can be transported to the vacuole in a Golgi-dependent way (Pimpl *et al.*, 2006).

In conclusion, we would like to define the 'minimal UPS pathway' as the short route followed by functional ER-resident proteins from their site of synthesis to their desired ER localization.

Direct delivery of proteins from the ER to the vacuole

Vacuoles contribute to plant growth and development because of their involvement in many cellular functions; for example, lytic vacuoles, mainly found in vegetative tissues, play an essential role in regulation of turgor pressure, defense against pathogens, degradation of cellular waste, and accumulation of ions and secondary metabolites (Marty, 1999; Bethke and Jones, 2000). On the other hand, protein storage vacuoles (PSVs) are the most abundant type of vacuoles in seeds, which accumulate storage proteins for plant growth. Sometimes these two types of vacuoles can co-exist in the same cell (Paris *et al.*, 1996; Zhang *et al.*, 2014). To cope with these multiple functions, plants have developed a complex vacuolar transport system that has been extensively reviewed (Vitale and Hinz, 2005; Hwang, 2008; Xiang *et al.*, 2013; Pereira *et al.*, 2014). Distinctly regulated routes have been reported according to the plant tissues analyzed, the vacuolar sorting signals used, and the different proteins that regulate vesicle formation and fusion, such as the RAB small GTPases and adaptor protein complexes (Bottanelli *et al.*, 2011; Ebine *et al.*, 2014; Robinson and Neuhaus, 2016). Furthermore, proteins with ER-targeting signals can be delivered from the ER to the vacuole by UPS pathways, and several examples of soluble proteins, membrane proteins, or proteins aggregated in large polymers have been described (Herman and Schmidt, 2004; De Marchis *et al.*, 2013*b*; Pedrazzini *et al.*, 2013*a*). The studies on the ER-to-vacuole direct trafficking routes will be updated in the following sections.

Insoluble ER protein aggregates directly transported to the vacuole

Insoluble protein aggregates can be formed in the ER and directly transported to the vacuole, where they are able to carry out various physiological functions (Fig. 2). We can schematically divide these ER-derived structures into two groups: those formed mainly by storage proteins and those formed mostly by enzymes.

During seed maturation, storage proteins can be accumulated in the ER as spherical bodies with diameters of 0.2–1.8 µm, which are released in the cytoplasm and then fused with the PSVs, as reported in wheat (Levanony *et al.*, 1992), rice (Takahashi *et al.*, 2005), maize (Reyes *et al.*, 2011), soybean (Mori *et al.*, 2004), pumpkin, and castor bean (Hara-Nishimura *et al.*, 1998). In this latter case, complex glycans are associated with the ER-derived bodies (designated precursor-accumulating vesicles, PACs), indicating that PACs on their way to the vacuolar compartment have somehow interacted with the Golgi apparatus as well. However, no detailed description of these vacuolar targeting machineries is available yet. According to Reyes *et al.* (2011), the Golgi-independent pathway observed for zeins, which in seed aleurone cells are sequestered in pre-vacuolar compartments before being delivered to the PSVs, can represent a special kind of selective autophagy. Alternatively, based on the available literature, the data presented by Reyes *et al.* are not explained as autophagy, but rather as an unconventional process in which membrane-containing vesicles bud from the ER and release their contents into the PSVs (Rogers, 2011). Michaeli *et al.* (2014) have recently reviewed in plants the involvement of autophagy in the direct ER-to-vacuole UPS trafficking route, concluding that many examples have shown that this route uses the autophagy machinery. In this regard, we believe that the only strong evidence has been described in Arabidopsis plants by Honig *et al.* (2012). They show that two closely related new proteins, ATI1 and ATI2, bind the autophagy-related Atg8f isoform using '*in vivo*' systems and that these proteins in normal growth conditions are partially localized to the ER membrane. Conversely, upon exposure to carbon starvation, ATI1 and ATI2 become associated with newly identified ER aggregates (ATI bodies). These spherical bodies (with diameters of 0.5–1.0 µm), moving dynamically along the ER network, are then transported directly to the vacuole, but they differ from the classical autophagosomes because of their lack of co-localization with classical autophagosomes labeled with the monomeric red fluorescent protein (mRFP)–Atg8f or the green fluorescent protein (GFP)–Atg8f autophagosomal markers (Honig *et al.*, 2012). Consequently, this is an alternative pathway to the conventional autophagy, so the autophagy process is not necessarily involved in UPS routes in plants. The binding between ATI1/ATI2 and the Arabidopsis ATG8f protein can only mean one thing: an autophagy-related protein (ATG8f) has an autophagy-independent effect on a UPS pathway, but the exact function of this effect is still unknown. Indeed, it remains unclear and has recently been disputed whether there is an inter-relationship between unconventional secretion and autophagy, which could be a possible source for double-membrane vesicles to transport specific cargo directly from the ER to the lysosome/vacuole or to the extracellular space/cell wall (Kulich and Žárský, 2014; Pompa *et al.*, 2017).

Some ER-derived structures accumulate mainly enzymes, such as the KDEL-tailed cysteine proteinase-accumulating vesicles (KVs), ricinosome vesicles in germinating seeds, and the ER bodies in roots and seedlings. Spherical KVs in mung bean (Toyooka *et al.*, 2000) as well as ricinosomes in castor bean (Schmid *et al.*, 2001) range in size from 0.2 µm to 1.0 µm, and they contain papain-type proteases responsible for protein degradation. In mung bean cotyledons, KVs are sorted to PSVs, while in castor bean endosperm there is no experimental evidence of ricinosome fusion with the vacuole. Being rod-shaped and larger than the previously described protein aggregates (~10 µm long and ~1 µm wide), ER bodies accumulate above all β-glucosidases and have been observed only in three families of the *Brassicales* order (Nakano *et al.*, 2017). In the presence of salt stress which makes the epidermis cells of *A. thaliana* seedlings die, these structures fuse to vacuoles, releasing the cysteine protease RD21 and vacuolar processing enzyme-γ (Hayashi *et al.*, 2001), showing the involvement of vacuolar processing enzymes in plant programmed cell death (Hatsugai *et al.*, 2015). Other functions for ER bodies have been suggested, from plant defense to abiotic stress resistance, but direct evidence of their physiological functions is still lacking (Matsushima *et al.*, 2003; Nakano *et al.*, 2014).

Depicted as a microdomain, a model of the ER body formation in Arabidopsis seedlings has been recently suggested (Nakano *et al.*, 2014). Core formation of these structures could be induced by β-glucosidase PYK10 protein interaction with another protein, NAI2, followed by the NAI2-dependent recruitment of two integral membrane proteins, MEB1 and MEB2, to form the ER body-specific membrane. Similar hypothetical models for formation and budding of other ER-derived bodies involve specific ER subdomains enriched in selected membrane and soluble proteins, which then bud as vesicles and traffic directly to the vacuoles (Hara-Nishimura *et al.*, 1998; Oufattole *et al.*, 2005; Rogers, 2011). The organization of microdomains in ER membranes implies that proteins and lipids constantly reshape ER architecture. One example is the characterization of the metabolon synthesizing the cyanogenic glucoside dhurrin at the cytosolic side of the ER membranes in sorghum (Laursen *et al.*, 2016). By studying the soluble UDP-glucosyltransferase UGT85B1 together with the membrane proteins cytochrome P450 oxidoreductase and cytochrome P450 (CYP79A1 and CYP71E1), Laursen and colleagues show that soluble UGT85B1 interacts with CYP79A1 and CYP71E1. These two integral membrane proteins (CYP79A1 and CYP71E1) dynamically diffuse along the ER network, but within the metabolon their interaction with cytochrome P450 oxidoreductase results in slower diffusion rates along the ER. In addition, the membrane itself is an integral part of the metabolon due to an enrichment of negatively charged phospholipids in its proximity. Therefore, plant ER membranes are heterogeneous and dynamic, with variable local lipid and protein composition that give rise to the required microdomains to conduct biological functions in various cell types or in response to different growth cues and conditions (Staehelin, 1997; Zhang and Hu, 2016).

Unconventional secretion of transmembrane proteins with ER localization to the vacuole

Synthesized in the ER, tonoplast-resident proteins are targeted to the vacuolar membrane through the secretory pathway or through UPS routes (Pedrazzini *et al.*, 2013; Viotti, 2014). Early studies performed in tobacco protoplasts showed a Golgi-independent traffic of the tonoplast intrinsic protein α-TIP (Gomez and Chrispeels, 1993; Jiang and Rogers, 1998). Other examples of tonoplast proteins, such as δ-TIP, trafficking along UPS pathways were produced later. The utilization of plant protoplasts combined with the immunofluorescence method and several chemicals to inhibit specifically the Golgi-dependent or the Golgi-independent vacuolar routes has helped to understand which pathway is followed by a certain protein (Rivera-Serrano *et al.*, 2012; De Marchis *et al.*, 2016). Isayenkov *et al.* (2011) investigated the mechanisms that define how rice two-pore K^+ channel (TPK) membrane proteins reach their vacuolar destination. They demonstrated that the TPKa isoform present in the lytic vacuoles reaches the tonoplast following the conventional secretion route, whereas another isoform located on PSV-type compartments, TPKb, traffics by a Golgi-independent transport. Moreover, three C-terminal amino acids are crucial for the differential targeting of TPKa and TPKb to the lytic or storage vacuoles. In Arabidopsis, the tonoplast calcineurin B-like (CBL) proteins CBL2, CBL3, CBL6. and CBL10 are targeted to the vacuolar membrane in a COPII-independent way. Although this strongly suggests that CBL proteins traffic by a Golgi-independent route, it is only the case for CBL6. CBL proteins do not seem to be translocated into the ER, so perhaps they reach the tonoplast directly from the cytosol (Bottanelli *et al.*, 2011; Batistič *et al.*, 2012). Unlike CBL proteins, another two tonoplast proteins (α-TIP and syntaxin Vam3) follow a Golgi-independent route for their export from the ER in a COPII-dependent manner. However, these two proteins are then delivered to the vacuolar membrane in a different way. Indeed, by using dominant-negative mutants of RAB GTPases, which block the vesicular transport in specific traffic steps in which a given RAB is involved, it has been shown that α-TIP requires both plant RAB5 (RABF2a/RHA1 and RABF2b/ARA7) and plant RAB7 (RABG3C), which are active in the pre-vacuolar compartment and the tonoplast, respectively (Bottanelli *et al.*, 2011; Pedrazzini *et al.*, 2013). Conversely, Vam3 transport to the vacuole is disputed because according to Bottanelli *et al.* (2011) it is RAB5 independent but RAB7 dependent. On the other hand, other authors suggest the opposite, that Vam3 is targeted to the tonoplast in a RAB5-dependent yet RAB7-independent pathway (Ebine *et al.*, 2014). Recently, Viotti *et al.* (2013) demonstrated in Arabidopsis root meristematic cells that the transport route of the proton pumps VHA-a3 and AVP1 is a direct ER to vacuole UPS pathway. Both pumps are localized in provacuoles, which can be distinguished from autophagosomes and are not formed by the core autophagy machinery. They suggested that provacuoles arise directly from ER microdomains incorporating tonoplast lipids and proteins through an uncharacterized mechanism. Not only VHA-a3 and AVP1, but also other tonoplast

proteins such as α-TIP could follow the same route (Viotti *et al.*, 2013; Viotti, 2014). Moreover, Feng *et al.* (2017) suggested that VHA-a3 delivery to the tonoplast can rely on a RAB5-dependent but RAB7-independent vacuolar trafficking route. Thus, the ER seems to be the main membrane source for the biogenesis of the plant lytic compartments in root meristematic cells. This hypothesis is corroborated by recent results obtained by studying the quantity of proteins with Golgi-modified glycans on the tonoplast (Pedrazzini *et al.*, 2016*b*). The scarcity of the entire spectrum of complex glycans on the Arabidopsis tonoplast could be explained if the major route to the tonoplast bypasses the Golgi apparatus. Interestingly, Zhuang *et al.* (2017) demonstrated in Arabidopsis that initiation of autophagosome formation largely involves the ER membrane. Therefore, the ER can be assumed to act as the main hub to sort plant membranes toward both the lytic vacuole tonoplast by UPS pathways and the autophagosomes, even if other cell compartments as alternative sources of membranes for these processes cannot be excluded.

Transport of soluble proteins from the ER to the vacuole bypassing the Golgi apparatus

Some secretory soluble proteins reach vacuoles or lysosomes by UPS, even though there are only a few examples. In mammalian cells, the soluble proteins EDEM1 and OS-9 are ER chaperones targeting misfolded glycoproteins for ERAD. To prevent premature interruption of folding, EDEM1 and OS-9 are segregated from the bulk ER into ER-derived vesicles (without a recognizable COPII coat) which regulate rapid EDEM1 and OS-9 turnover by the subsequent action of lysosomal proteases (Zuber *et al.*, 2007; Calì *et al.*, 2008). The existence of these UPS pathways for soluble proteins has also been confirmed in transgenic plants by expressing pharmaceutical glycoproteins with heterologous signal peptides for translocation into the ER (De Marchis *et al.*, 2011; Ocampo *et al.*, 2016). A functional human lysosomal α-mannosidase MAN2B1 has been produced in plants of the genus *Nicotiana* in both stable and transient expression systems, and it localizes in leaf lytic vacuoles or seed PSVs (De Marchis *et al.*, 2011). The correctly processed α-mannosidase fragments in the tobacco lytic vacuoles have not been modified by Golgi enzymes, suggesting that MAN2B1 follows a Golgi-independent vacuolar sorting pathway (De Marchis *et al.*, 2013*b*). Ocampo *et al.* (2016) demonstrated that another glycoprotein, the mouse IgG1 14D9, reaches the lytic vacuole of transiently transformed *N. benthamiana* leaves bypassing the Golgi apparatus. Two different protein versions were prepared, in which the monoclonal antibody 14D9 was fused to different plant vacuolar targeting motifs, one typical of lytic vacuole proteins and the other distinctive for storage proteins. The results, similar in the two gene constructs, showed that vacuolar sorted 14D9 glycoproteins were correctly assembled and they carried two types of glycans, predominantly oligomannosidic structures (75%) and complex glycans (25%). This indicates that a large part of the 14D9 glycoproteins can be delivered to the vacuole by direct transport from the ER, whereas a minor part of these glycoproteins traffic through the Golgi where the complex oligosaccharides originate.

Does direct protein transport between the ER and the plasma membrane exist in plants?

Studies conducted on mammalian and *Drosophila* cells describe several proteins unconventionally secreted from the ER to the plasma membrane or the extracellular space. These include the cystic fibrosis transmembrane conductance regulator (CFTR) (Gee *et al.*, 2011), a member of the integral plasma membrane αβ heterodimers (integrins), integrin αPS1 (Schotman *et al.*, 2008), the receptor protein-tyrosine phosphatase CDC45 (Baldwin and Ostergaard, 2002), a mutated form of pendrin (Jung *et al.*, 2016), lysyl hydroxylase 3 (Wang *et al.*, 2012), and others. Conversely, very few plant proteins are delivered to the plasma membrane or are secreted to the apoplast from the ER by means of a UPS pathway. Kunze *et al.* (1995*a*, *b*) reported that secretion of two vacuolar proteins, a class I chitinase termed SN11 and a lectin-like protein designated SN9, is not blocked by brefeldin A in suspension-cultured tobacco cells. This chemical inhibits the bidirectional membrane trafficking between the ER and the Golgi; thus they suggested the existence of an unconventional route that transports these proteins from the ER to the apoplast, without showing additional details of this pathway. In plants, to date, we have no convincing experimental evidence that proteins travel from the ER to the plasma membrane or the extracellular space bypassing the Golgi complex (Ding *et al.*, 2012, 2014). Recently, Watanabe and colleagues demonstrated, using GFP-fused protein constructs, that two pathogenesis-related proteins, PDF1.2 and PR1, are stored in Arabidopsis ER bodies and released into the apoplast after pathogen inoculation. However, there is no indication in the study of whether the intracellular route responsible for the secretion of PDF1.2a–GFP and PR1–GFP is Golgi dependent or Golgi independent (Watanabe *et al.*, 2013).

Intriguingly, data presented by our group during the ‘Unconventional Protein and Membrane Traffic (UPMT)’ meeting held in Lecce (4–7 October 2016) show that an Arabidopsis protein, CLAVATA (CLV) 3, harboring a signal peptide, does not seem to follow the conventional pathway to be secreted into the apoplast. One possible explanation is that, once it has entered the ER lumen, CLV3 is retrotranslocated to the cytosol where, processed by the proteasome machinery, it can form the CLV3 active peptide secreted by an unknown unconventional route (Pompa *et al.*, 2017). Many plant biological processes are strictly regulated by peptide signaling pathways, and a large number of these peptides derive from processing of secretory protein precursors (Matsubayashi, 2014; De Coninck and De Smet, 2016). Therefore, we can infer that other active peptides can be secreted in a way similar to CLV3 secretion.

Almost 30 years ago, contact sites between the ER and the plasma membrane were identified in plants (Hepler *et al.*, 1990), termed ER–plasma membrane contact sites (EPCSs; Fig. 2), being a common feature in eukaryotic organisms. EPCSs have well-established functions in the movement of lipids and ions between the ER and plasma membrane, as well as in the regulation of membrane transport and cell

signaling pathways (Stefan *et al.*, 2013), but EPCS structure and function remain to be fully elucidated in plants (Wang *et al.*, 2017). Recent studies have started to localize proteins in plant EPCSs, including NETWORKED 3C (NET3C), members of the VAP27 protein family, and synaptotagmin 1 (SYT1). These proteins create a complex together with microtubules and actin filaments; in particular, NET3C forms a link between actin filaments and the plasma membrane (Wang *et al.*, 2014). SYT1 also seems to be involved in cell mechanical stability, by stabilizing the ER network and the EPCSs (Pérez-Sancho *et al.*, 2015; Siao *et al.*, 2016). The role of VAP27 in plants is still not clear. In Arabidopsis, SYT1 and VAP27-1 interact with the reticulons RTN3 and RTN6, proteins resident in the plasmodesmata (i.e. the plant channels involved in cell-to-cell transport; Kriechbaumer *et al.*, 2015). Moreover, Wang *et al.* (2017) suggested that VAP27 might mediate the oxysterol transport in EPCSs, which could also be the sites where cuticular lipid secretion takes place. These lipids are synthesized in the ER and secreted through the ATP-binding cassette (ABC) transporters localized in the plasma membrane. Interestingly, although the subcellular compartments involved in wax secretion remain unknown, this transport seems to be Golgi independent, with wax moving directly from the ER to the plasma membrane via non-vesicular lipid traffic (McFarlane *et al.*, 2010). Based on all these findings, we believe that it is only a matter of time before solid evidence can support the existence of UPS pathways from the ER to the plasma membrane in plant cells. Further studies on the characterization of proteins belonging to the EPCS complex in plants will certainly help.

UPS routes between the ER and the endosymbiotic organelles

Mitochondria and plastids are the semi-autonomous organelles of the plant cell. More than 1.5 billion years ago, they evolved from two distinct endosymbiotic events in which free-living bacteria, probably an aerobic and a photosynthetic prokaryote, were engulfed by a primordial eukaryote cell. During evolution, two processes led these endosymbionts to become integrated cell organelles: one was the dramatic reduction in their genome size due to gene loss or transfer to the host nucleus, and the other was the acquisition of mechanisms enabling the nuclear-encoded proteins to re-enter chloroplasts (the green photosynthetic plastids) and mithocondria (Martin and Herrmann, 1998). Indeed, it is calculated that >95% of the genes encoding the ~3000 chloroplasts proteins have been transferred to the host nucleus, while the residual ~100 genes have been encoded by the plastid genome (Martin *et al.*, 2002; Timmis *et al.*, 2004). As a result, the majority of mitochondrion and chloroplast polypeptides are now post-translationally imported from the cytoplasm. During targeting, organellar proteins are kept in an unfolded state by interaction with different cytosolic chaperones, such as Hsp70, Hsp90, and 14-3-3 proteins. Such interaction allows translocation of the organellar proteins across the envelope membranes, which is, in the majority of cases, facilitated by two distinct translocation complexes (Duncan *et al.*, 2013; Sjuts *et al.*, 2017). The two sophisticated translocase structures present on the organelles double membranes are the plastidial Tic/Toc and the mithochondrial Tim/Tom complexes (Soll and Schleiff, 2004; Bedard and Jarvis, 2005). These elaborate protein machineries enable the translocation of the cytoplasmically synthesized protein due to the recognition of an N-terminal cleavable targeting signal: the presequence and the transit peptide for the mitochondrion and chloroplast, respectively. After import, processing peptidases cleave off the N-terminal targeting signals and the remaining mature proteins undergo folding and insertion or further direction to intraorganeller targets (Duncan *et al.*, 2013; Sjuts *et al.*, 2017).

We consider the biological material, including plastid thylakoids and mitochondrial cristae, surrounded by the outer membrane of the organelles as an ‘external space’, regulated by internal protein trafficking machineries (Schünemann, 2007; Brandizzi, 2011). This concept only appears to be in contrast to the opinion that internal organelle matrices are cytoplasm like and therefore protein transport systems located to the mitochondrial inner membrane and the chloroplast thylakoid membrane can be considered export routes (Settles and Martienssen, 1998). The reason is that mitochondrial matrix and chloroplast stroma now appear very different from the cytoplasm of their eubacterial symbiont ancestors, because these internal matrices have acquired typical ER characteristics during evolution. First, proteins imported from the cytosol to the two organelles must go through double-membrane envelopes by a translocation process requiring an N-terminal cleavable targeting signal with a similar function to the signal peptide used for ER insertion. Secondly, during protein import into the ER lumen/chloroplast stroma/mitochondrial matrix, membrane translocation occurs in an unfolded state, with molecular chaperones and enzymes ensuring proper folding of newly translocated proteins. Consequently, the lumen of the two organelles, like the ER lumen, can be imagined as an ‘external space’ in comparison with the cytoplasm. Therefore, we can define routes transporting proteins directly from the ER to the mitochondria and chloroplasts as UPS pathways.

Is there any protein trafficking from the ER to endosymbiotic organelles? Based on the current literature, there is no mention of polypeptides directly moving from the ER to plastids or mitochondria in plants (Fig. 1C). However, in 1998, a 45 kDa glycoprotein involved in the respiratory chain was identified in the rat liver mitochondrion inner membrane. The analysis of its *N*-linked glycans confirmed a high mannose structure and sensitivity to endoglycosidase-H digestion, indicating that traffic of this protein depends on ER-mediated delivery (Chandra *et al.*, 1998). The authors suggested that the contact regions of the ER and the mitochondrion, the so-called MAM (mitochondria-associated membrane), could be involved in protein transfer between the two cell compartments (Fig. 2). Indeed, despite their viral origin, proteins from human cytomegalovirus and hepatitis C virus traffic sequentially from the ER into mitochondria, probably through the MAM junctions (Williamson *et al.*, 2012). Instead of being

an isolated compartment, the ER is part of a communication network, through membrane contact sites, with other cell compartments, including the mitochondria and plastids (Wang and Benning, 2012; Prinz, 2014). The importance of the MAM connections has been the subject of numerous studies in yeast and animal cells. Today they are known as regulators of lipid biosynthesis, Ca^{2+} exchange, mitochondrial fission and dynamics, autophagosome formation, and cell survival (Rowland and Voeltz, 2012; Vance, 2014). Four MAM tethering complexes have been described in mammalian cells, which simultaneously bind the two apposing ER and mitochondrion membranes (Prinz 2014). For example, the ER-resident protein inositol trisphosphate receptor (IP3R) enables Ca^{2+} movement from the ER to mitochondria, facilitated by the voltage-dependent anion channel (VDAC) located in the outer mitochondrial membrane and the cytosolic chaperone Grp75. The three proteins form a complex that links the ER and the organelle, allowing Ca^{2+} exchange (Szabadkai *et al.*, 2006). Another example is the dynamin-like protein mitofusin-2, which not only acts as a tether by residing both in the outer mitochondrial membrane and in the ER, but also mediates mitochondrial fusion (de Brito and Scorrano, 2008). Conversely, MAM significance and molecular constitution remain mostly unknown for plants, except for a dual-localized protein, named the protein mitochondria–ER-localized LEA-related LysM domain protein1 (MELL1). When overexpressed in the model moss *Physcomitrella patens*, the association of the ER and the mitochondria is increased (Mueller and Reski, 2015). There is a growing literature on the existence of ER/chloroplast contact sites, or plastid-associated membranes (PLAMs; Fig. 2) (Hanson and Köhler, 2001; Andersson *et al.*, 2007; Griffing, 2011). Moreover, stroma-filled tubules extending from plastids, the so-called stromules, seem to have a role in the communication between plastids for exchanging metabolites. They have been shown to occupy channels between ER tubules possibly through multiple membrane contact sites (Schattat *et al.*, 2011). Furtermore, transorganellar experiments have demonstrated the possibility for ER-localized enzymes to access plastid-localized substrates, and vice versa (Mehrshahi *et al.*, 2013; Pérez-Sancho *et al.*, 2016). However, the structure and the functional significance of PLAMs remain to be determined, even if a reasonable role for PLAMs in non-vesicular lipid exchange between ER and plastids can be hypothesized (Wang and Benning, 2012). Based on these studies, UPS pathways between the ER and the plant organelle of endosymbiotic origin involving vesicles or the MAM and PLAM sites will probably be identified in the future.

Chloroplast proteins reaching the organelle through Tic/Toc-independent pathways have already been described (Baslam *et al.*, 2016). These proteins traffic along the secretory pathway from the ER to the Golgi and reach the plastid envelope. Villarejo *et al.* (2005) identified a stromal protein, the α-type carbonic anhydrase, targeted to the Arabidopsis chloroplast via the Golgi. The carbonic anhydrase, harboring an N-terminal signal peptide for translocation into the ER, is glycosylated and its plastidial localization is impaired by brefeldin A. In the same year, Asatsuma *et al.* (2005) described the plastidial localization of the rice α-amylase I-1, which was targeted to the chloroplasts through the ER–Golgi system in a vesicle-mediated manner, as confirmed some years later by Kitajima *et al.* (2009). Subsequently, other glycoproteins were identified in the rice chloroplast as the manganese superoxide dismutase 1 (Shiraya *et al.*, 2015) and the nucleotide pyrophosphatase/phosphodiesterases 1, 2, and 6 (Nanjo *et al.*, 2006; Kaneko *et al.*, 2016). The discovery that chloroplast transport could occur independently from the Tic/Toc system has raised some questions about the evolution of the symbiotic organelle protein targeting mechanism. Two theories are currently proposed. According to Bhattacharya *et al.* (2007), the traffic mediated by the endomembrane system evolved during the early stages of plastid evolution. Cyanobacterial genes transferred to the host nucleus acquired an ER-targeting signal peptide, which guaranteed their correct relocation to the endosymbiont via the conventional secretory pathway. Only later did the signal peptide become a transit peptide for plastid localization, along with an efficient Tic/Toc translocation system on the chloroplast envelope. A recent study on *Paulinella chromatophora* (Nowack and Grossman, 2012) seems to confirm this theory. This amoeba contains two photosynthetic organelles, the chromatophores, of more recent evolutionary origin (~60 million years ago), and some proteins of the photosynthetic apparatus are transported to the chromatophores by a Golgi-mediated traffic. The other theory regards the secretory traffic to chloroplasts as a characteristic of only a few proteins. According to Gagat *et al.* (2013), many of the endosymbiont proteins were initially relocated to the primordial chloroplast via the transit peptide owing to a simple translocon system of cyanobacterial origin, which successively evolved into the multisubunit Tic/Toc complex. It is commonly believed that proteins trafficking through the conventional secretory pathway to reach plastids should be glycosylated to exert their functions. Therefore, the traffic along the ER/Golgi route might be imposed by the proteins' need to acquire proper folding by glycosylation before being localized in the chloroplast. Until now, no case of a conventional secretory route to mitochondria in plant cells has been reported.

Theoretically, based on our definition of UPS pathways, the organellar proteins synthesized in the cytosol and post-translationally translocated into plastids and mitochondria through the Tic/Toc and Tim/Tom complexes also follow a UPS route. In fact, these proteins are transported from the cytoplasm to an 'external space', as well as the leaderless secretory proteins, such as FGF2 or IL-1β, move from the cytoplasm to the extracellular space, bypassing the Golgi (Fig. 1C). However, this statement may be too extreme, so let us assume that all the typical transport routes followed by chloroplast and mitochondrial polypeptides which are synthesized on cytoplasmic 80S ribosomes, and then post-translationally translocated into these organelles, are only 'conceptually' similar to a UPS pathway. Indeed, the work of Paultre *et al.* (2016) seems to support our idea. Using different GFP-tagged chloroplast transit peptides expressed in micrografted Arabidopsis scions, they demonstrated that some plastid-targeted proteins exit from the companion cells and enter into the phloem translocation

stream. Although many of these proteins are considered by the authors as part of a default process, it is interesting to note that, once unloaded laterally from the phloem in the apical root stele, these fusion polypeptides are adequately imported by the Tic/Toc machinery of the stelar cell chloroplasts. This phenomenon is an atypical long-distance transport of plastidial proteins inside the symplast that probably may be considered as a UPS route.

Conclusions

In this review, we update the published data on the UPS pathways followed by plant proteins with ER-targeting signals (Fig. 2). We consider the ER as a sorting point for proteins that start their traffic from this compartment versus different 'external' locations, with the distinction between conventional secretory and UPS routes essentially based on the involvement or non-involvement of the Golgi apparatus in the protein transport. Direct protein deliveries from the ER to the vacuole, plasma membrane, and apoplast have been summarized. Membrane contact sites formed between the ER and other cell compartments or organelles have been studied in many cell types and different organisms for several years. The functions of many of these domains are still unclear but, based on recent investigations, they play critical roles in intracellular signaling and trafficking of metabolites (Prinz, 2014). We believe that in the near future the knowledge of how membrane contact sites perform their cellular functions will help to understand the protein traffic between cell compartments. The concept of 'external space' in this review is extended not only to the extracellular space but also to the lumen of the secretory route compartments and to the volumetric space contained inside plastids and mitochondria. Our assumption comes from a previous idea that the lumen of the secretory route compartments (ER, Golgi apparatus, etc.) can be considered as the cell exterior, because soluble secretory proteins translocated across the ER membrane, or membrane secretory proteins inserted into the ER membrane, leave the cytoplasm to be exposed in an 'external space' (Cooper, 2000). Here, we also include the lumen of the endosymbiotic organelles in the definition of 'external space'. From an evolutionary point of view, it seems advantageous that certain proteins follow an UPS pathway to be transported efficiently and to enable a very fast cell reaction to different biotic and abiotic stresses. However, the real advantage of these secretion routes over the conventional secretory pathway is still to be discovered. The fundamental role of the ER in orchestrating conventional and unconventional trafficking routes is allowed by its dynamic structure made up of several functional microdomains. Powerful tools such as new microscopy or biochemical techniques will help us to characterize these microdomains to elucidate the molecular details of this complex biological hub.

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