

An Overview of Protein Secretion in Plant Cells

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Abstract

The delivery of proteins to the apoplast or protein secretion is an essential process in plant cells. Proteins are secreted to perform various biological functions such as cell wall modification and defense response. Conserved from yeast to mammals, both conventional and unconventional protein secretion pathways have been demonstrated in plants. In the conventional protein secretion pathway, secretory proteins with an N-terminal signal peptide are transported to the extracellular region via the endoplasmic reticulum–Golgi apparatus and the subsequent endomembrane system. By contrast, multiple unconventional protein secretion pathways are proposed to mediate the secretion of the leaderless secretory proteins. In this review, we summarize the recent findings and provide a comprehensive overview of protein secretion pathways in plant cells.

Key words Conventional protein secretion, Unconventional protein secretion, Coat protein complex I, Coat protein complex II, Exocyst-positive organelle

1 Conventional Protein Secretion in Plants

1.1 Endoplasmic Reticulum—The Port of Entry to the Secretory Pathway

In higher eukaryotes, the synthesis of nascent secretory proteins is initiated on cytoplasmic ribosomes before their translocation across the endoplasmic reticulum (ER) membrane through a channel formed primarily by the Sec61 protein. Although Sec61 paralogs are encoded in plant genomes, the function of Sec61 has remained uncharacterized in plants. After translocation into the lumen of the endoplasmic reticulum, secretory and membrane proteins achieve their native conformations through interactions with distinct molecular chaperones, lectins, as well as folding enzymes. Incompletely folded or unassembled proteins are recognized by a constitutively active ER-mediated protein quality control (ERQC) system that recognizes aberrant proteins and targets them for destruction in the cytosol via an evolutionarily conserved degradative process known as ER-associated degradation (ERAD) that involves multiple steps including ubiquitination, retrotranslocation, and the cytosolically located proteasome [1]. Conserved and unique functions (involved in stress pathways and pathogen

defense) of plant ERQC/ERAD mechanisms have been revealed in recent studies combining biochemical approaches and genetic analysis [2, 3].

1.2 ER-to-Golgi Anterograde Transport in Plants—Vesicles Versus Tubules?

If correctly folded, the secretory proteins exit the ER and move to the Golgi apparatus for further modifications. The molecular basis for ER protein export has been built on the isolation and characterization of *sec* mutants in yeast by the Schekman laboratory that accumulate ER membranes at the nonpermissive temperature [4, 5]. Combining this genetic approach with biochemical assays (in vitro reconstitution), the vesicle coat proteins responsible for ER–Golgi transport, collectively termed the coat protein complex II (COPII), were discovered first in yeast and later in animal by the Rothman laboratory [6, 7]. The COPII vesiculating machinery mainly consists of five cytosolic components: Sar1, Sec23, Sec24, Sec13, and Sec31. The small GTPase Sar1 is first activated and recruited onto the ER membrane by the guanosine nucleotide exchange factor (GEF) Sec12 [8–11], which is an ER-localized integral membrane protein [12]. Subsequently, a GTPase activating protein (GAP) Sec23 that stimulates the enzymatic activity of Sar1 [13] and the adaptor protein Sec24 [14], are recruited to ER membrane as a heterodimer by Sar1-GTP to form the prebudding complex [15]. This complex in turn recruits a Sec13/Sec31 heterotetramer, which forms the outer layer of the COPII coat, completing the vesicle formation process. Eventually, secretory proteins recruited by Sec24 or cargo receptors into the nascent COPII vesicles accumulate at ER export sites (ERESs) and will further transport to the Golgi apparatus (Fig. 1).

Despite increasing progress being made in our understanding of COPII function in the early secretory pathway of yeast and mammals, comparable studies on plants are still in their infancy. In higher plants, Sar1 and Sec12 were the first COPII components to be being characterized in ER to Golgi trafficking [16, 17]. Recently, other COPII proteins have also been studied in terms of their functions in the early secretory pathway as well as in plant development and stress responses [18–26]. However, the existence of large numbers of *Arabidopsis* COPII isoforms as opposed to other eukaryotes remains a mystery. In *Arabidopsis*, there are five Sar1, two Sec13, two Sec31, seven Sec23, and three Sec24 isoforms encoded in the genome [27]. However, the significance of this diversification remains poorly understood, but raises the question whether tissue specificity or stress-related functional diversity exists for plant COPII isoforms. Several recent studies have pointed toward the functional diversity for COPII paralogs in ER protein export and stress pathways in *Arabidopsis*. Genetic screening has identified a recessive missense point mutation (R693K) in Sec24A, which induces the formation of ER and Golgi membrane clusters leading to a redistribution of Golgi and secretory proteins into

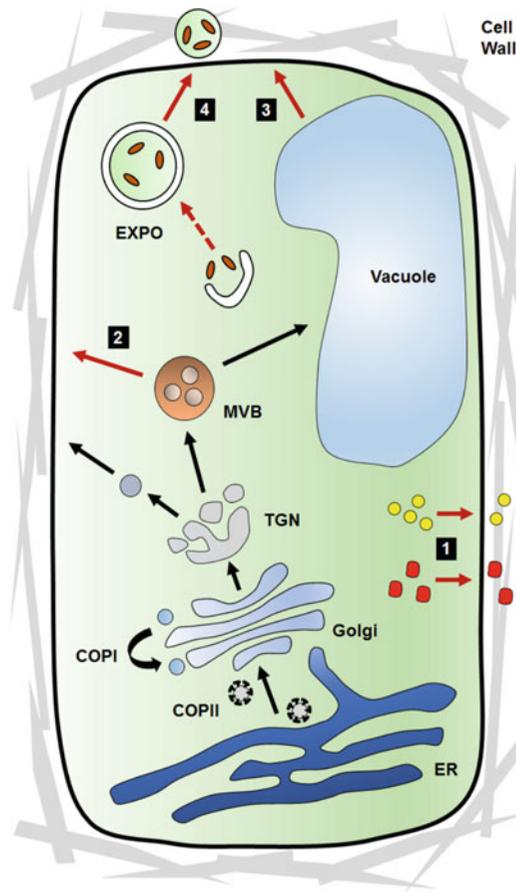


Fig. 1 Overview of protein secretion pathways in plant cells. In the conventional protein secretion pathway (indicated by *black arrows*), proteins with signal peptides are translocated into the ER. Properly folded proteins are exported from the ER and anterogradely transported to the Golgi and subsequently to the TGN. In the TGN, certain proteins are sorted to the plasma membrane, while others are sorted to the MVB which are then targeted to vacuole. In the unconventional protein secretion pathway (indicated by *red arrows*), leaderless secreted proteins are delivered to the extracellular space via different routes: (1) Golgi-bypass pathway; (2) secretion through MVB–plasma membrane fusion; (3) secretion through vacuole–plasma membrane fusion and (4) EXPO-mediated secretion pathway

these clusters [20, 21]. Interestingly, the expression of Sec24B and Sec24C are incapable of complementing the missense mutation phenotype, indicating the existence of functional diversity among the *Arabidopsis* Sec24 paralogs. More strikingly, the *Arabidopsis* Sar1 homolog AtSar1A was reported to exhibit distinct inhibitory effects on ER protein export in both tobacco protoplasts and *Arabidopsis* plants [19, 25]. Cell biology, biochemistry, and structural approaches have revealed the functional heterogeneity of

AtSar1A through an evolutionary amino acid substitution, which is crucial for the recognition of unique Sar1-GAP AtSec23a [25]. Further microarray analyses have demonstrated the specific pairing of AtSar1A and AtSec23A, and their potential role in the plant ER stress pathway [26].

Besides COPII-mediated protein ER export, nascent secretory cargos have also been found to exit the ER in a COPII-independent manner in mammals [28]. In higher plants, direct ER–Golgi tubular connections have been proposed to mediate ER-to-Golgi protein traffic (Fig. 1). Using osmium impregnation, ER–Golgi connections were observed in tobacco leaves [29]. Although the presence of tubules in electron micrographs does not prove they are directly involved in protein transport, recent findings in mammals have indicated that the ER–Golgi contact sites may be involved in cargo protein as well as lipid transport in the early secretory pathway [30].

1.3 Intra-Golgi Transport in Plants

In mammals, newly synthesized secretory proteins are delivered to an ER–Golgi intermediate compartment (ERGIC) in COPII vesicles before arriving at the *cis*-Golgi and progressing through the Golgi complex [31]. However, the presence of ERGIC in higher plants remains doubtful, as Golgi stacks are closely associated with ERES in plant cells. Nevertheless, a recent study suggests that the *cis*-most Golgi cisternae are biosynthetically inactive and may function as a mammalian ERGIC equivalent, which is the site of membrane assembly and cargo sorting [32]. Indeed, Brefeldin A (BFA) treatments in tobacco BY-2 cells show that punctate structures containing some *cis*-Golgi components near the ERES act as scaffolds for Golgi stack regeneration, suggesting their ERGIC-like properties in plants [33, 34]. Thus, in contrast to mammals, the *cis*-Golgi may function as a bona fide ERGIC in plants. Once arriving at the *cis*-Golgi, secretory proteins then undergo carbohydrate modifications and proteolytic processing in a sequential manner as the cargo passes through distinct Golgi compartments. In mammals and yeast, there exists a long-lasting debate about how secretory proteins are transported through the Golgi stack. There are two major models: one is the COPI-dependent vesicular transport (stable compartments) model, and the other is the cisternal maturation model [35–37]. The stable compartment model, which describes the Golgi as consisting of discrete unconnected subcompartments retaining distinct sets of matrix proteins that establish Golgi compartmental identity and maintain Golgi architecture in each stack, was first postulated by Rothman and colleagues. Such a scenario is supported by the observation of COPI transport vesicles at the cisternal rims [38, 39] as well as biochemically through cell-free reconstitution assays [40]. The model was further modified by having COPI vesicles move bidirectionally between intra-Golgi cisternae, with anterograde vesicles carrying secretory proteins

and retrograde vesicles recycling trafficking components [41]. Nevertheless, the basic concept of the cisternal maturation model appears to be widely accepted for the following reasons: (1) Cargoes much larger than conventional COPI vesicles can travel across the Golgi stacks [35]. For instance, procollagen aggregates in mammalian fibroblasts have been shown to progress across the Golgi stack without entering small vesicles [42, 43]. (2) Retrograde COPI-dependent cargo concentration and transport is favored, while the existence of anterograde vesicles remains to be confirmed [44]. In higher plants, the existence of COPI vesicles was first demonstrated using a cell-free reconstitution assay [45]. Later, distinct populations of COPI vesicles, COPIa and COPIb which bud exclusively from *cis*-cisternae and exclusively from *medial*- and *trans*-Golgi cisternae respectively, were observed using a combination of electron tomography and immunolabeling techniques in plants [46]. These studies support the cisternal maturation model in plants. Recently, observations of Golgi regeneration after BFA treatment and removal in plant cells have revealed that the Golgi stacks regenerate in a *cis*-to-*trans* manner, which is consistent with the cisternal maturation model [33]. Therefore, the basic concept of the cisternal maturation model appears to be more applicable in higher plants as in other eukaryotes.

While the secretory proteins passing through distinct Golgi compartments undergo carbohydrate modifications and proteolytic processing, the immature cargos, escaped ER resident proteins, as well as Golgi resident proteins need to be retrieved by COPI vesicles to their proper loci. COPI-interacting signals such as the canonical dilysine motif (KKXX and KXXXX) were first identified on the cytoplasmic C terminus of adenoviral E3 19 kDa (E19) protein in mammals [47, 48]. In plants, dilysine motifs can also be found in many Type I integral membrane proteins, such as Cf-9 in *Lycopersicon esculentum* and the p24 family proteins in *Arabidopsis thaliana* [49–51]. Interestingly, a novel COPI-interacting signal, the KXD/E motif, is responsible for the Golgi retention of polytopic integral membrane proteins in *Arabidopsis thaliana* [52]. Sequence alignment analyses and further studies suggested the conserved nature of the KXD/E motif function in COPI-dependent Golgi retention in all eukaryotes [53, 54]. For tethering of intra-Golgi COPI vesicles to designated membranes, distinct proteins and complex such as coiled-coil tethers p115, golgin-84, CASP, as well as multisubunit complexes like TRAPP II and COG have been characterized in plants. For instance, an *Arabidopsis* p115 homolog, identified as GOLGIN CANDIDATE 6 (GC6) and MAIGO4, was shown to localize to the restricted domain of *cis*-Golgi cisternae as well as COPII vesicles in *Arabidopsis* root and tobacco BY-2 cells by confocal and immunoelectron microscopy [55, 56]. Mutant analysis has shown that the *maigo4* mutant accumulates seed storage proteins in the ER, indicating its essential role in the secretory pathway. Similarly, golgin-84 (identified as GC1

and GC2) and AtCASP have also been shown to localize at the rims of *cis*-Golgi cisternae [55]. However, the physiological roles and tethering functions of p115, golgin-84, and AtCASP remain largely uncharacterized in plants. Meanwhile, the distinct components of the COG tethering complex, namely, COG7, COG3, and COG8, have been shown to be responsible for plant Golgi morphology maintenance, embryo development as well as pollen tube growth [57, 58]. Nevertheless, the TRAPII complex components and their functions in COPI vesicle tethering are still elusive. Further studies are needed to elucidate how the tethering proteins mediate COPI vesicle targeting to the designated membrane and their physiological impacts on the plant development.

1.4 Post-Golgi Trafficking of Secretory Proteins to the Plasma Membrane in Plants

After modification in Golgi apparatus, secretory proteins continue to be processed by resident enzymes and are eventually sorted at the *trans*-Golgi network (TGN), which is defined as a specialized compartment on the *trans*-most cisterna of the Golgi and is composed of tubular-like membrane structures [59]. In plant cells, the ultrastructure of the TGN was observed in the EM as a branched and tubular membrane structure with clathrin-coated buds [60, 61]. Intriguingly, besides acting as a sorting station for post-Golgi pathways, the plant TGN may also function as an early endosome (EE), and is therefore distinct from yeast and mammals [62]. Furthermore, live-cell analysis using spinning disk confocal and superresolution confocal microscopy shows the existence of two types of TGN in plant cells [63]: (1) a Golgi-associated TGN, which is located at the *trans*-side of the Golgi apparatus, (2) a Golgi-released TGN, which is a mobile and independent organelle located away from the Golgi apparatus. The functional significance of these distinct TGN populations in plants is unclear. Furthermore, unlike in mammalian cells, the trafficking of secretory proteins en route to the plasma membrane in plants remains underinvestigated. Using fluorescently tagged SCAMP2, a secretory vesicle cluster (SVC) generated from TGN that moves toward and eventually fuses with plasma membrane has been identified. SVCs have been found in *Arabidopsis thaliana* as well as in rice (*Oryza sativa*) cells and move to the cell plate in dividing tobacco cells, indicating that the SVC is a dynamic mobile structure [64, 65]. In addition, a recent study on the *Arabidopsis* adaptor protein complex 1 (AP1), a conserved protein complex that participates in TGN to PM protein trafficking in mammals, has shown that loss of function of the AP-1 adaptins lead to defects in secretory protein trafficking to the plasma membrane and cell plate formation [66]. These studies point to the conserved nature of the TGN to PM trafficking pathway in higher plants (Fig. 1). However, the vesicles mediating this trafficking process and the underlying mechanism remain elusive. Future study on the TGN to PM trafficking pathway in plant cells will certainly shed light on plant cell polarity and development.

2 Unconventional Protein Secretion in Plants

2.1 *Conventional vs. Unconventional Protein Secretion in Plants*

Being highly conserved in yeast, animals, and plants, conventional protein secretion is a classical and well-defined pathway mediating the extracellular delivery of proteins via endomembrane system. However, analysis of the plant secretome has revealed that more than 50% of the secreted proteins lack a signal peptide [67]. These leaderless secretory proteins (LSP) are not translocated into the ER and do not enter the conventional protein secretion pathway, thus raising the possibilities of an alternative route termed unconventional protein secretion (UPS). In general, LSP bypassing the ER–Golgi protein transport pathway, (1) traffic unaffected by BFA and (2) are without posttranslational modification, probably make use of the UPS pathway for their extracellular delivery [68, 69]. Proteomic studies have revealed that the majority of the LSP are related to stress or pathogen infection [70], implicating the essence of the UPS pathway in dealing with various environmental cues in plants.

2.2 *Direct Translocation of LSP Across the Plasma Membrane via a Golgi-Bypass Pathway*

Different types of UPS pathway have been described in plants over the last decade. For instance, certain LSPs may be secreted directly from cytosol without the involvement of other organelles, while in some cases the secretion of LSPs are mediated by the fusion of vacuoles, multivesicular bodies (MVB) or exocyst-positive organelles (EXPO) with the plasma membrane [71] (Fig. 1). Although the direct translocation of LSPs across the plasma membrane has not yet been proven in plants, previous studies have suggested that the leaderless cytosolic enzyme mannitol dehydrogenase (MTD) is directly secreted into the apoplast in response to salicylic acid, a plant defense hormone, in tobacco [72]. It was shown that MTD secretion is Golgi-independent since BFA treatment did not interfere with its extracellular trafficking [73]. Similarly, another cytoplasmic enzyme hygromycin phosphotransferase (HYG^R), which is commonly used for the selection of hygromycin B resistance, was found to be secreted into the extracellular space in a BFA-insensitive manner in *Arabidopsis thaliana* [74]. In yeast, it has been shown that farnesylated peptides such as α -factor and M-factor are transported via a plasma membrane-localized ABC-transporter based secretion [75]. However, it is not clear whether any protein or channel present on the plasma membrane could assist in the translocation and secretion of the cytoplasmic MTD and HYG^R in plants. Nevertheless, MTD and HYG^R represent LSPs that utilize a Golgi-independent pathway for secretion and therefore belong to the UPS category.

2.3 UPS via Vacuole- and Multivesicular Bodies-Plasma Membrane Fusion

In addition to direct translocation through the plasma membrane, LSPs could be secreted with the help of other organelles as carriers. Upon pathogen infection, fusion of the vacuole with the plasma membrane at the site of attack has been reported in *Arabidopsis thaliana* [76, 77]. UPS mediated by vacuole–plasma membrane fusion enables the release of vacuolar enzymes in response to pathogen attacks, thus representing a strategy for plant survival [78]. During fungal infection, it has also been reported that MVBs accumulate at the site of the invasion papillae [79]. It is plausible that the fusion of MVB with the plasma membrane causes the release of the intraluminal vesicles (known as exosomes) into the fungal haustorium [80]. Indeed, previous studies have implicated the presence of exosome-like structures in the papilla matrix [81–83]. In barley leaves infected by powdery mildew fungus, vesicle-like bodies were frequently observed, and some of them were identified as MVB and paramural bodies (PVB). It was suggested that antimicrobial compounds were contained in the MVB and PVB and their subsequent discharge could be used to prevent fungal penetration [79]. However, how the LSPs that block papilla building block and other antimicrobial compounds get sequestered into the MVB and undergo UPS is still unclear.

2.4 Exocyst-Positive Organelle (EXPO)-Mediated Secretion Pathway

UPS is not only involved in pathogen response but may also be responsible for plant growth and development. S-adenosylmethionine synthetase 2 (SAMS2), an enzyme involved in lignin biosynthesis which contributes to cell wall architecture, has been suggested to be secreted into the extracellular space via EXPO [84, 85]. EXPO is a novel double-membrane compartment that is characterized by the exocyst subunit AtExo70E2 [86]. Although the origin and the mechanism for EXPO formation are still currently unknown, it is proposed that cytosolic LSP cargos are sequestered into the forming EXPO, and the completed EXPO would then eventually fuse with the plasma membrane and release a single-membrane bound vesicle to the extracellular space [69]. Interestingly, EXPO showed no response to BFA, wortmannin (known to affect MVB) nor concanamycin A (known to affect TGN) treatment, suggesting its distinctive nature and is independent of the conventional protein trafficking pathway. Consistently, fluorescence signals of AtExo70E2-GFP did not overlap with other fluorescence-tagged organelle-specific markers including the Golgi, TGN, and MVB. Transmission electron microscopy and immunogold labeling revealed the ultrastructure of EXPO and confirmed its presence in the extracellular space, indicating that EXPO together with its LSP cargos are ultimately secreted [87].

2.5 Techniques and Approaches for Future UPS Studies

Although the mechanisms underlying the UPS pathway in plants remain elusive, enormous efforts have been made toward advancing our knowledge in this field. Amongst the various approaches, proteomic studies on the plant secretome represent a major technique

in studying plant UPS. However, it should be noted that the purity of the fractions obtained for analysis is a matter of the utmost importance. Contamination with cytosolic proteins caused by the breakage of cells during homogenization would cause deviation and discrepancy of the secretome data. Strikingly, a recent study reported a successful purification of the extracellular vesicle (EV) fraction from the leaf apoplast [88]. Proteomic data suggested that proteins involved in biotic and abiotic stress were enriched in the purified EV, while the amount of secreted EV is increased upon pathogen attack and salicylic acid treatments [88]. It is reasonable that plant secretome and the secretion activity are altered in response to different conditions. Thus, by exposing plants toward various environmental cues and comparing the corresponding protein secretome data may aid in the identification of specific LSPs in plants. On the other hand, in-depth studies of the proteins involved in UPS, for instance AtExo70E2, are required to elucidate the molecular mechanisms for EXPO biogenesis. Superresolution in vivo real time imaging can be used to monitor the LSP trafficking and their dynamic behavior with the UPS machinery. For instance, the sequestration of SAMS2 into EXPO may be able to be followed using advanced imaging techniques. In the ultrastructural perspective, cryo-electron microscopy is a promising tool in studying the architecture of EXPO as well as the structure of the exocyst complex, and could provide insight into the formation of EXPO.

In summary, more and more evidence points to the existence and importance of UPS in plants. UPS has not only been reported in model plant *Arabidopsis thaliana* but also in many plant species such as tobacco, barley and sunflower, indicating that it is an efficient protein trafficking pathway which is widely adopted. Due to the complexity and lack of genetic mutant information, UPS studies in plants remain challenging and are underinvestigated. Nevertheless, combining the proteomic data with the latest cell biological techniques would certainly help to elucidate the detailed mechanisms for UPS in plants.

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