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From the Endoplasmic Reticulum to the Golgi Apparatus:

In Vitro and In Vivo Approaches to

Understanding COPII Vesicle Function in Plant Cells

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Zusammenfassung

Der Transport zwischen ER und Golgi wird in Hefe und in Säugetierzellen durch COPII-Vesikel vermittelt. Der COPII-Proteinmantel besteht aus der kleinen GTPase Sar1p und den Heterodimerproteinkomplexen Sec23/24 und Sec13/Sec31. Das COPII vermittelte Sorting kommt zustande, wenn die Proteintransporter das ER verlassen. Obwohl die Vorgänge des ER-GA-Transports in Pflanzen ähnlich zu sein scheinen wie in Hefe- und Säugetiersystemen, ist kein Beweis vorhanden, der diese These unterstützt. Mehr noch, es gibt deutliche Hinweise, die nahe legen, dass Unterschiede im Ablauf dieser Prozesse auftreten, wie zum Beispiel das Fehlen des intermediären Kompartiments in Pflanzen, eine große Zahl von Golgiapparaten bewegen sich entlang des Endoplasmatischen Retikulums sowie die Unterschiede in Aufbau und Organisation des Cytoskelets, das an der Interaktion zwischen ER und Golgi beteiligt ist. In dieser Arbeit werden in vitro und in vivo Ergebnisse zum Verständnis der Funktion von COPII-Vesikeln in Pflanzen herangezogen.

In den in vitro vorgenommenen Studien gelang es uns ein Bindungsassay zu entwickeln mit der wir die in vitro Formation von COPII-Vesikeln aufzeigen können. Hierzu wurden ER-reiche Microsomen, 30% $(NH_4)_2SO_4$ Zytosol, GMP-PNP und ein ATP regenierendes System verwandt. Die Vesikelbildung wurde verstärkt, wenn ER-reiche Mikrosomen eines Sec12 Überproduzenten und zusätzliches Sar1p in der Bindungsmixtur verfügbar sind. Potentielle COPII Vesikel wurden von floatierenden Gradienten in der 41 % Saccharosefraktion isoliert, unter dem Elektronenmikroskop stellen sich diese als 50 nm große Vesikel heraus.

Die Fähigkeit des cytosolischen Teils eines pflanzlichen p24 Proteins COPI und COPII-Untereinheiten aus pflanzlichen und tierischen Quellen zu binden, konnte ebenfalls in vitro nachgewiesen werden. Wir fanden ein dihydrophobes Motiv an der -7,-8 Position (relativ zum zytosolischen Carboxyterminus), der für die Bindung der COPII-Untereinheiten sowohl in Arabidobsis als auch im Zytosol von Ratten Leberzellen verantwortlich zu sein scheint. Wie auch immer, anders als im Rattenleberzytosol haben COPI Vesikel aus pflanzlicher Quelle eine stärkere Affinität zum cytosolischen Teil des p24 Proteins als COPII. Nur bei Fehlen des Dilysine-Motivs in der -3,-4 Position (die sehr stark mit dem dihydrophobischen Motiv in der -7,-8 Position interagiert) oder nach dem Entfernen von COPI konnten wir die Bindung von COPII an den p24-Schwanz mit Pflanzenzytosol nachweisen.

Mit dem Hintergrund ERESs in Tabak BY2-Zellen zu visualisieren haben wir zwei verschiedene Methoden entwickelt: a) die direkte Darstellung von endogenen COPII Proteinen (Sar1, Sec13, Sec23) durch Immunfluoreszenzmikroskopie in stabilen Zellinien, die ER- und Golgi lokalisierte GFP-Markerproteine expremieren, und die Darstellung von membrangebundenem Sec13 durch Expression eines Sec13-GFP-Konstrukts in Zellen, die transient ER- und Golgi-lokalisierte RFP-Marker expremieren. In beiden Fällen begrenzen die ERESs erwartungsgemäß die Golgi-Stapel und einige ERESs sind mit Dictyosomen kolokalisiert. Dual-wavelength live cell imaging von ERESs (Sec13-GFP) und Golgi Stapeln (Man1-RFP) zeigt, dass bei Bewegung die Golgi-Stapel ERESs an ihrer Peripherie sammeln. ERES verschwinden nach BFA-Behandlung nicht, stattdessen kommt es zu deutlichen morphologischen Veränderungen im Golgi-Apparat. Die Verhinderung des ER-Exports durch Expression einer Sar1-Mutante, die im GDP-Status angehalten ist, führt zu einem teilweisen Verlust der sichtbaren ERESs.

Summary

Endoplasmic reticulum (ER) to Golgi transport is mediated by COPII vesicles in yeast and mammalian cells. COPII coats consist of the small GTPase Sar1p and the heterodimeric protein complexes Sec23/24 and Sec13/31. COPII-mediated sorting occurs when protein cargo exits the ER. Although the principles of ER-to-GA transport in plant cells are supposed to be similar to those in yeast and mammalian systems, evidence in support of such an assertion is largely circumstantial. Moreover, there is a substantial body of evidence that emphasizes the differences, such as the apparent absence of the intermediate compartment in plants, large numbers of GA stacks moving along the ER and the differences in the organization of the cytoskeleton involved in interrelationships between the ER and GA. In this study, in vitro and in vivo approaches were employed to understand the function of the COPII vesicles in plant cells.

In vitro, we have set up a budding assay which we could monitor the in vitro formation of COPII vesicle using ER-rich microsomes, 30% $(NH_4)_2SO_4$ cytosol, GMP-PNP and an ATP regenerating system. The vesicle budding was enhanced when ER-rich microsomes from a Sec12 overproducer and extra Sar1p are available in the budding mixture. Putative COPII vesicles were isolated from a flotation gradient at 41% sucrose fraction and observed as 50 nm in diameter vesicle under the electron microscope.

The ability of the cytosolic tail of a plant p24 protein to bind COPI and COPII subunits from plant and animal sources *in vitro* was also examined. We have found that a dihydrophobic motif in the -7,-8 position (relative to the cytosolic carboxy-terminus) is responsible for binding of COPII subunits from both *Arabidopsis* and rat liver cytosol. However, unlike rat liver cytosol, COPI from plant sources has a stronger affinity for p24 cytosolic tails than COPII. Only in the absence of the dilysine motif in the -3,-4 position (which strongly cooperates with the dihydrophobic motif in the -7,-8 position in binding COPI) or after COPI depletion could we observe COPII binding to the p24 tail with plant cytosol.

In order to visualize ERESs in tobacco BY-2 cells we have employed two different approaches: a) direct visualization of endogenous COPII proteins (Sar1, Sec13, Sec23) in cell lines stably expressing ER- and Golgi-localized GFP-markers by immunoflourescence microscopy, and b) visualization of ER-bound Sec13 by expression of a Sec13-GFP construct in cells transiently expressing ER- and Golgi-localized RFP markers. In both cases ERESs considerably outnumber Golgi stacks, and some ERESs colocalize with Golgi stacks. Dual wavelength live cell imaging of ERESs (Sec13-GFP) and Golgi stacks (Man1-RFP) demonstrates that, as they move, Golgi stacks collect ERESs at their periphery. ERESs do not disappear as a result of BFA treatment, despite considerable morphological changes in the Golgi apparatus. Prevention of ER-export through expression of a Sar1 mutant locked in the GDP state leads to the partial loss of visible ERESs.

1. INTRODUCTION

The eukaryotic secretory pathway sorts and delivers a tremendous variety of proteins to their proper intracellular locations. Delivery proceeds through a series of events including directed membrane translocation, membrane budding and membrane fusion that guide secretory proteins to their ultimate destination. Involved in this process are the normal endomembrane organelles of the endoplasmic reticulum (ER), the Golgi stacks, a cell-delimiting plasma membrane (PM), various endosomal compartments, and a vacuole/lysosome equivalent. Over the past few years, a vast amount of research has illuminated the workings of the secretory system of eukaryotic cells. The bulk of this work has been focused on the yeast *Saccharomyces cerevisiae* and on mammalian cells. At a superficial level, plants are typical eukaryotes with respect to the operation of the secretory system. In detail, however, important differences emerge in the function and appearance of these organelles in plants. In plants, the ER is at least partially a storage organelle, whereas the Golgi stacks have devoted a major part of their existence to the creation of cell wall precursors. Most visibly, the vacuole has expanded to occupy the majority of the cell volume in mature cells. In addition, the vacuole also has extended beyond the role of a "garbage dump" accorded to the equivalent organelles in other eukaryotes, and has attained major role in the storage of ions, metabolites, and even proteins.

1.1. An overview of the secretory pathway

The endomembrane system is composed of many organelles, each of which must maintain a unique composition of membrane and cargo proteins. Traffic flows in both directions through the secretory system. Anterograde traffic flows from the ER to the PM or vacuole; while retrograde traffic flows counter to the biosynthetic pathway. The transfer of transmembrane proteins and lumenal macromolecules (proteins, glycoproteins, and polysaccharides) between organelles or compartments of the endomembrane system takes place by vesicle transport. Many of the vesicles involved are formed with a protein covering 'coat' on their cytoplasmic surface, which later has to be removed to allow for vesicle fusion. These vesicles bud from a donor compartment and then travel to a specific destination where they fuse with the target compartment. Clearly, some manner of regulation is required to prevent mistargeting of vesicles to an incorrect compartment.

Intracellular transport between early compartments of the secretory pathway relies on a series of protein-sorting events that are accomplished by coat protein complexes

(COPs). In general, activated small guanine triphosphatases (GTPases) recruit coat proteins to specific membrane export sites, thereby linking COPs to export cargos. As coat proteins polymerize, vesicles are formed and bud-off from membrane-bound organelles. Transport between the endoplasmic reticulum (ER) and Golgi, traffic is bidirectional, ensuring that proteins required to form and fuse vesicles with organelles are recycled as secretory cargo advances. COPII vesicles bud from the ER for anterograde transport, whereas the COPI coat appears to be responsible for retrograde transport of recycled proteins from Golgi and pre-Golgi compartments back to the ER (Figure 1.1).

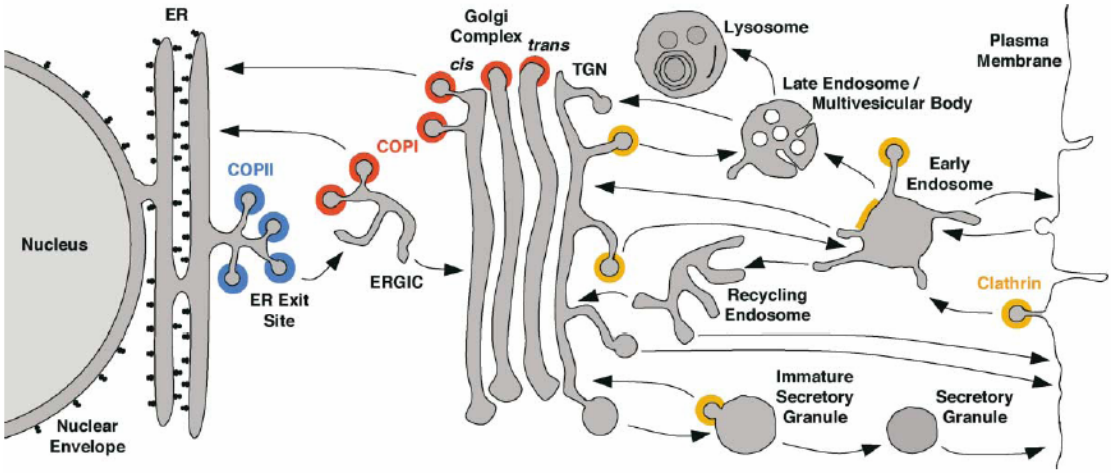

Fig. 1.1 Intracellular Transport Pathways

The scheme depicts the compartments of the secretory, lysosomal/vacuolar, and endocytic pathways. Transport steps are indicated by arrows. Colors indicate the known or presumed locations of COPII (blue), COPI (red), and clathrin (orange). Clathrin coats are heterogeneous and contain different adaptor and accessory proteins at different membranes. Only the function of COPII in ER export and of plasma membraneassociated clathrin in endocytosis are known with certainty. Less well understood are the exact functions of COPI at the ERGIC and Golgi complex and of clathrin at the TGN, early endosomes, and immature secretory granules. The pathway of transport through the Golgi stack is still being investigated but is generally believed to involve a combination of COPI-mediated vesicular transport and cisternal maturation (Pelham and Rothman, 2000). Additional coats or coat-like complexes exist but are not represented in this figure. (From Bonifacino and Glick 2004)

1.2. Secretion-related Organelles and Compartments in Plant Cells

1.2.1. Endoplasmic Reticulum (ER)

Proteins intended for the endomembrane system typically contain N-terminal signal peptides, or have analogous transmembrane domains at other places within the protein that engage the ER-translocation machinery in a similar way (Martoglio and

Dobberstein, 1998). The overall mechanism and the proteinaceous machinery is remarkably conserved across eukaryotes. Most eukaryotes are capable of both co- and post-translational translocation, and often similar proteins may use either method depending on the species in which it is expressed (Kalies and Hartmann, 1998). Regardless, signal peptides can be successfully exchanged between different organisms (Gierasch, 1989), indicating the overall conservation of the process.

Once engaged by the translocation machinery, proteins that lack membrane-spanning domains are released into the lumen of the ER, whereas the membrane proteins are threaded into the membrane (often multiple times) depending upon the folding information within the peptide sequence of the membrane protein. In either case, chaperones and other factors assist in the folding, disulfide bond formation, core-glycosylation, and oligomerization of the proteins. During this process, misfolded proteins are selectively retained by a quality control process which either completes the folding and releases the protein, or marks it for destruction (Vitale and Denecke, 1999).

The ER is comprised of a three-dimensional network of continuous tubules and sheets that underlies the plasma membrane, courses through the cytoplasm, and links up with the nuclear envelope. It is the most versatile and adaptable organelle of eukaryotic cells. Its principal functions include the synthesis, processing and sorting of proteins, glycoproteins and lipids, ER associated protein degradation, as well as the regulation of cytosolic calcium levels. The classical literature distinguishes three ER subcompartments: rough ER, smooth ER, and the nuclear envelope. However, it has been suggested that the ER has many distinct domains based upon metabolic, morphological, and other criteria (Figure 1.2. Staehelin, 1997). The tER is the domain where secretory cargo proteins become concentrated for packaging into COP-II transport vesicles which will carry them to the Golgi stacks. tER has been best studied in mammals and fission yeast, where clear morphological and cytochemical evidence for tER “exit sites” is found (Hammond and Glick, 2000, Rossanese et al., 1999). Similar sites have seldom been reported in plant cells (Staehelin, 1997). The tER-type domain may not be strictly necessary, as baker’s yeast does not appear to produce specific tER sites, and instead, it is believed that cargo may exit from any point in this type of yeast (Rossanese et al., 1999).

Packaging of cargo in the tER still remains somewhat controversial. While some of the proteinaceous components of the vesicle forming machinery have been characterized, it is still unclear whether there is a selective packaging or simple “bulk-flow” of lumenal contents towards the Golgi. Many so called ER-to-Golgi “cargo

receptors" have been identified in yeast and mammals suggesting specific packaging (Lavoie et al., 1999, Denzel et al., 2000, etc.). Whether or not a bulk-flow or specific mechanism exist in plant cells has recently been investigated (Phillipson et al., 2001; Törmäkangas et al., 2001), and further work will be required to implicate one over the other. Despite some level of specificity in packaging, ER-residents are often reported to escape as far as the trans-Golgi network, and an efficient Golgi-to-ER recycling system (KDEL-receptor) has been found for their retrieval (Vitale and Denecke, 1999). Thus, it is likely that some selective process is functioning for particular proteins, whereas some nonselective sampling of the lumenal contents also occurs. Packaging of membrane proteins is likely to be more selective and is probably dependant on peptide signals within the membrane spanning domain.

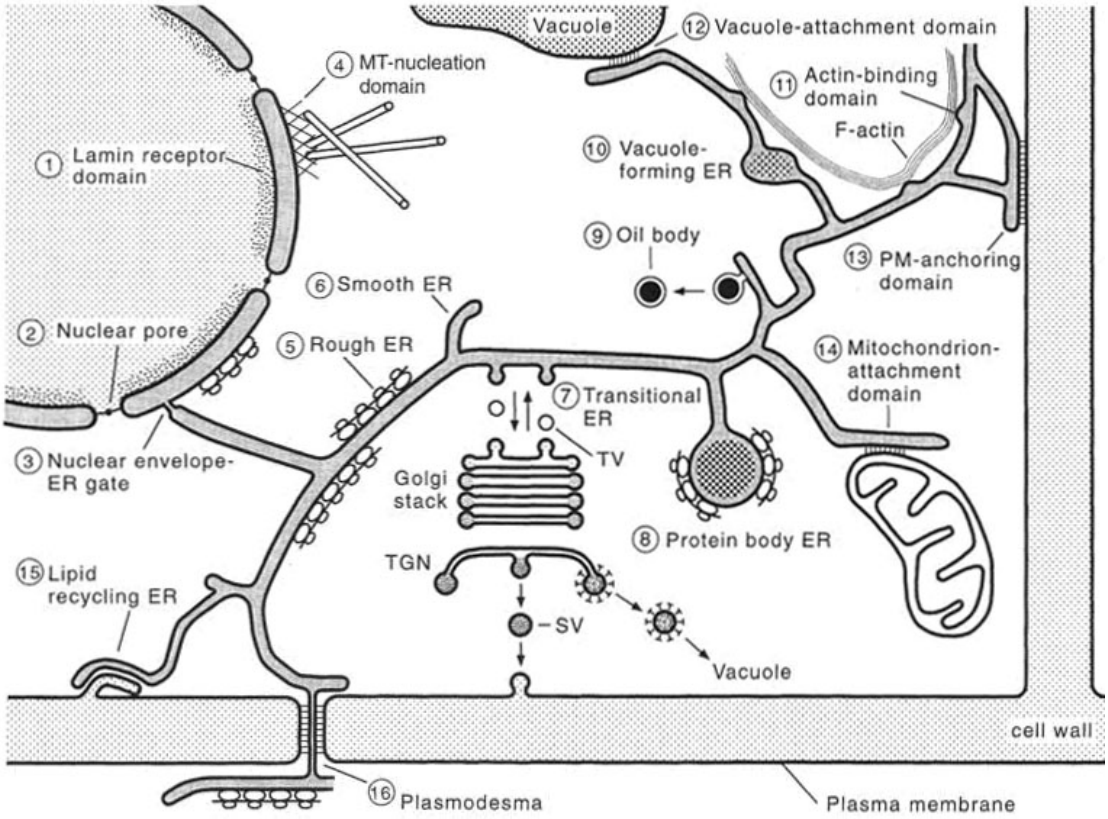

Fig. 1.2. Schematic diagram of a plant cell depicting the sixteen types of ER domains.

PM, plasma membrane; TV, transport vesicle; SV, secretory vesicle; TGN, *trans* Golgi network.(From L.A.Staehelin，1997)

The Golgi is not the only destination for cargo exiting from the ER. Many plant species create unique protein bodies of seed storage proteins by selective distention of an ER subdomain (Herman and Larkins, 1999). Evidence for a direct (non-Protein Body) pathway from the ER to the vacuole has also been reported (Jiang and Rogers, 1998), although mechanistically, little else is known about this type of trafficking. It

has recently become clear that peroxisomes may also be formed (at least partially) by budding from a sub-domain of the ER (Mullen et al., 2001).

1.2.2. Golgi Apparatus (GA)

The plant Golgi apparatus shares many features with its animal counterpart, but also has unique characteristics. The most important difference concerns its structure. Whereas in animal cells the Golgi apparatus occupies a rather stationary perinuclear position, in plant cells the Golgi is divided into individual Golgi stacks, which are generally considered to functionally independent (Staehelin and Moore, 1995). Each stack is typically formed of 5-10 individual cisternae. The number of Golgi stacks per cell and the number cisternae per stack vary with the species and cell type, but also reflect the physiological conditions, the developmental stage and the functional requirements of a cell (Staehelin and Moore, 1995; Andreeva et al., 1998). Despite these variations, each individual Golgi stack can be described as a polarized structure in term of cisternal morphology and with enzymatic activities changing gradually from the ER-adjacent *cis*-face to the trans-face (Fitchette al., 1999). Proteins destined for secretion enter the Golgi at the *cis*-face and subsequently move towards the trans-face where the majority of proteins exit the stack. Although no matrix proteins surrounding the plant Golgi stack have yet been identified, the existence of a matrix has been predicted from the appearance of a clear zone, excluding ribosomes, around each Golgi on micrographs from ultra-rapidly frozen root cells (Staehelin et al., 1990). The Golgi matrix has been suggested to play an important role in the maintenance of stack organization against the shearing forces during cytoplasmic streaming (Staehelin and Moore, 1995)

Confocal microscopy of Golgi-targeted proteins or peptides fused to green fluorescent protein (GFP) has revealed that individual stacks are highly mobile within the plant cell, moving over the ER on an actin-network (Boevink et al., 1998; Nebenführ et al., 1999; Brandizzi et al., 2002). This has resulted in them being christened ‘stacks on tracks’ (Boevink et al., 1998) or ‘mobile factories’ (Nebenführ and Staehelin, 2001). The fact that the plant Golgi apparatus is divided into highly mobile biosynthetic subunits certainly poses major problems when trying to elucidate mechanisms for controlled protein import into and targeted product export out of the stacks.
How the cargo passes through the cisternae is still a matter of some controversy (Pelham and Rothman, 2000). Some evidence suggests that the cisternae are static organelles, and that all traffic between the stacks is vesicle-mediated by COP-I coated vesicles. Other evidence indicates that the stacks may simply be transient structures

that are formed new from ER-derived vesicles at the *cis*-side, then sequentially mature into medial- and trans-cisternae (cisternal maturation model). New *cis*-stacks form behind the maturing cisternae creates an "assembly-line"-like formation of stacks. The *cis*-Golgi is the first cisterna encountered following the ER. Within the cis-cisterna, modifications to the core N-glycosylation of proteins begin. Enzymes such as α-mannosidase remove the terminal mannose residues, creating substrates for other glycosyltransferases which act in the later cisterna to produce the unique glycosylation patterns found on many proteins. This enzyme, α –mannosidase I, has been fused to green fluorescent protein (GFP) by the Stahaelin group, and has been used as an *in vivo* marker for the Golgi stacks, revealing some interesting behavior in dividing cells (Nebenführ et al., 2000).

The cis-cisternae is also the site of recapture of escaped ER residents. The KDEL-receptor is a transmembrane protein whose lumenal domain specifically recognizes a C-terminal K/HDEL motif found at the C-terminus of ER-resident soluble proteins (Pelham, 1996). Upon binding of proteins with these signals, the KDEL-receptor recruits the COP-I machinery and mediates the return of these proteins to the ER. Recent work has indicated that the plant KDEL receptor appears to exceptionally efficient, and rarely allows any ER residents to escape past *cis*-cisterna (Phillipson et al., 2001). In contrast, experiments in mammalian cells have indicated that ER residents may sometimes be allowed to pass as far as the TGN before recapture.

Since the number of cisternae in a Golgi apparatus can vary significantly, definition of a "medial" stack is equally variable. Enzymatically, these stacks can often have activities that differ from that of the earlier and later cisternae. Certainly, the spectrum of glycosyltransferases must be somehow distinct, but how this distinction is setup and maintained remains unclear. Some evidence suggests that synthesis of some cell wall glycans is initiated in the medial stacks, whereas the trans-cisterna is the typical site of xyloglucan assembly (Dupree and Sherrier, 1998). Some of the molecular details of the glycosyltransferasese involved in these processes have begun to be worked out, though much remains to be done (Steinkellner and Strasser 2003).

The ELP/BP-80 class of vacuolar sorting receptors (VSR) are found concentrated in the trans-cisternae and the TGN (Paris et al., 1997; Sanderfoot et al., 1998). These proteins specifically recognize one particular class of vacuolar sorting signal (NTPP or sequence-specific VSS), leading to a concentration of these proteins to specific domains of the TGN (Kirsch et al., 1994 ; Cao et al., 2000 ; Ahmed et al., 2000). The VSR then recruits a clathrin-type coat to the membrane, and directs the packaging of the cargo into CCVs. These CCVs travel onto the prevacuolar compartment. Storage proteins are not recognized by the ELP/BP-80 class. In certain cell types, these types

of proteins are packaged into “Dense Vesicles” (Hara-Nishimura et al., 1998; Hinz et al., 1999; Hillmer et al., 2001). The molecular details of this pathway are not yet clear, but a condensation-sorting mechamism as known for regulated secretory proteins in mammals has been suggested (Robinson and Hinz 1999).
Another aspect of Golgi mediated targeting occurs during cytokinesis. Plants have a unique method of cell division whereby a novel membrane structure (the cell plate) is synthesized by Golgi-derived vesicles at the point of cytokinesis. The cell plate is a site of intense vesicle fusion and formation, and eventually, a new plasma membrane is formed by the fusion of the cell plate with the maternal plasma membranes. Plant cytokinesis is a distinct membrane trafficking process in at least three ways. First, this process is confined to the mitotic phase of the cell cycle whereas other trafficking processes occur during interphase when the cell is growing in size. Second, unlike vesicles destined to fuse with target membranes in the non-dividing cell, cytokinetic vesicles initially fuse with one another to form a new membrane compartment, the cell plate, which becomes the target membrane for fusion of later arriving vesicles. Third, vesicle trafficking during is assisted by a plant-specific cytoskeletal array, the phragmoplast, that forms only in late anaphase and disassembles upon the completion of cytokinesis (Jürgens and Pacher 2003).

1.2.3. Prevacuolar Compartment (PVC)

Prevacuolar compartments (PVCs) are defined as organelles that receive cargo from transport vesicles and subsequently deliver that cargo to the vacuole by fusion with the tonoplast (Bethke and Jones 2000). Based on precedents from mammalian and yeast systems (Lemmon and Traub 2000), they are intermediate organelles on the pathways to vacuoles from both Golgi and from endocytosis of the plasma membrane. In mammalian cells, they have been identified as the prelysosomal compartment (PLC). It is a 0.5-1 μm diameter organelle, which typically has internal 60-80 nm vesicles (Dunn et al., 1986, van Deurs et al., 1993).

There is increasing evidence suggesting that PVCs exist in plant cells and they play a similar role in protein trafficking in the plant secretory pathway (Robinson and Hinz 1999, Bethke and Jones 2000). Identification of these PVCs will enable functional definition of their roles in the complex plant vacuolar system and the vesicular pathways leading to multiple vacuoles (Jiang and Rogers 1999, Vitale and Raikhel 1999, Robinson et al., 2000).

Compared to the yeast and mammalian cell, plant cells contain two functionally distinct vacuoles: the protein storage vacuole (PSV) and lytic vacuole (Hoh et al., 1995, Paris et al., 1996 Vitale and Raikhel 1999). Putative PVCs for the PSVs have been identified and characterized. In developing pea seeds, multivesicular bodies (MVBs) rangeing in size from 0.5 to several μm containing storage proteins have been postulated as PVCs for PSVs (Robinson et al., 1998). Although, there are some evidences support the existence of lytic PVC (Li et al., 2002). Whether or not there are two functional distinct types of PVCs in plant cells is not clear. Several proteins markers like BP-80 (Kirsch et al., 1994), AtPep12 (Conceicao et al., 1997) and AtELP (Sanderfoot et al., 1998) specific for the PVC have been reported. However, due to the complexity of the plant late endosome system, there are multiple pathways using distinct transport vesicles for transporting proteins to the PSV and lytic vacuole in plant cells (Hara-Nishimura et al., 1998, Hinz et al., 1999, Jiang and Rogers 1999, Vitale and Raikhel 1999). The PVC is still not well defined.

Receptor-mediated endocytosis is a well-documented feature of mammalian cells (Trowbridge et al., 1994, Mukherjee et al., 1997), and has also been established for yeast cells (Geli and Riezman, 1998). In both case, the biosynthetic (TGN-PLC/PVC-lysosome/vacuole) and endocytotic pathways to the vacuole converage at the PLC/PVC. However, receptor-ligand complexes which are internalized at the plasma membrane via CCV are not delivered directly to the PLC/PVC since they are on the way to the degradatic compartment. At certain point the receptor must be recycled to the plasma membrane. This occurs at the compartment called early endosome, in contrast to the PLC/PVC, which is termed the late endosome.

Three possibilities exist for the arrival of the contents and the membrane of the PLC/PVC into the lysosome/vacuole: maturation and gradual transformation of the PLC/PVC into lysosome/vacuole (Murphy, 1991); fusion of the PLC/PVC with a pre-existing lysosome/vacuole (Griffiths and Gruenberg, 1991); and by vesicle transport.

1.2.4. Vacuoles

In a plant cell, the most obvious organelle is the large central vacuole (Marty, 1999). Initially thought of as a large empty space, the central vacuole often occupies as much as 90% of the cell volume in a mature cell (Cutler et al., 2000). The vacuole is also the equivalent of the lysosome of mammals, in that it contains the various hydrolyases capable of degrading and recycling proteins, lipids, carbohydrates. In concert with the plasma membrane, the vacuole also has the essential role of maintaining turgor

pressure in the plant cell. This turgor is essential for homeostasis and also for growth and development in plant. Recently, this has been underlined is a paper by Rojo et al. (2001) with the *vacuoleless* mutant of Arabidopsis. Plants where the *Vacuoleless* gene is disrupted by a T-DNA insertion were found to completely lack a central vacuole, and die as embryos.

Recent research has rediscovered an old observation in plant cytology: a single plant cell can have more than a single type of vacuole (Hoh et al., 1995; Paris et al., 1996; Di Sansebastiano et al., 1998; Swanson et al., 1998). It has long been observed that in the storage cells of embryo-derived tissue a new organelle develops that contains storage proteins. This structure was called either a protein body (PB) or a protein storage vacuole (PSV), depending on the species of plant. In garden pea (*Pisum satium*), the storage proteins destined for the PSV begin to condense in the early stacks of the Golgi and form "Dense vesicles" (Hohl et al., 1996; Hinz et al., 1999; Hillmer et al., 2001). These DVs travel from stack-to-stack at the distal ends of the cisterna – or alternately, are carried along the maturing cisterna as it travels forward. Either way, the DV expands as additional cargo is packaged within, until finally budding free from the TGN. The DV is subsequently trafficked to a structure similar to a MVB, before eventually fusing with the PSV (Robinson et al., 2000).

In other species (the cucurbits), storage proteins are instead packaged into a DV-like organelle (called a PAC-precusor accumulating) at the ER (Hara-Nishimura et al., 1998). The ER distends until a PAC buds free into the cytoplasm. A single PAC may fuse with other PACs, receive additional cargo delivered from the Golgi, eventually form an MVB-like organelle, and then fuse with a PSV.

1.3. Vesicles mediating cargo transport in the early secretory pathway

Intracellular protein transport in eukaryotic cells is mediated by small transport vesicles that are defined by their coat proteins: COPII-coated vesicles allow exit from the endoplasmic reticulum (ER), COPI vesicles carry proteins within the early secretory pathway (i.e. the ER and Golgi apparatus) and clathrin-coated vesicles mediate transport from the *trans*-Golgi network (TGN) and endocytic transport from the plasma membrane (Schekman and Orci, 1996; Rothman and Wieland, 1996; Schmid, 1997; Barlowe, 1998). Vesicular transport intermediates not only perform delivery of cargo to various destinations: at the same time they regulate the steady state of the endomembrane system of a eukaryotic cell (Wieland and Harter 1999).

Formation of a transport vesicle is not a spontaneous event. A mechanism for cargo selection and concentration is required to decide what belongs in which vesicle. Secondly, some signal must be sent from the donor membrane into the cytoplasm (where a coat must form) to indicate a site for vesicle formation. Most importantly, some method of membrane deformation and scission from the donor compartment is needed to actually produce the vesicle. This is the job of a large collection of proteins that are referred to as coat proteins. Each type of coat is distinct, though some are related, and a particular coat is responsible for vesicle formation at a particular type of organelle. An overall similarity in the coating mechanism is found, even though the protein content of the coats may vary. The first step requires recruitment of the cargo to a site on the donor membrane, the details of such a step is still a matter of debate. However, this step most likely involves the cytoplasmic tails of integral membrane proteins. These proteins may themselves be cargo, or may serve as cargo receptors. Coincident with this step is the GTP-cycle of a coat-GTPase which helps to coordinate the coating process. The coat-GTPases typically exist as a soluble GDP-bound state while inactive. A GTP-exchange factor (GEF) localized to the coating site serves to recruit the coat-GTPase to the donor membrane and then triggers a nucleotide exchange, such that the coat-GTPase assumes its active GTP-bound state. The GEF for the Sar1p-type G-protein is a member of the Sec12-family, a group of ER-integral membrane proteins found in all eukaryotes. The GEF for the ARF-type G-protein vary somewhat, since the ARF-type coat-GTPase is involved in many different coating steps (Kirchhausen, 2000).

Subsequently, a GTPase Activating Protein (GAP) triggers the intrinsic GTPase activity of the coat-GTPase. This step occurs either directly after vesicle formation (COP-II) (Antonny et al., 2001), or can be triggered by some other event (COP-I or clathrin) (Tanigawa et al 1993; Cukierman et al., 1995). The GDP-bound G-protein now triggers uncoating of the vesicle, releasing the coatomers back to the cytoplasm for future coating steps. The uncoated vesicle is now free to travel to its target membrane where the SNARE-mediated fusion process occurs.

1.3.1. COPI Vesicles

COP I-coated vesicles were first identified from an intra-Golgi transport assay (Balch et. al., 1984). In this assay their formation was observed when Golgi membranes were incubated in the presence of cytosol, fatty acyl-coenzyme A, and nucleotides (Balch et. al., 1984; Pfanner et al., 1989). They accumulated under conditions known to block protein transport such as the addition of non-hydrolysable GTP analogues (Melancon et al., 1987). This led to the hypothesis that they were the vesicles mediating forward

transport through the Golgi complex. Subsequently these vesicles, now known as COP I vesicles, were purified to homogeneity (Malhotra et al., 1989) and the cytosolic components forming their coat characterized (Serafini et al., 1991; Water et al., 1991). The complex these components form in the cytosol was named the 'coatomer' and consisted of 7 polypeptides plus the small GTP binding protein ARF1 that is not part of the coatomer itself. There are two conformations of coatomer: that of the soluble coatomer; and that of coatomer in COPI-coated vesicles or aggregates. Coatomer aggregation in the presence of tetrameric p23-CT thus appears to be related to COPI coat polymerization on native membranes, which suggests that the interaction of p24 proteins with coatomer is a critical trigger of COPI coat assembly (Harter and Wieland, 1998; Reinhard et al., 1999).

COPI vesicles instead appear to be involved in both biosynthetic (anterograde) and retrograde transport within the Golgi complex (Orci et al., 1997), as well as mediating the recycling of proteins from the Golgi to the ER (Cosson and Letourneur, 1994; Letourneur et al., 1994; Sönnichsen et al., 1996).COP-I coats are typically associated with Golgi trafficking, although they may also be involved in forming coated vesicles at the ER and endosomal compartments (Kirchausen, 2000). The COP-I coatomer is made up of seven components (α /Rer1p, β/Sec26p, β'/Sec27p, γ/Sec21p, δ/Ret2p, ε/Sec28p, and ζ/Ret3p; listed in the mammalian/yeast nomenclature) that are conserved across eukaryotes (Table 1.1). As with COP-II coatomers, the COP-I-coatomers are encoded by multiple genes (except for γ and δ) in Arabidopsis. The GTPase that drives COP-I coat formation is from the ARF-family, a large family of small GTPases (at least 18 in Arabidopsis) that also function in other coating processes. The COP-I coatomer also plays a role in cargo selection, with various members of the complex interacting specifically with motifs present in cytoplasmic domains of cargo proteins. COP-I coated vesicles seem to be involved in both anterograde and retrograde trafficking between the Golgi stacks – though a role in anterograde trafficking would be unnecessary in a cisternal maturation mode of Golgi functioning.

Table 1.1. Coat proteins of COPI vesicles (Wieland and Harter1999)

COPI					
Coatomer	α-COP	Ret1p	~140 kDa	WD-40 repeats	β′-COP, ε-COP
	β-COP	Sec26p	~107 kDa		δ-COP, ARF1
	β′-COP	Sec27p	~102 kDa	WD-40 repeats	α-COP
	γ-COP	Sec21p	~97 kDa		ζ-COP, ARF1, KKXX motifs, p24 family
	δ-COP	Ret2p	~57 kDa		β-COP
	ε-COP	Sec28p	~35 kDa		α-COP
	ζ-COP	Ret3p	~20 kDa		γ-COP
ARF 1	ARF 1	yARF 1/2/3	~20 kDa	Ras family of GTPases	β-COP, γ-COP

The initial event in the COPI pathway that leads to recruitment of the coat requires the association of the GTPase ARF1 (ADP-ribosylation factor 1) in its active form to the membrane.The ARF protein family has many members and targeting of ARF1 to the correct membrane involves specific association with its appropriate GEF. Several GEFs have been identified that stimulate the guanine nucleotide exchange rate of specific ARFs, and each possesses a 200-amino acid segment referred to as the 'Sec7 domain' (Chardin et al., 1996; Chardin et al., 1999). Interestingly, the ARF1–Sec7 domain complex resembles more closely the GTP and not GDP conformation, suggesting that some form of ARF1 activation precedes interaction with the Sec7 domain.

Recent progress has also been made in understanding the molecular detail of brefeldin A (BFA) effects on COPI coat assembly. It had been shown that BFA addition to certain cell types leads to a disassembly of the Golgi complex and redistribution into the ER (Lippincott-Schwartz et al., 1989; Klausner et al., 1992). Specifically, BFA inhibits the rate of guanine nucleotide exchange exhibited by ARF1, and interferes with ARF1 function in recruiting the COPI coat (Donaldson et al., 1992; Helms and Rothman 1992). In essence, BFA acts by sequestering specific Sec7 domain-containing proteins, which in turn prevent the activation of ARF1 and ultimately blocks COPI coat formation.

ARF-specific GAPs have been identified that accelerate the rate of GTP hydrolysis. A founding member of this class of proteins contains a zinc finger motif that is essential for ARF GAP activity and stimulates the rate of GTP hydrolysis (Cukierman et al., 1995).

COPI-coated vesicles efficiently capture proteins carrying in their cytoplasmic carboxyterminal domain sorting signals of the form KKXX (the dilysine motif) or KXKXX (X is any amino acid). The KDEL receptor, a multiple-spanning membrane protein that binds and retrieves lumenal proteins containing the KDEL carboxy-terminal sequence, is also transported along this pathway. The γ subunit seems to be the component responsible for cargo recognition because it recognizes the KKXX and KXKXX motifs, but it is not known whether it also recognizes the KDEL receptor.Members of the p24 protein family also interact with COPI coatomers in addition to COPII and might facilitate the recruitment of COPI coatomers to Golgi membranes (Dominguez et al., 1998). p24 proteins not only bind to coatomer and alter its conformation but are also abundant residents of the intermediate compartment (IC) and the Golgi (Sohn et al., 1996; Rojo et al., 1997; Dominguez et al., 1998),

which are the intracellular sites of COPI vesicle biogenesis (Griffiths et al., 1995; Orci et al., 1997).

Therefore, it has been proposed that the minimal machinery for COPI vesicle formation consists of p23/p24, ARF-GTP and coatomer (Figure 1.3; Nickel et al., 2002; Aniento et al., 2003).

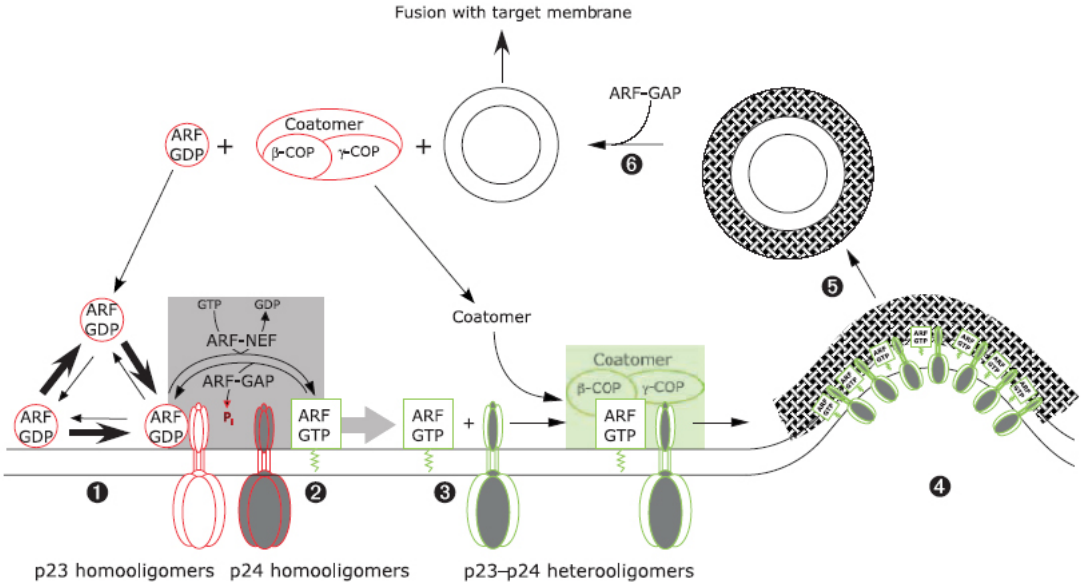

Fig. 1.3 The core machinery of COPI recruitment to membranes, coat polymerization, vesicular budding and uncoating.

Recruitment of coat proteins is initiated by ARF-GDP binding to p23 (1). Upon nucleotide exchange, ARF-GTP dissociates from p23 resulting in its stable association with the membrane (2). Multiple cycles of GTP hydrolysis and GDP to GTP exchange are likely to occur, possibly causing rearrangements of p23/p24 oligomers (3). The products of these processes are ARF-GTP and presumably a p23/p24 heterooligomer, which triggers coatomer binding and coat polymerization (4). Following budding (5), the catalytic domain of ARF-GAP is sufficient to trigger uncoating (6). Active components are shown in green; inactive components are shown in red. (From Nickel et al., 2002)

1.3.2. COPII Vesicles

The second class of vesicle is the COP II vesicle identified throught a combination of genetical and biochemical approaches in the yeast *Saccharomyces cerevisiae* (Antonny and Schekman, 2001). These vesicles mediate the transport of a subset of secretory proteins from the ER to the Golgi complex.

The COP-II coat is conserved in all eukaryotes and is involved in transport from the ER. In most organisms, ER-exit sites are discrete points along the ER membrane (Bannykh and Balch, 1997; Rossanese et al., 1999), although in some organisms (like *S. cerevisiae*) the entire ER membrane seems capable of acting as ER-exit sites (Glick,

2001). The exit sites are points at which the COP-II machinery is concentrated (Hammond and Glick 2000), and also where anterograde cargo is collected. How cargo becomes concentrated remains unclear, with some arguing for a "bulk-flow" model, whereas other invoke specific cargo receptors. In a bulk-flow model, all cargo not specifically retained in the ER is subject to COP-II-directed (Herrmann et al., 1999) transport to the Golgi. On the other hand, some propose that a large class of conserved proteins are involved in clustering cargo for packaging at exit sites. Evidence for both models has accumulated, and it is possible that both occur depending on the cell-type or developmental state or particular cargo molecule.

1.3.2.1. COPII coat components

The COP-II coat is made of 5 proteins (Antonny and Schekman, 2001). Having been first described in yeast, the components are typically referred to according to the yeast nomenclature. Once activated by the Sec12-GEF, Sar1p-GTP recruits two coatomer protein complexes from the cytosol: the Sec13/31p and the Sec23/24p complexes (Table 1.2). The Arabidopsis coatomer subunits are each encoded by multiple genes, though whether this indicates redundancy has yet to be investigated. The coatomer complexes then assemble into higher order structures which drive membrane deformation and eventual membrane budding. The COP-II coated vesicles are quite unstable as the GAP for Sar1p turns out to be a component of the coat itself (Sec23p), thus the vesicles are uncoated soon after budding. These vesicle quickly fuse with each other into larger vesicles which either fuse with the cis-Golgi (or form a de novo cis-Golgi stack in cisternal maturation models), or with a compartment called the ER-Golgi Intermediate complex (ERGIC) found in most mammals. Upon uncoating, Sar1p and the coatomers are released into the cytoplasm for further rounds of vesicle budding.

Table 1.2. Coat proteins of COPII vesicles (*Arabidopsis isoforms)

(Adapted from Wieland and Harter1999 with modification)

Coat proteins of COPII							
	Subunits						
Proteins Complex	Mammals	Yeast	Arabidopsis	*	Size	Feature	Interactions
Sec13 complex	hSec13p	Sec13p	AtSec13p*	2	33 kDa	WD-40 repeats	
	hSec31p	Sec31p	AtSec31p*	2	150 kDa	WD-40 repeats	
Sec23 complex	hSec23p	Sec23p	AtSec23p	5	85 kDa	GAP for Sar1p	Bos1p,Bet1p
	hSec24p	Sec24p	AtSec24p*	5	105 kDa		
Sar1	hSar1p	Sar1p	AtSar1p	4	21 kDa	GTPase	Bos1p

Sar1p

The Sar1 GTPase is an essential component of COPII vesicle coats involved in export of cargo from the ER. The GTPase activity of Sar1 function as a molecular switch to control protein-protein and protein-lipid interactions that direct vesicle budding from the ER (Springer et al., 1999). Activation from the guanosine 5′-diphosphate (GDP) to the GTP-bound form of Sar1 involves the membrane-associated guanine nucleotide exchange factor (GEF) Sec12, first identified in yeast (Nakano et al., 1988; Barlowe et al., 1993). A homologue of yeast Sec12 has also identified in plants (Bar-Peled and Raikhel, 1997) and in mammalian cells (Weissman et al., 2001). During or after fission of vesicle from ER, GTP hydrolysis is stimulated by the recruited Sec23/24 GTPase-activating protein (GAP) complex to promote coat disassembly and subsequent delivery of cargo to the Golgi complex (Barlowe et al., 1994).

The GDP-binding site includes residues found in all of the highly conserved binding motifs diagnostic of GTPases.Thr39 is found in the conserved $GxxxxGKT_{39}$ motif. It is involved in the co-ordination of a Mg2+ ion, which is also co-ordinated to oxygen atoms of the *β*- and *γ*-phosphates of GTP (Pai et al., 1990) and is essential for Sar1 function. In particular, the Sar1 dominant negative mutant Sar1 [T39N] is a potent inhibitor of COPII vesicle formation (Kuge et al., 1994; Aridor and Balch, 1996; Rowe et al., 1996). Sar1 [T39N] inhibits wild-type Sar1 function in vivo and in vitro by interfering with its interaction with mammalian Sec12 (mSec12), the ER-associated GEF that is required for Sar1 activation and COPII vesicle formation (Weissman et al., 2001).

A second mutation, Sar1 [H77L], abolishes the His residue that presumably coordinates the water molecule participating in GTP hydrolysis, and blocks the GTPase activity of Sar1 leading to its stabilization in the GTP-bound activated form. The Sar1-[H77L] mutant supports vesicle budding but displays no overall ER-to-Golgi transport in cell –free assays. It is insensitive to the GAP function of the sec23/24 complex (Saito et al., 1998).

Sec23/24 complex

The Sec23p/24p complex is a heterodimer**.** Sec23p/24p elutes on gel filtration chromatography as a single species corresponding to a globular protein of about 200 kDa. The masses of Sec23p and Sec24p deduced from their cDNAs are 85 and 104 kDa, and the molar ratio of Sec23p to Sec24p in the complex is 1:1, as assessed by a

combination of SDS/PAGE and quantitative amino acid analysis. The Sec23/24 complex has a bone-like outline, the outside dimensions of which are about 11x17 nm (Lederkremer et al., 2001).

The Sec23p–Sec24p complex is probably the component responsible for cargo recognition (Springer and Schekman 1998; Peng et al., 2000) but the sorting signals recognized by the complex remain to be identified. Members of the p24 family of transmembrane proteins bind to Sec23p through a cytosolic diphenylalanine motif. As these proteins are required for efficient ER-to-Golgi traffic of some cargo proteins (Schimmoller et al., 1995), it is thought that they might serve as cargo adaptors (Kaiser 2000). In addition to recruiting the Sec23p–Sec24p complex, the GTP-bound form of Sar1p activates Sec23p to bind SNARE proteins involved in the specificity of targeting and in the fusion reaction of vesicles with acceptor membranes (Springer and Schekman 1998). ER membranes with Sec23p–Sec24p and Sar1p can then recruit Sec13p–Sec31p. The complex is likely to act as a scaffold to drive membrane deformation and to complete vesicle budding. Sec23p also acts as a GTPase-activating protein (GAP) for Sar1p. It is thought that, after GTP hydrolysis, Sar1p–GDP is released, leading to uncoating before fusion of the vesicle to the target membrane and formation of a new coated vesicle.

Sec13/31 complex

The Sec13p/31p complex is a heterotetramer**.** During gel filtration chromatography, Sec13p/31p eluted as a single monodisperse species corresponding to a globular protein of about 700 kDa in mass. Because of the marked disparity with the added molecular masses of its Sec13p and Sec31p subunits (33 and 140 kDa, respectively), it has been proposed that the Sec13p/31p complex might be a heterodimer of elongated shape (Salama et al., 1997), although the possibility that the complex could contain more subunits was not explored. Sec13p/31p is a relatively asymmetric heterotetramer. The simplest organization that is consistent with the elongated shape of Sec13p/31p is a side-by-side arrangement of its subunits, such that the globular domain at one end of the complex corresponds to two Sec13p subunits, and the globular domain at the opposite end contains the carboxyl terminal part of Sec31p (Lederkremer et al., 2001).

1.3.2.2. COPII formation and disassembly

Vesicular transport can be reconstituted by using three cytosolic components containing five proteins: the small GTPase Sar1p, the Sec23p/24p complex, and the

Sec13p/Sec31p complex (Salama et al., 1993). These proteins will support a cargo-carrying budding reaction from isolated ER membranes. Sar1p, a GTP-binding protein, initiates coat formation (Matsuoka et al., 1998). The GDP-bound form of Sar1p is normally cytosolic. It is recruited to the ER membrane by interaction with Sec12p, an ER-bound membrane protein that serves as its guanine exchange factor (Barlowe et al., 1993). Sar1p-GTP then recruits cytosolic Sec23p/24p complex, most likely through its interaction with Sec23p (Yoshihisa et al., 1993).

In addition to recruiting Sec23p/24p, the GTP-bound form of Sar1p stabilizes Sec23p and binds to certain ER and Golgi SNARE proteins involved in the specificity of targeting and in the fusion reaction of vesicles with acceptor membranes (Springer and Schekman 1998). The interaction of Sar1p-GTP with Sec23p also facilitates the association of the Sec23p/24p complex with cargo proteins (Kuehn et al., 1998); Sec24p is probably the component responsible for cargo recognition (Shimoni et al., 2000). ER membranes with Sec23p/24p and Sar1p can then recruit Sec13p/31p, a complex that is likely to act as a scaffold, like clathrin, to effect membrane deformation and vesicle budding. Completing the cycle, Sec23p acts as a GTPase activating protein for Sar1p (Yoshihisa et al., 1993). It is thought that on GTP hydrolysis; Sar1p-GDP is released, leading to uncoating before fusion of the vesicle to the target membrane and recycling of COPII components.

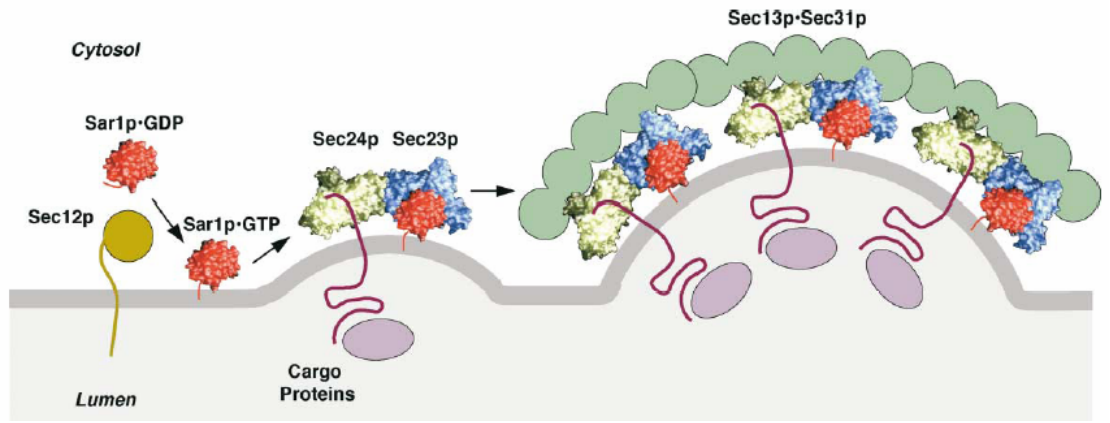

Fig. 1.4. Assembly of COPII

Cytosolic Sar1p-GDP is converted to membrane bound Sar1p-GTP by the transmembrane protein Sec12p. Sar1p-GTP recruits the Sec23p/Sec24p subcomplex by binding to Sec23p, forming the "pre-budding complex". Transmembrane cargo proteins gather at the assembling coat by binding to Sec24p. The Sec13p/Sec31p subcomplex polymerizes onto Sec23p/Sec24p and crosslinks the pre-budding complexes. Cargo proteins are further concentrated. The depictions of Sar1p, Sec23p, and Sec24p are surface representations from the crystal structures of these proteins (Bi et al., 2002). The Sec13p/Sec31p complex is represented as an elongated, five-globular domain structure based on electron microscopy (Lederkremer et al., 2001). Sec16p and Sed4p also participate in the assembly of COPII, but are not represented here because their roles are less well understood. See text for additional details. (From Bonifacino and Glick 2004)

At present, it is unclear what marks ER exit sites for COPII recruitment. A candidate for this role is Sec16p, a large peripheral ER membrane protein (Espenshade et al., 1995). Sec16p interacts with Sec23p, Sec24p, and Sec31p via different domains (Espenshade et al., 1995; Shaywitz et al., 1997) and may serve as scaffold for the nucleation or stabilization of the assembling coat (Supek et al., 2002). It is likely that Sec16p acts in conjunction with the transmembrane protein Sec12p to recruit GTP bound Sar1p to the ER membrane. Sar1p-GTP associates with the lipid bilayer through a hydrophobic amino-terminal extension and recruits its effector, the Sec23p/Sec24p subcomplex, through interactions with two "switch" regions characteristic of Ras superfamily proteins (Huang et al., 2001; Bi et al., 2002). The initiation of COPII assembly thus involves both GTP-independent and GTP-dependent reactions that cooperate to deposit the coat at ER exit sites.

Sar1p-GTP together with Sec23p/Sec24p constitutes the so-called "pre-budding complex," which has recently been analyzed by electron microscopy (Lederkremer et al., 2001; Matsuoka et al., 2001) and X-ray crystallography (Bi et al., 2002). This complex has the appearance of a bow tie with one side corresponding to Sec23p and the other to Sec24p (Bi et al., 2002). Sec23p makes direct contact with Sar1p-GTP (Bi et al., 2002), while Sec24p participates in cargo recognition. Once assembled onto membranes, the pre-budding complex recruits the Sec13p/Sec31p subcomplex, which consists of two Sec13p and two Sec31p subunits (Lederkremer et al., 2001). Sec13p/Sec31p appears by electron microscopy as a flexible, elongated structure that polymerizes to form a meshlike scaffold (Lederkremer et al., 2001; Matsuoka et al., 2001).

Sec23p stimulates the GTP hydrolysis activity of Sar1p (Yoshihisa et al., 1993) by contributing an "arginine finger" that pokes into the GTP binding site and aids catalysis (Bi et al., 2002). This activity of Sec23p as a GTPase-activating protein (GAP) is augmented approximately ten-fold by addition of Sec13p/Sec31p (Antonny et al., 2001). A paradoxical implication of this mechanism is that COPII coat assembly should trigger disassembly by promoting GTP hydrolysis. How can the COPII coat polymerize to cover a forming vesicle if the basic unit of the polymer is unstable? A possible explanation is that the kinetics of GTP hydrolysis might be slower than the kinetics of vesicle budding, in which case there would be time for a vesicle to form before the coat fell apart. Alternatively, GTP hydrolysis might cause Sar1p to be released from the coat while the other subunits remained assembled on the membrane. The polymeric nature of the coat could provide kinetic stability in the absence of Sar1p-GTP. In addition, the cytosolic domains of transmembrane cargo

proteins could act as secondary membrane tethers or could modulate the GAP activity of Sec23p. Any of these alternative explanations would imply that Sar1p-GTP is dispensable for the integrity of the central area of the coat and is required only to stabilize the coat edges (Antonny and Schekman, 2001).

1.3.2.3. Protein Sorting into COPII vesicles

Export of many secretory proteins from the endoplasmic reticulum (ER) relies on signal-mediated sorting into ER-derived transport vesicles. Experimental evidence demonstrates that certain cargo, in order to be included into COPII vesicles, possess a binding affinity for subunits of the coat. More specifically, pre-budding complexes consisting of Sar1–GTP and Sec23–Sec24 bound to cargo can be isolated under conditions that preserve the Sar–GTP-bound configuration (Kuehn et al. 1998; Aridor et al. 1998). Pre-budding complexes of Sar–Sec23–Sec24–cargo form on the surface of ER membranes. These pre-budding cargo complexes are then gathered by the Sec13–Sec31 complex into nascent vesicles to extract specific cargo from the ER. Presumably, the Sar1 GTPase is regulated in a manner to allow for productive incorporation of pre-budding cargo complexes into the polymerized coat before hydrolysis of bound GTP.

The structure of the pre-budding Sar1–Sec23–Sec24 complex is ‘bowtie-shaped’ and forms a concave surface that apparently faces the ER membrane. Therefore, domains of Sar1 and Sec23–Sec24 could well be available for binding to integral membrane cargo proteins (Bi et al., 2002)

The so called di-acidic sequence (DXE) motif contained within the VSV-G tail sequence is found in many other secretory proteins 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 (Malkus et al., 2002; Kappeler et al., 1997). Moreover, Sys1p depends on its di-acidic residues for direct binding to Sec23–Sec24 (Votsmeier and Gallwitz 2001) and Gap1p requires its di-acidic motif to form pre-budding complexes with Sar1 and Sec23–Sec24 (Malkus et al., 2002) However, there are many other membrane proteins that are efficiently exported from the ER but do not contain apparent di-acidic motifs. For example, the membrane protein ERGIC53, which cycles between the ER and Golgi compartments, has been well studied. This type I transmembrane protein possesses a cytoplasmic tail sequence of 16 residues that is required for properlocalization. More specifically, a conserved pair of aromatic residues at the extreme C-terminus of ERGIC53 is necessary for transport from the

ER (Kappeler et al., 1997). 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 the COPII subunits (Kappeler et al., 1997; Nufer et al., 2002). ERGIC53 homologs in yeast also possess bulky hydrophobic residues at their C-termini (LL) that are required for export from 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 (Nufer et al., 2002; Nakamura et al. 1998) although not to rates observed for endogenous ERGIC53.

An additional conserved ER export signal has been identified more recently in the tail sequence of the ERGIC53 family of proteins (Sato and Nakano 2002). This tyrosine-containing motif is ~12 amino acids from the C-terminal signal. Both motifs are required for assembly into COPII pre-budding 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) and the Erv41–Erv46 complex (Otte and Barlowe 2002). Interestingly, many of these proteins, including ERGIC53 and VSV-G, 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 for multiple signals in secretory proteins might be an important element in ER quality control. Recent study has shown that the oligomerization of Emp47p at ER is essential for Emp47p and Emp46p exit from ER (Sato and Nakano 2004).

Several lines of evidence indicate that a family of Sec24 proteins functions in cargo recognition. Furthermore, the presence of multiple Sec24 homologs appears to expand the variety of cargo that must be efficiently exported from the ER. Yeast cells express two additional Sec24-like proteins, termed Lst1 and Iss1. Higher eukaryotes are endowed with at least four Sec24 isoforms (Pagano et al., 1999). In yeast, the Lst1 subunit is not essential for COPII-dependent export but is required for efficient export of specific transmembrane cargoes 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). More recently, a functional sorting assay demonstrated 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 (Votsmeier 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.

Soluble secretory proteins are efficiently exported from the ER and cannot directly contact the COPII coat. Two non-exclusive models, known as the 'bulk flow' and 'receptor-mediated' export models, have been described in studies addressing export of soluble cargo from the ER. ERGIC53, p24 proteins and Erv29p are proposed to recognize and bind to specific export signals contained within distinct soluble cargo molecules. Presumably, binding is regulated such that fully folded secretory proteins are bound by the receptor in the ER and then released in post-ER compartments. Possible changes in lumenal pH and/or $Ca2^{+}$ concentration within distinct membrane compartments could regulate receptor–cargo interactions. Alternatively, COPII-dependent oligomerization of membrane receptors could influence receptor–cargo interactions.

1.3.2.4. COPII vesicles in plants

Although the principles of ER-to-GA transport organization in plant cells are supposed to be similar to those in yeast and mammalian systems, evidence in support of such an assertion is largely circumstantial. Moreover, there is a substantial body of evidence that emphasizes the differences, such as the apparent absence of the intermediate compartment in plants, large numbers of GA stacks moving along the ER and the differences in the organization of the cytoskeleton involved in interrelationships between the ER and GA. Although the existence of both anterograde and retrograde ER-to-GA transport in plant cells is generally accepted, no direct evidence about the carriers involved is available. As to COPII vesicles mediating anterograde ER-to-GA transport in yeasts and mammals, they have not been unambiguously identified in plants (Movafeghi et al., 1999; Robinson et al., 1998). However, the existence of Sar1 and other plant homologues of the proteins necessary for their function (d'Enfert *et al*., 1992; Bar-Peled *et al*., 1995; Bar-Peled and Raikhel, 1997), as well as microscopical and recent biochemical (Movafeghi et al., 1999) evidence do suggest their existence and a similar role in plants. In this respect, the ability of Sar1 mutants to block ER-to-GA transport *in vivo* in plant cells

described above is good evidence in support of COPII function in this pathway (Takeuchi et al., 2000).

1.4. SNAREs and Membrane Fusion

Intracellular membrane trafficking in eukaryotic cells utilizes transport vesicles and tubulovesicular structures to deliver cargo proteins and lipids from one internal compartment to the next (Rothman 1994, Salama and Schekman 1995). Membrane fusion is a fundamental biochemical reaction and the final step in all vesicular trafficking events. An ever-widening variety and number of factors are required to mediate the controlled transport of cargo along the secretory pathway. Factors necessary to confer fusion between donor and acceptor compartments (or organelles) include the Rab GTPases, tethering complexes, AAA-type ATPases, and SNAREs (Pfeffer 1999; 2001; Vale 2000). These evolutionarily conserved factors populate the sites of membrane fusion and confer an ordered chain of events that ultimately leads to bilayer fusion. Multiple homologs of these factors are found in eukaryotic cell.

1.4.1. SNAREs

SNAREs were identified after more than a decade of intensive biochemical effort. Using an in vitro trafficking assay developed during the early 1980s, Rothman and colleagues (Balch et al. 1984) were able to purify two soluble proteins required to reconstitute efficient transport. These proteins, *N*-ethylmaleimide sensitive factor (NSF) and an adaptor protein called soluble NSF attachment protein (SNAP) (Block et al. 1988, Clary et al. 1990), act in many intracellular trafficking pathways. They were subsequently used to affinity purify their membrane receptors from brain, a strategy that yielded proteins crucial for vesicle fusion–mediated neurotransmitter release (Söllner et al. 1993). The receptors were termed SNAREs (for SNAP receptors). Subsequent efforts by a number of groups established that members of the SNARE protein superfamily act not only in neurotransmitter release at the synapse, but also in most, if not every, other intracellular trafficking pathway (Chen & Scheller 2001, Jahn et al. 2003, Kavalali 2002, Pelham 2001, Rizo & Südhof 2002).

The synaptic SNAREs identified by Rothman and colleagues (Söllner et al., 1993) had already been localized either to synaptic vesicles or to the presynaptic membrane (Bennett et al. 1992, Elferink et al. 1989, Südhof et al. 1989). This localization immediately suggested that there were two types of SNAREs, v- (vesicle) SNAREs and t- (target membrane) SNAREs (Söllner et al. 1993). A second nomenclature that

categorizes SNAREs in terms of a single key residue that is usually either arginine (R-SNAREs) or glutamine (Q-SNAREs) has been developed (Fasshauer et al. 1998). Both naming schemes—one functional, the other structural—are still in common use. Because the fusion of two membranes generally requires four SNAREs, at least one of the two membranes must contribute multiple SNAREs. Most intracellular membrane fusion reactions involve one R-SNARE and three Q-SNAREs (Bock et al. 2001, McNew et al. 2000). In many cases, the R-SNARE is contributed by the vesicle, and three Q-SNAREs are contributed by the target organelle.

Assembled SNARE complexes that bridge two membranes are called *trans*-SNARE complexes. Membrane fusion converts these *trans* complexes to *cis* complexes, complexes in which all the SNAREs are associated with the same membrane. The extreme kinetic and thermodynamic stability of *cis*-SNARE complexes means that their disassembly requires a specialized chaperone machinery (Fasshauer et al. 2002, Fasshauer et al. 1997). This machinery, the chaperone NSF and the cochaperone SNAP, has already been introduced because it was discovered prior to SNAREs and was instrumental in their identification (Söllner et al. 1993a). NSF/SNAP utilizes the energy of ATP hydrolysis to disassemble *cis*-SNARE complexes, freeing SNAREs for productive *trans*-complex assembly and allowing the recycling of SNAREs that have already mediated one round of membrane fusion (May et al. 2001).

SNARE proteins are architecturally simple, characterized by the presence of SNARE motifs approximately 60–70 residues in length (Weimbs et al. 1997). In most SNAREs, a single SNARE motif is located immediately adjacent to a C terminal transmembrane anchor. The SNARE motif is most notable for its repeating heptad pattern of hydrophobic residues, spaced such that the adoption of an α-helical structure places all the hydrophobic side chains on the same face of the helix. SNARE motifs assemble into parallel four-helix bundles stabilized by the burial of these hydrophobic helix faces in the bundle core (Antonin et al. 2002b, Poirier et al. 1998, Sutton et al. 1998). The parallel arrangement of SNARE motifs within complexes brings the transmembrane anchors, and the two membranes, into close proximity (Hanson et al. 1997).
In addition to SNARE motifs and membrane anchors, many SNARE proteins have autonomously folding N-terminal domains. The structures of a number of these domains have been determined by using crystallography and/or NMR (Dulubova et al. 2001, Fernandez et al. 1998, Gonzalez et al. 2001, Lerman et al. 2000, Lu et al. 2002, Munson et al. 2000, Tochio et al. 2001). Autonomously folded domains can regulate SNARE assembly. The three-helixbundle domain of syntaxin 7 and the profilin-like

domain of yeast Ykt6p have modest abilities to inhibit SNARE assembly (Antonin et al. 2002a, Tochio et al. 2001). A much more dramatic inhibitory effect is observed for Sso1p. The yeast ortholog of syntaxin, Sso1p, has a three-helix-bundle domain near its N terminus that binds intramolecularly to the SNARE motif. This intramolecular interaction generates a closed conformation that strongly inhibits entry of Sso1p into SNARE complexes, both in vitro and in vivo (Fiebig et al. 1999, Munson et al. 2000, Munson and Hughson 2002).

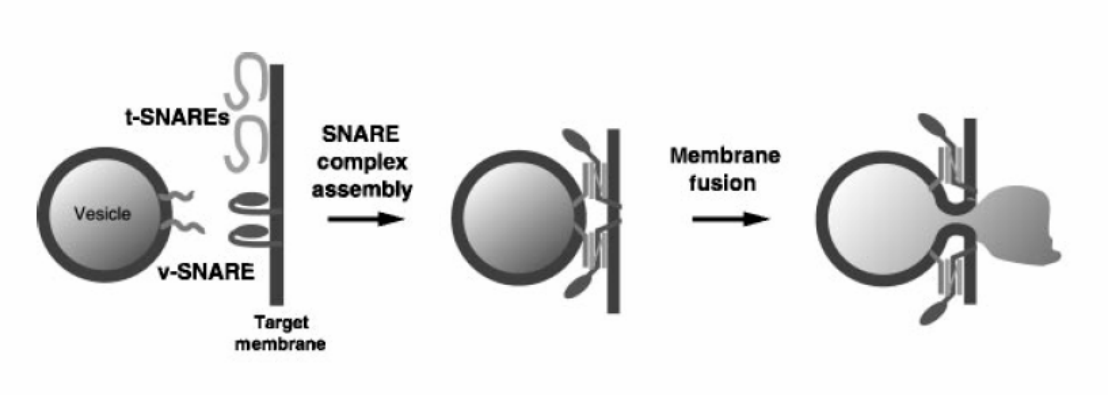

Fig. 1.5. A general SNARE-mediated fusion reaction. (From Ungar and Hughson 2003)

Most SNAREs are integral membrane proteins with a single transmembrane helix at their C-terminal ends. Within *trans*-SNARE complexes, at least one of the participating SNAREs is anchored via a transmembrane domain in each membrane.

1.4.2. Other proteins

Other proteins important for vesicle docking and fusion interact either directly or indirectly with SNAREs. Investigators have focused particularly intensively on three families-the rab, tethering, and SM proteins.

1.4.2.1. SM Proteins

A member of the Sec1/Munc18 family (SM) proteins appears to be essential for every intracellular fusion reaction and to act in conjunction with SNAREs (Gallwitz and Jahn 2003).

The Sec1-family of proteins is known to interact with the syntaxin family of SNAREs (Hanson, 2000). This interaction is believed to trigger a change of conformation in the syntaxin (from “closed” to “open”) allowing formation of SNARE complexes (Dulubova et al., 1999). Plants have 6 members of the Sec1 family (Sanderfoot et al., 2000). This number is four-fold lower than the number of syntaxins in Arabidopsis

(Sanderfoot et al., 2000), so clearly, the Sec1-family members must interact with more than one syntaxin, or other mechanisms for triggering the conformational change in syntaxins must exist. Two members of the Arabidopsis Sec1- family have been investigated. VPS45 is a Sec1-family member that is localized to the TGN in Arabidopsis (Bassham et al., 2000). On the other hand, KEULLE, a second Sec1-homologue, interacts with the cytokinesis-specific syntaxin KNOLLE (Assad et al., 2001), and likely regulates the function of this syntaxin since *keulle* mutants have similar phenotypes to *knolle*, and double mutants display an additive phenotype (Waizeneggar et al., 2000).

1.4.2.2. Rab Proteins

Like other members of the small GTP binding protein family, rabs activate downstream effector proteins when in their GTP-bound state, and their activation and deactivation is in turn controlled by GTP exchange factors and GTPase activating proteins. Geranylgeranyl groups attached to the C terminus of rab proteins mediate membrane attachment, although binding to a protein called GDP dissociation inhibitor can solubilize rabs (Araki et al. 1990). Rab proteins are associated with both trafficking vesicles and other cellular membranes; their localization is specified by interactions with other proteins and lipids (Pfeffer 2003, Pfeffer 2001, Zerial and McBride 2001). Efforts to understand their roles in intracellular trafficking have emphasized the identification of effector molecules via the use of genetic and biochemical approaches.

In their activated state, rabs bind to a variety of effector proteins. These include components of motor complexes involved in vesicle movement (Hammer and Wu 2002), vesicle cargo proteins (van IJzendoorn et al. 2002), and even GTP exchange factors for other rab proteins (Ortiz et al. 2002, Wang and Ferro-Novick 2002). Several early reports suggested that rab proteins interact directly with either vor t-SNAREs and activate them for *trans*-SNARE complex assembly (Lian et al. 1994, Lupashin and Waters 1997). Recent work has focused increased attention on the idea that rabs regulate SNARE assembly and function indirectly through interactions with effector proteins known as tethering factors (Pfeffer 1999, Waters and Hughson 2000, Zerial and McBride 2001). Overall, despite their centrality for membrane trafficking and organization, the molecular role of rab proteins and the multifaceted machinery they regulate is still incompletely understood.

1.4.2.3. Tethering Factors

The initial interactions between two membranes destined to fuse appear to be mediated by tethering factors (Guo et al. 2000, Pfeffer 1999, Waters and Hughson 2000, Whyte and Munro 2002). In addition to providing the first physical linkage between the membranes, tethering factors may also trigger the engagement of *trans*-SNARE complexes. Tethering factors are structurally diverse but can be divided into two main classes: long coiled-coil proteins and large hetero-oligomeric complexes. Members of the first class, which include the Golgi protein p115/Uso1 (Nakajima et al. 1991, Waters et al. 1992) and the endosomal EAA1 (Simonsen et al. 1998), form elongated dimers (Callaghan et al. 1999, Dumas et al. 2001). Members of the second class are large hetero-oligomers of 4–10 subunits, including the COG (Ungar et al. 2002), GARP/VFT (Conibear and Stevens 2000, Siniossoglou and Pelham 2001), and TRAPP (Sacher et al. 1998) complexes at the Golgi; the exocyst or Sec6/8 complex (Hsu et al. 1996, Terbush et al. 1996) at the plasma membrane; the HOPS/C-Vps complex (Sato et al. 2000, Seals et al. 2000) at the vacuole; and the Dsl1p complex at the ER (Reilly et al. 2001). Homologies have been noted among some subunits of the COG, GARP, and exocyst complexes (Whyte and Munro 2001, Whyte and Munro 2002), although in general the large hetero-oligomeric complexes are related to one another primarily in a functional sense.

Many tethering factors interact with SNAREs. For example, subunits of the yeast COG complex (formerly known as the Sec34/Sec35p complex) interact genetically and/or physically with five intra-Golgi SNARE proteins: Bet1p, Sec22p, Ykt6p, Gos1p, and Sed5p (Ram et al. 2002, Suvorova et al. 2002, VanRheenen et al. 1998, VanRheenen et al. 1999). In addition, COG interacts genetically and physically with the rab protein Ypt1p and the Golgi vesicle coat complex COPI (Kim et al. 2001, Ram et al. 2002, Suvorova et al. 2002). Because tethering factors can facilitate SNARE assembly (McBride et al. 1999, Sato et al. 2000, Seals et al. 2000, Shorter et al. 2002), it is possible that they orchestrate a sequential process, triggered by one or more activated rab proteins, that begins with initial membrane tethering and finishes with the assembly of *trans*-SNARE complexes.

The ability of tethering factors to functionally interact with SNAREs, rabs, and vesicle coat proteins is strongly suggestive of a central role in membrane trafficking.

1.5. Objectives

Endoplasmic reticulum (ER) to Golgi transport is mediated by COPII vesicles in yeast and mammalian cells. COPII-mediated sorting occurs when protein cargo exit the ER. Although the principles of ER-to-GA transport organization in plant cells are supposed to be similar to those in yeast and mammalian systems, evidence in support of such an assertion is largely circumstantial. Moreover, there is a substantial body of evidence that emphasizes the differences, such as the apparent absence of the intermediate compartment in plants, large numbers of GA stacks moving along the ER and the differences in the organization of the cytoskeleton involved in interrelationships between the ER and GA.

COPII vesicles were first identified in yeast almost 10 years ago. However, little is known about COPII vesicles in plant cells. It is believed that such vesicles could present in plants as well. All COPII related genes can be found in palnt databases like COPII coats, ER/Golgi Snare, p23/24 and so on.

The main goal of my study has been to uncover the COPII machinery and its possible function in the early secretory pathway of plants. I adopted the following approaches. First, an in vitro approach to reconstitute vesicle formation using ER-enriched microsome and cytosol from Arabidopsis cell cultures. (1) The immediate goal was to isolate the putative COPII vesicles formed by this produce. (2) In parallel, the role of p24 proteins in ER-to-Golgi transport was to be inverstigated by peptide-binding experiments using plant vs. animal cytosolic extracts.

Second, an in vivo approach designed to visualize ERESs in plants. For this two different methodologies were to be employed: a) direct visualization of endogenous COPII proteins (Sar1, Sec13, Sec23) in tobacco BY-2 cell lines stably expressing ER- and Golgi-localized GFP-markers through immunoflourescence microscopy, and b) visualization of ER-bound Sec13 by expression of a Sec13-GFP construct in cells transiently expressing ER- and Golgi-localized RFP markers.

2. MATERIALS and METHODS

2.1. Culture media

2.1.1. Arabidopsis culture media:

2.1.1.1. Seed germination media (1/2MS)

0.5X(2.2 g) Murashige and Skoog basal salt mix (MS) salt (Sigma M5524); 0.5 g 2-N-morpholino ethanesulfonic acid (MES); agar 8 g. adjust to pH5.7 with 1M KOH and make up to 1 litre. Autoclave and pour on Petri Dish.

2.1.1.2. Callus induction medium and culture media

3.86g Gamborg´s B5 Medium including vitamins (Sigma G5893);
20 g glucose;
0.5g MES;
0.5 mg 2, 4-Dichloro-phenoxyacetic acid (2, 4-D);
0.05 mg kinetin
Adjust to pH5.7 with KOH and make up to 1 litre and sterilize by autoclaving.
For plating: add 8g/litre agar

2.1.2. Tobacco Bright Yellow 2 (BY-2) culture medium:

4.3g MS(Sigma M5524)
0.2 mg 2,4-D
200 mg KH_2PO_4
100mg myoinositol
1mg thiamine
30 g sucrose
Adjust to pH5.7 with KOH and make up to 1 litre and sterilize by autoclaving.
For plating: add 8g/litre agar

2.1.3. Bacteria culture meium:

1. Luria Bertani (**LB**) medium (1litre)

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

2. Low salt LB (**LSLB**) medium (1litre)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

All bacteria culture medium was sterilized by autoclaving.

For plating: add 15g/litre agar

2.2. Organisms and Culture Techniques

2.2.1. Bacteria

Bacterial strains were grown in the described media (see 2.1.3) at 37°C or at lower temperatures. For short-time storage, bacteria on plates were sealed with Parafilm and stored at 4°C. For long time storage, 0.9 ml of overnight cultures were mixed with an equal volume of 50% sterile glycerol (50% glycerol / 50% LB medium), rapidly frozen in liquid nitrogen, and then kept at –80°C.

2.2.2. Arabidopsis

2.2.2.1. Seed germination

20-30 seeds were sterilized in a solution containing 20% bleach and 0.05% Tween-20. Let stand for 5-10 min with occasional mixing. Draw off the liquid by aspiration and wash thoroughly with sterile double distilled water (DDW) five times. The seeds were plated on 0.5xMS plates and sealed with micropore tape. Incubate the plate at 4°C for 24 hours and the move the plates to the light and at 25°C for germination.

2.2.2.2. Callus induction

When plantlets had grown for 10 days, the plantlets were removed and sliced with a sterile razor blade. Place slices and dice them then put them on the callus induction medium plates (see 2.1.1.2). The plates were incubated at low light at 25°C. After three to

four weeks, small calli had grown. Transfer them to the fresh medium. The calli were subcultured every 3-4 weeks.

2.2.2.3. Preparation of cell suspension cultures

When the calli were pea sized and had been subcultured at least twice, they were ready to start growth in liquid. Transfer the calli in culture medium (see 2.1.1.2). The culture was maintained in dark at 25°C and swirl at 100 rpm.

2.2.3. BY-2 cell suspension culture

BY-2 cells were cultured in the dark by shaking at 100 rpm in suspension at 27°C in BY-2 medium (see 2.1.2). Cells were maintained in the log phase by subculturing weekly into fresh medium at a dilution of 1:50.

2.3 Molecular Biological Methods

2.3.1. Bacterial plasmid DNA preparation

2.3.1.1. Minipreps of plasmid DNA

1.5 ml bacterial culture was centrifuged 5000 x g for 3 min to pellet the cells. Resuspend the pellet in 200µl solution A (50 mM Tris-HCl, 10 mM EDTA, 100 µg/ml RNase A, pH8.0). Add 200µl of solution B (200 mM NaOH, 1% SDS and gently mix, then stand at room temperature not more than 5 min. Add chilled solution C (2.8 M KAc, pH5.1) mix and then stand on ice for 10 min. Centrifuge the mixture at 20,000 x g at 4°C for 10 min. About 550µl of supernatant was transfer to a clean tube, add 400µl iso-propanol, and then the mixture was centrifuged at 20,000xg for 25 min. The pellet was washed with 70% ethanol. The dry pellet was resuspended in TE (10 mM Tris-HCl, pH8.0; 1mM EDTA) buffer.

2.3.1.2. Midipreps of plasmid DNA

Inoculate 100 ml LB containing the appropriate antibiotic with a single colony. Incubate the culture at 37°C overnight on a shaker with the speed set at 250 rpm. Harvest cells by centrifuging at 4000 x g for 20 min at 4 °C.

The midipreps were performed using Plasmid midiprep kit from QIAGEN (Hilden, Germany) or Macherey-Nagel (Düren, Germany) and according to the manufacturer's recommended procedure.

2.3.2. Preparing samples for sequencing

Add 10µl 3M NaOAc to 1 to 2 µg plasmid DNA in solution (adjust to 100 µl with H2O). And then, add 220 µl (2 volumes) of 100% ethanol. Mix them and stand at room temperature for 30 minutes. Pellet the DNA by centrifugation at 20,000 x g for 20 minutes. Remove the supernatant and dry the pellet. The sample is then ready for sequencing. In this study, the DNA sequencing was done by MWG Biotech in Ebersberg, Germany or IBA in Göttingen, Germany.

2.3.3. Spectrophotometric estimation of DNA purity and quantitation

As described in "Molecular Cloning" (Sambrook *et al*., 1989), it is possible to quantify nucleic acids and to evaluate their purity by spectrophotometric analysis.
DNA and RNA absorb light of 260 nm wavelength, proteins (aromatic amino acids) absorb light of 260 nm wavelength too, but absorption is much stronger at 280 nm.
The ratio A260/A280 gives an estimation of DNA purity. For pure DNA, the A260/A280 ratio is about 1.8.
Spectrophotometric conversion: 1A260 of double-stranded DNA = 50 mg/ml
1A260 of single-stranded DNA = 33 mgml
1A260 of single-stranded RNA = 40 µg/ml

2.3.4. Enzymatic treatment of DNA

Restriction enzyme digestion was carried out according to standard procedures (Sambrook *et al*., 1989). Depending on the enzymes used and their cutting sites, sticky-ended (5'- or 3'-protruding single strand DNA) or blunt-ended DNA fragments were generated. Restricted DNA fragments were purified either by gel electrophoresis and extraction using a QIAGEN gel extraction kit (Hilden, Germany), or by using a QIAGEN PCR and nucleotide purification kit (Hilden, Germany).

DNA fragments with compatible cohesive ends were ligated using T4 DNA ligase which catalyses the ATP-dependent ligation of blunt or complementary sticky ends of DNA.

Sticky-end ligations were carried out at room temperature for 2-4 h using a 1:1 - 1:5 vector:insert molar ratio. Blunt-ended ligations were carried out at 14°C overnight, with a 1:5 molar ratio of vector:insert. A typical 20 µl reaction mixture contain: 50-100 ng insert DNA, 10-50 ng vector DNA, 1x ligase buffer, 0.5-1 mM ATP, 1U T4 DNA ligase, water.

2.3.5. Transformation of *E. coli*

2.3.5.1 Preparation of competent cells and transformation by heat shock

The method of Hanahan (Hanahan *et al.*, 1991) was employed to render the cells competent. Upon reaching an OD600 of 0.5-0.9, a 50 ml culture was harvested by centrifugation (10 min at 4.000*g*, 4°C). Cells were resuspended in 20 ml of cold RF1 buffer and left on ice for 15 min, and then the cells were centrifuged again and resuspended in 4 ml RF2 buffer. 70 µl aliquots were taken, rapidly frozen in liquid nitrogen and stored at –80°C.

RF1

100 mM RbCl
50 mM MnCl2
30 mM KOAc
10 mM CaCl2
15% (v/v) Glycerol

(pH adjusted to 5.8 with 0.2 M acetic acid; sterilized by filtration through 0.2 µm filters)

RF2

10 mM MOPS
10 mM RbCl
75 mM CaCl2
15% (v/v) Glycerol

(pH adjusted to 6.8 with NaOH; sterilized by filtration through 0.2µm filters)

Transformation: 1-10 ng of plasmid DNA or 20 µl ligation mixture were added to 100 µl competent cells (thawed on ice). Cells were incubated for 40 min on ice, then heat-shocked for 90 sec at 42°C. Thereafter, 0.5 medium was added and the samples were

incubated with agitation at 37°C for 45 min. Finally, cells were plated onto LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.3.5.2. Preparation of competent cells and transformation by electroporation

10 ml of overnight culture of *E. coli* were used to inoculate 1l of fresh LB medium.
The culture was grown at 37 with agitation until an OD600 of 0.5-0.9 was achieved. Cells were harvested by centrifugation (10 min at 4.000*g*, 4°C). The cell pellet was washed 2 times with 1 volume sterile cold water and once with 20 ml sterile cold 10% glycerol. Finally, cells were resuspended in 2 ml sterile cold 10% glycerol, dispensed in 40 µl aliquots and frozen in liquid nitrogen. The frozen cells were stored at –80°C.

Transformation: 40 µl electro-competent cells were thawed on ice and transferred to a chilled 0.2 cm electroporation cuvette. 1-2 µl of ligation mixture was added and the sample was kept on ice for 1 min. Thereafter, the cuvette was transferred to a Gene Pulser electroporation chamber (BioRad, München, Germany) and pulsed once with 25 µF, 2500 V, 200 Ohms. 1 ml SOC medium was added immediately after the pulse and the sample transferred to a 2 ml Eppendorf tube and incubated with agitation at 37°C for 45 min. Cells were then plated onto LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

2.3.6 Transformation of *Agrobacterium tumefaciens*

Inoculate 50 ml of LSLB contained the appropriate antibiotics with a single colony of LBA4404 or GV3101. Grow at 28°C till OD600=0.5-0.6. Spin the cell at 6000xg for 10 min and resuspend in 10 ml of 0.15M CaCl2. Pellet the cell again and then resupend the cell in 1ml ice-cold 20 mM CaCl2. Transfer 0.2 ml of cells to a 1.5 ml tube. Add 1 µg plasmid DNA. Incubate on ice for 30 min and the freeze the mixture in liquid N2 for 1 min. Thaw the mixture in a 37°C water bath. Immediately add 1 ml of LSLB and then incubate at 28°C for 2 to 4 hours with gentle shaking. Pellet the cell and resuspend it in 100µl LSLB and then plate out. The plates were incubated at 28°C for 2 to 3 days.

2.3.7 BY-2 Stable Transfromation

A Co-culture method was used to create stable BY-2 transformants. Four ml of a 3-day-old BY-2 culture were co-cultivated with 100 µl of an overnight culture of Agrobacterium in Petri dishes in the dark at 25°C for two days. Cells were then collected and washed three times with BY-2 medium contained 250µg/ml carbenicillin by centrifugation and were plated on solid medium supplemented with carbenicillin (300µg/ml) and appropriate antibiotics as a selection factor for transformation. Successfully transformed BY-2 cells grew as small calli in about three weeks. The calli were then transfer to fresh medium with the appropriate antibiotic.

2.3.8 PCR amplification of DNA

The polymerase chain reaction (PCR) is a very useful technique that allows to produce high yields of specific DNA target sequences (Saiki *et al.*, 1988).

Most PCR protocols are performed at the 25 µl -100 µl scale.

A typical 50 µl reaction mixture consisted of:

1-10 ng plasmid DNA or 50-100 ng genomic DNA

20 pmol forward primer

20 pmol reverse primer

1x nucleotide mix (200 µM of each dNTP)

1x PCR buffer with $MgCl_2$ (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$)

1 U DNA polymerase (Taq or a mixture Taq/Deep-Vent 5/1)

dH2O

The reaction was incubated in a thermocycler device where the temperature could be changed rapidly. Usually there is a preheating step of 5 min at 95°C during which the template DNA is denatured. This is followed by 30-32 cycles of:

denaturing 30-60 sec at 95°C

annealing 30-60 sec at 45-60°C

elongation 30-120 sec at 72°C

The last cycle is followed by an extra elongation step of 5-10 min at 72°C.

The annealing temperature is dependent on the primer composition, on their Tm (melting temperature) and on their homology with the template. The primers may have modifications such as extensions at their 5' ends or point mutations.
PCR can be done directly from bacterial colonies. Bacteria were recovered with a toothpick from agar plates, dissolved in 60 µl PCR buffer 1x and boiled at 95°C for 5 min, after that 5 µl of the mixture were used as template for the PCR reaction. PCR products were purified by using a QIAGEN PCR purification kit (Hilden, Germany) according to the manufacturer's instructions.

Table 2.1. PCR reaction stock mixture (µl)

Volum(µl)	80	140	280	560	600
10x buffer(µl)	8	14	28	56	60
Primer1(5µM)	5	10	20	40	43
Primer2(5µM)	5	10	20	40	43
dNTP(10mM)	3	5	10	20	22
DNA polymerase	0.5-1	1	1.5-2	3	3
DDWater	58	100	200	401	429

Colony PCR allows for rapid detection of transformation success when primers are available. 1. Making mini-culture: pick up a small colony and inoculate in 200µl LB media. The media was incubated at 37°C on a shaker for about 2 hours. 2. Make PCR reaction stock mixture (See table 2.1). 3. Assembling and cycling reaction: take 20 µl from stock mixture and add 0.5µl from bacterial mini-culture for each sample Run PCR with appropriate conditions for the primers and expected product, Make sure to begin the PCR protocol with an extended time at 95°, (e.g. 5 minutes). Run 20µl of the PCR reaction on an agarose gel to identify which cultures to keep for plasmid DNA isolation.

2.3.9 Plasmid constructions

1. pGEX-GST::AtSec13:

The EST clone of putative AtSec13 (Genbank Acc: R30472) was ordered from Arabidopsis Biological Resource Center, Columbus, OH. U.S.A. It contained the full length of AtSec13 which was verified by sequencing. AtSec13 was amplified from the EST clone using AtSec13For and AtSec13Re1 as primers (Table 2.2) and cloned into the

EcoRI, SamI sites in pGEX4T3 (Amershan Biosciences europen GmbH, Freiburg, Germany).

2. pASK-IBA3-AtSec13::strep-tagII:

AtSec13 was amplified from EST clone using AtSec13For and AtSec13Re2 as primers (Table 2.2) and cloned into the EcoRI, SmaI sites in pASK-IBA3 vector (Institut für Bioanalytik GmbH, Germany).

3. pGEX-GST::AtSec22:

The EST clone of AtSec22 (Genbank Acc: F14097) was ordered from Laboratoire de Biologie Cellulaire, INRA centre de Versailles, France. It contained the full length of AtSec22 which was verified by sequencing. AtSec22 was amplified from the EST clone using AtSec22For and AtSec22Re1 as primers (Table 2.2) and cloned into the EcoRI, SamI sites in pGEX4T3

4. pASK-IBA3-AtSec22::strep-tagII:

AtSec22 was amplified from EST clone using AtSec22For and AtSec22Re2 as primers (Table 2.2) and cloned into the EcoRI, SmaI sites in pASK-IBA3 vector (Institut für BioanalytikGmbH,Germany).

5. pGEX-GST::AtSec12b:

AtSec12b was amplified from a cDNA library CD4 using AtSec12bfor and AtSec12bRe as primers (Table 2.2) and cloned into the EcoRI, SamI sites in pGEX4T3.

6. pQE326xHis::AtSec12b:

AtSec12b was amplified from a cDNA library CD4 using AtSec12bfor2 and AtSec12bRe2 as primers (Table 2.2) and cloned into the EcoRI, SamI sites in pQE3 vector.

7. pGEX-GST::AtBet1:

AtBet1 was amplified from a cDNA library CD4 using AtBetfor and AtBet1Re as primers (Table 2.2) and cloned into the EcoRI, SamI sites in pGEX4T3.

8. LeSec13::EGFP:

Tomato (*Lycopersicon esculentum*) LeSec13 EST (Genbank acc# AI776423) clone with the AtSec13 homologs inserted was ordered from Clemson University Genomics Institute (Clemson, U.S.A). The full length of LeSec13 was amplified by PCR. It was cloned into pCK(X/S) LTEV-EGFP to pCK-LeSec13-EGFP. LeSec13-EGFP was sub-cloned into the pTA7002 vector for creating LeSec13-EGFP stable transformation in BY-2 cells. pTA7002-LeSec13-EGFP was induced in LBA4404 by the Freeze-Thaw method (2.3.6.).

9. ManI::MonoRFP

The monomeric red fluorescent protein (RFP) (Campbell et al., 2002) gene was amplified using DSR2-30For and DSR2-30Re as primers (Table 2.2) and sub-cloned into BP30 (Nebenführ et al., 1999) to get pBP30-ManI::MonoRFP for transient expression.

10. Sp::MonoRFP

pGreen0029 Sp::GFP-HDEL was created by subclone the BamHI SacI fragment from pBIN m-GFP5-ER plasmid into pGreen0029 vector. PCR amplified monoRFP using DsRed2-SpFor and DsRed2-SpRe as primers (Table 2.2) was clone into pGreen0029 Sp::GFP-HDEL vector to replace GFP. The result vector is pGreen0029 Sp::MonoRFP without HDEL tail. The BamHI SacI fragment was cut from this vector and subcloned into p35S vector to create p35S-Sp::MonoRFP vector for transient expression in BY 2 cell.

Table 2.2. The primers used in this study

Primers	Sequences 5´ to 3´
AtSec22For	GGAATTCATGGTGAAAATGACATTGATAGCTCGTG
AtSec22Re1	TCCCCCGGGTTCGGATCAAAGCCTGACG
AtSec22Re2	TCAGATCAAAGCCTGACGGTTCAAATCTTTAGC
AtSec13For	GGAATTCCCAGGTCAGAAGATTGAAACGGGTC
AtSec13Re1	TCCCCCGGGCCTCAACAGCAGTAACTTGTTCC
AtSec13Re2	TCCCCCGGGCTAAGGCTCAACAGCAGTAAC
AtSec12bFor	CGGGATCCCGCGAGTAATCAACAACCA
AtSec12bRe	CTAACGTTTTTCACCCTTTTGTTTG
AtSec12bFor2	GGAATTCGCGAGTAATCAACAACCA
AtSec12bRe2	TCCCCCGGGTACGTTTTTCACCCTTTTG
AtBet1For	GGAATTCATGAATCCTAGAAGG
AtBet1Re	TCCCCCGGGTCATCCTCCGGCTTG
AtBet1For2	GGAATTCGGCAATCCTAGAAGG
BP30For	TGAAGGATGTTAATAGTGGAG
BP30Re	TGCGGGACTCTAATCATAAAAAC
DSR2-30For	GATCGGATCCGGAGGTGGCATGGCCTCCTCCGAG
DSR2-30Re	GATCCGCGGCCGCTACAGGAACAGGTGG
DSR2For	ATGGCCTCCTCCGAGAACGTCATCAC
DSR2Re	CTACAGGAACAGGTGGTGGCGGC
pCKFor	AGATGAACTTCAGGGTCAGCTTG
pCKRe	AAATTTTCACCATTTACGAACGAT
FeSec13ReGGG	CATGCCATGGCTCCACCTCCTTGGTCAACAGTGG
DsRed2-SpFor	GGAATTCGCCTCCTCCGAGAACG
DsRed2-SpRe	CGAGCTCCTACAGGAACAGGTGG
FeSeC13For	CCGCTCGAGATGCCAGCCCAGAAGAATG
FeSeC13Re	CTAGCCATGGCTTGGTCAACAGTGGAGG

2.4 Biochemical Methods

2.4.1 Polyacrylamide gel electrophoresis (PAGE)

The principle of polyacrylamide gel electrophoresis is the separation of a large range of proteins of varying molecular masses under the influence of an electrical field by means of a continuous, cross-linked polymer matrix. Here, the polymer is polyacrylamide and the cross-linking agent bis-acrylamide. Cross-linking is effected trough a radical-induced pathway by the addition of APS and TEMED (Ogden and Adams, 1987). In

polyacrylamide gel electrophoresis, proteins migrate in response to an electric field through pores in the gel matrix. The pore size decreases with higher
acrylamide concentration. The combination of gel pore size and protein charge, size and shape determines the migration rate of the proteins (Coligan *et al*., 1997; Sambrook *et al*., 1989).

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional gel electrophoresis under denaturing conditions (in presence of 0.1% SDS) separates proteins on the base of their molecular size. The mobility of the proteins is inversely proportional to the logarithm of their molecular mass. SDS is employed to effect denaturation of the proteins, to dissociate protein complexes and to impart upon the polypeptide chains net negative charge densities proportional to the length of the molecule. A reducing agent such as DTT or 2-ME is used to reduce any existing disulphide bond. The method used is that described by Laemmli (Laemmli, 1970). Two gels are employed: a "stacking gel" with a low level of crosslinkage and low pH, allowing proteins to enter the gel and collect without smearing, and a "resolving gel" with a higher pH, in which the proteins are separated. For an 8x10x0.1 cm gel the following volumes were used:

5% Stacking gel (5 ml)

2.98 ml	H2O
0.8 ml 30%	Acrylamide stock solution
1.25 ml 0.5M	Tris-HCl, pH 6.8
50 µl 10%	SDS
25 µl 10%	APS
5 µl	TEMED

Resolving gel (10 ml)

10%	12%	15%	
3.88 ml	3.21 ml	1.88 ml	H2O
3.33 ml	4 ml	5 ml 30%	Acrylamide stock sol
2.67 ml	2.67 ml	2.67 ml	1.5 M Tris-HCl, pH 8.8
100 µl	100 µl	100 µl	10% SDS

50 µl	50 µl	50 µl	10% APS
10 µl	10 µl	10 µl	TEMED

2.4.2. Preparative gel electrophoresis and electro-elution

To obtain up to milligrams amounts of proteins from an impure mixture, preparative gel electrophoresis was employed. The principle is the same as above (3.4.1), here however, a gel of larger dimensions is poured, and a comb with a single slot is used that allows the application of larger amounts of sample. The band of interest is excised from the gel after standard Coomassie staining and eluted using an electro-elution chamber. Fusion protein was further purified by BIOTRAP electro-separation system (Schleicher & Schuell, Germany).

2.4.3. Western blotting and immunological detection of proteins on nitrocellulose filters

Proteins in both fractions were precipitated out using the chloroform/methanol procedure of Wessel and Flügge (1984).

Proteins were separated by SDS-PAGE and electrophoretically transferred from the polyacrylamide gels to nitrocellulose membranes as described by (Burnette, 1981). The transfer was carried out at 100 mA constant current for 1 hour or at 30 mA overnight.

Transfer buffer 20 mM Tris-base
150 mM Glycine
20% (v/v) Methanol

After the transfer onto nitrocellulose the proteins were stained with Ponceau S solution. Then the membranes were washed briefly with washing buffer-A, treated with blocking solution for 1 h at RT and incubated with the primary antibody in blocking solution for 1 h at RT. After 2x 5 min washes in buffer-A, 2x 5 min in buffer-B and once again for 5 min in buffer-A, the membrane was incubated for 1 h at RT with horseradish peroxidase-coupled secondary antibody (1:10.000 dilution). Finally, the membranes were washed as described above. Detection by chemiluminescence was performed using the ECL detection system as recommended by the manufacturer.

Ponceau S solution 2.5 g/l Ponceau S

15% (v/v) Glacial acetic acid
40% (v/v) Methanol

Blocking solution 5% (w/v) low fat milk powder in washing buffer A

Washing buffer-A 10 mM Tris-HCl, pH 7.4
0.9% (w/v) NaCl
0.05% (v/v) Tween 20

Washing buffer-B 0.2% (w/v) SDS
0.9% (w/v) NaCl
0.5% (v/v) Triton X-100
0.5% (w/v) BSA

2.4.4. Protein quantitation

Protein concentrations were estimated according to the method of Bradford (Bradford, 1976). The method is based on the observation that the absorbance for the protein-specific dye, Coomassie brilliant blue G-250 shift from 465 nm to 595 when binding to protein occurs. Therefore, the A595 yields good linear concentration dependence for most soluble proteins. 800 µl of a proteins solution of unknown concentration was mixed with 200 µl of the dye solution (BioRad, Müchen, Germany) and the measured A595 was plotted against a reference curve obtained with known concentrations of BSA.

2.4.5. Concentrating proteins

Protein solutions were concentrated using Centricon spin columns (Millpore, Bedford, MA. USA), or membra-spin PES columns (membraPure, Bodenheim, Germany) as recommended by the manufacturer.

2.4.6. Protein expression and purification

Genes can be cloned into expression vectors, and expressed in the appropriate cell systems. An expression vector is a vector that contains the necessary regulatory sequences for gene expression. Both prokaryotic and eukaryotic expression vectors exist, many of them are shuttle vectors (cloning vectors that can replicate in two or more dissimilar hosts). Many factors (number of copies of the gene per cell, promoter strength and regulation, translation initiation, codon usage and protein stability) can influence the level of expression of a gene. In addition a suitable host must be used in which the

expression vector is most effective. Often it is advantageous to express proteins as a fusion product with a protein or an epitope. Some of the sequence "tags" can facilitate detection and purification of the target proteins, others increase the probability of biological activity by affecting the solubility in the cytoplasm or the export into the periplasm.

2.4.6.1. Protein extraction from bacteria

Bacteria were harvested by centrifugation, suspended in cold lysis buffer (2-5 volumes per gram of wet weight) and sonicated 3 times on ice (1 min bursts/1 min cooling/200-300 Watt). The lysis buffer composition depended on the subsequent use of the protein extracts (see 3.4.9). After sonication the cell debris was separated from the solubilized proteins by centrifugation (2x10 min at 5.000*g*). Alternatively, total protein extracts for SDS-PAGE and western blotting were obtained easily by resuspending the pellet from 1 OD600 cells with 100 µl Mg2+/SDS buffer (Tris-HCl, pH 6.8 / 0.1 M MgCl2 / 4% SDS / 10% glycerol/ 5% 2-ME / 0.01% bromophenol blue) and subsequently centrifuging for 2 min at 14000 rpm to remove the precipitate.

2.4.6.2. Protein purification

Different chromatography techniques were used to purify the target proteins from total bacterial protein extracts.

a) 6xHis-fusion protein purification

6xHis-fusion proteins can be purified on Ni-NTA metal affinity chromatography matrices (Janknecht *et al.*, 1991). Purification can be performed under native or denaturing conditions. Purification under native conditions: a 5 ml cell pellet from 1 l IPTG-induced bacteria were dissolved in 20 ml lysis buffer and sonicated 3x1 min. Cell debris was eliminated by centrifugation for 10 min at 2.000*g* (4°C), then the supernatant was further centrifuged at 10.000*g* for 30 min (4°C). The 10.000*g* supernatant was transferred to a new tube containing 0.5-1 ml of a 50% slurry of Ni-NTA resin (prewashed with lysis buffer), and incubated at 4°C for 1-2 h with end-over-end rotation. After that the resin

was loaded onto a column and washed with 200 ml buffer-1, 200 ml buffer-2, and 200 ml buffer-3. Finally, the protein was eluted with elution buffer.

Lysis buffer:

20 mM Tris-HCl pH 7.5-8.00
500 mM NaCl
10 mM CHAPS
Proteinase inhibitors EDTA free (cocktail tablets, Roche, Mannheim, Germany)

Buffer-1

20 mM Tris-HCl
500 mM NaCl
20 mM Imidazole (pH 7.5)

Buffer-2

20 mM Tris-HCl pH 7.5
300 mM NaCl
20 mM Imidazole
(pH 7.5)

Buffer-3

50 mM NaH2PO4
300 mM NaCl
10 mM Imidazole
(pH 7.5)

Elution buffer

50 mM NaH2PO4
300 mM NaCl
250 mM Imidazole
(pH 7.5)

Purification under denaturing conditions: The cell pellet from 100 ml culture was dissolved in 10 ml buffer-B and sonicated. The lysate was centrifuged for 30 min at 10.000*g*. The supernatant was transferred to a new tube containing 250-500 µl of a

50% slurry of Ni-NTA resin and incubated at 4°C for 1-2 h with end-over-end rotation. After that the resin was loaded onto a column and washed twice with 10 ml buffer-C. Finally the protein was eluted with Laemmli buffer and analyzed by SDS-PAGE.

Buffer-B

100 mM NaH2PO4
10 mM Tris-HCl
8M Urea
(pH 8.0)

Buffer-C

100 mM NaH2PO4
10 mM Tris-HCl
8M Urea
(pH 6.3)

b) GST-fusion protein purification

Inoculate 50 ml LB medium (containing 100µg/litre ampicillin) with a bacterial colony transformed with a plasmid containing the GST-fusion protein construct of interest. Incubate overnight at 37°C with vigorous shaking. Inoculate 0.5 litre of LB broth with 50ml of cell culture (1:10 culture and LB dilution). Grow the cells till OD reaches 0.6 at 600nm fixed wave length. Induce cells with IPTG final concentration of 1mM for 3 hours at 28°C. Cool the bacterial culture on ice for 15 min. pellet the bacteria by centrifuging at 4000 x g for 20 min. at 4 °C. resuspend the bacterial pellet in 10 ml Lysis buffer. Freeze cell suspension at -80°C. Then thaw cell in an ice-water bath. Sonicate lysate 4 times with 25 sec bursts. Keep the lysate on ice during sonication and leave lysate on ice 1 min between bursts. Centrifuge the lysate at 20,000 x g for 20 min at 4 °C. During the centrifugation step, pellet 1ml of glutathione agarose (Pharmacia Biotech AB, Uppsala, Sweden) by centrifuging at 500 x g for 5 min. then wash the agarose beads with 1x PBS for 4 times. Resuspend the beads in 1 ml of PBS. Transfer bacterial lysate supernatant to a new 50 ml conical tube. Add 1 ml beads to the lysate. Incubate for 1 hour at 4 °C with rotation. Transfer the mixture to a empty column and allow the liquid to pass through. Then wash the agarose beads with 1x PBS for 10 times. Elute the bound GST-fusion

protein by incubating the agarose beads in 1ml elution buffer for 5 min at room temperature. Repeat the elution step for three times.
Alternatively, the agarose beads with the fusion proteins can be resuspend in sample buffer for SDS-PAGE and electroelution.

0.5 M Isopropyl beta-D-thiogalactopyranoside (IPTG)

0.12 g/ml IPTG
Filter sterilize and store aliquots at -20°C

Lyisis Buffer:

1 mM EDTA
50 mM HEPES, pH 7.9
15 mM KCl
5 mM $MgCl_2$
5 mM DTT (added just before use)
10 mg/ml Lysozyme (added just before use)

PBS:

137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.8 mM KH_2PO_4
pH 7.2

Elution Buffer:

5 mM Glutathione
50 mM Tris-HCl, pH 8.0

c) Strep-tag II-fusion protein purification

Strep-tag II system offers the efficient and fast purification of recombinant proteins in E. coli. The engineered interaction between Strep-tag II (NH2-WSHPQFEK-COOH; Schmidt et al., 1996) and StrepTactin (Voss & Skerra, 1997) guarantees optimal results in recombinant protein purification.

The expression of Strep-tag fusion protein was induced by 200 µg/l of anhydrotetracycline. After harvesting the cells by centrifugation (4°C, 4500 g, 15 min), discard the supernatant. Resuspend the pellet in 1 ml (for 100 ml bacterial culture) of precooled buffer W (100 mM Tris/HCl pH8.0, 1mM EDTA) at 4°C. sonificate the suspension under the ice-cooling. To remove insoluble components, centrifuge the suspension at 20000 g for 15 min at 4°C in a microcentrifuge. Equilibrate the streptavidin column by adding 5 ml of buffer W. Add 1 ml of cell extract to the column (1ml bed volume). After the sample has completely entered the colum, wash the column 5 x with 1 ml of buffer W. The strep-tag fusion protein was eluted with 6x0.5 ml buffer E, buffer W containing 2.5 mM desthiobiotin (Sigma D1417). The purified strep-tag II fusion protein usually elutes in the 3rd to 5th fractions.

For regeneration, wash the column 3x with 5 ml buffer R (=buffer W containing 1 mM 4-hydroxyazobenzene-2-carboxylic acid (HABA) (sigma H5126).

2.4.7. Antibody purification

Anti-AtSec13 was purified using GST-AtSec13 as an antigen. The antigen was coupled on the CNBr-activated Sepharose 4 B (Pharmacia Biotech AB, Uppsala, Sweden). After coupling the beads were incubated with blocking buffer (0.2 M glycine and 0.9 % NaCl, pH8.0) on rocker for 2 hours at room temperature. The beads were further wash 4 cycle of acetate buffer (0.1M acetate, 0.9% NaCl, pH4.0) and coupling buffer (0.1 M $NaHCO_3$, 0.9% NaCl, pH8.3). Pour suspended beads into column; wash the column 3 times with PBS buffer. Mix 1 volume of antiserum with 1 volume PBS, then load the mixture to the column and incubate on rocker at room temperature for 1 hour. Then the beads were washed with 6 times with PBS buffer. The anti-AtSec13 was eluted with 0.2 M glycine, pH 2.3 and neutralized with 1/10 volume of 1M tris pH8.0. The purified antibody was concentrated by using centricon (Millpore, Bedford, MA. USA) and the buffer was replaced with PBS contained 0.02% of NaN3. The antibody was stored at 4°C for later use.

2.4.8. Subcellular fractionation

2.4.8.1. Preparation of cytosol and microsomal fractions

Arabidopsis cells were harvest and resuspended in prechilled buffer (25 mM Hepes/KOH, pH 8.0, 300 mM sucrose,10 mM KCl, 3 mM EDTA, 1 mM DTT, 2 mM *o*-phenanthroline,1.4 mg/ L pepstatin, 0.5 mg/ L leupeptin, 2 mg/ L aprotinin, and 1 mg mL21 *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)-butane by using a Waring Blendor in three 15-sec bursts. The slurry was then passed through two layers of Miracloth (Calbiochem) and four layers of gauze. After pre-centrifugation at 5000 g for 20 min, microsomal membranes were pelleted at 100,000g for 1 hour. The concentration of protein was determined by dye binding (Bradford, 1976). BY-2 cytosol and microsomes were prepared as above except that the cells were broken in a Jeda press (Linca-Lamon Instrumenttation Co. Ltd, Israel).

2.4.8.2. Sucrose-density-gradient centrifugation

5000xg supernatant (see above) was pelleted at 60% sucrose cushion. The pellet was diluted with buffer and then load onto a linear 20% to 55% (w/w) sucrose gradient. After centrifugation at 100,000*g* in a swing out rotor for overnight, 1.5-mL fractions were harvested, and the proteins in 100-mL aliquots from each fraction were precipitated with 10% (w/v) TCA. The protein pellets were washed with cold acetone, dried and then resuspended in SDS-PAGE sample buffer for electrophoresis and western blotting.

2.4.8.3. Flotation gradient:

Load the sample on the bottom of the tube. Overlay with different sucrose solution: 2M, 1.8M, 1.6M, 1.4M, 1.2M, 1.0M, 0.8M, 0.6M, 0.4M 0.5 ml each one by one. The gradient was centrifuged in a swing out rotor at 100,000 x g overnight. 0.5 ml fractions were recovered. The proteins were precipitated with 10% (w/v) TCA, and analyses by western blotting.

2.4.9. COPII In vitro budding assay:

2.4.9.1. Preparation of 30% $(NH_4)_2SO_4$ cut off cytosol:

Arabidopsis cytosol was prepared as described in (2.4.8.1.). $(NH_4)_2SO_4$ in form of a saturation solution was added to increase $(NH_4)_2SO_4$ in the solution to 30%. The solution was stirred gently at 4°C for 1 hour. The salted out protein was pelleted by centrifugation at 20,000 x g for 20 min at 4°C. Remove the supernatant; the pellet was resuspended in 3 ml buffer 88 we call 30% $(NH_4)_2SO_4$ "cytosol". Undissolved materal were removed by centrifugation at 20,000 x g for 10 min at 4°C. To desalt, the cytosol was allow to pass through a PD-10 column pre-equilibrated with buffer 88. The cytosol was washed out with 3.5 ml of buffer 88. After desalting, the cytosol was centrifuged at 20,000 x g for 10 min to remove protein aggregates. The clear supernatant was frozen in liquid nitrogen and stored at -80°C.

2.4.9.2. Preparation of ER-enriched microsomes:

The Arabidopsis homogenate was prepared as described above and pre-centrifuged at 5,200 x g for 15 min at 4°C. Load the result supernatant on a step gradient consist of 20%, 36% 46% and 50% sucrose step. The gradient was centrifuged at 100,000 x g for 2 hours at 4 °C. The membranes at the 36% and 46% interface were recovered and diluted by adding an excess of buffer. The ER-rich microsomes were pelleted by centrifugation at 20,000 x g for 20 min at 4°C. The pellets were resuspended in buffer 88, then frozen in liquid nitrogen and stored at -80°C.

2.4.9.3. Budding conditions

The budding assay mixture consist of 30% $(NH_4)_2SO_4$ cytosol, ER rich microsome, GMP-PNP and ATP-RS. The mixture was incubated at 30°C for 30 min. Then the donor microsomes were removed by centrifugation at 20,000 x g for 25 min at 4°C. The resulting pellet called MSP and the supernatant called MSS. The MSS was further centrifuged at 120,000 x g for 30 min at 4°C to separate the cytosol and the induced vesicles. The resulting pellet was called HSP. The MSP and HSP were resuspended in sample buffer for western blotting analysis.

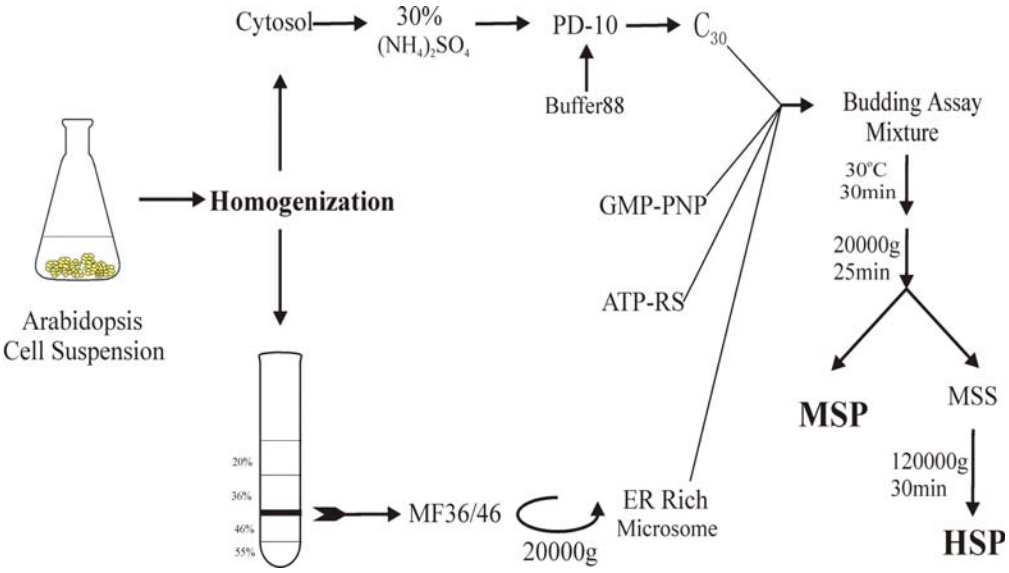

Fig. 2.1. Flow Chart in COPII-Budding Experiments.

MSP=Medium Speed Pellet
HSP=High Speed Pellet

2.4.10. In vitro binding of COPI and COPII coats to sorting motifs in p24 proteins

Synthetic peptides were generated (Gramsch Laboratories, Schwabhausen, Germany) corresponding to the carboxy-terminal cytoplasmic tail of a member the p24 family in *Arabidopsis thaliana* (Atp24), with an amino terminal cysteine residue to allow binding to activated thiol-sepharose. Atp24 peptides with lysines replaced by serines (Atp24-YFSS, Atp24-AASS) or hydrophobic residues (tyrosine/phenylalanine) replaced by alanines (Atp24-AAKK, Atp24-AASS) were also generated (Figure 1). All the peptides were coupled, via their amino terminal cysteine residue, to activated thiol-sepharose (Amersham Pharmacia Biotech), according to the recommendations of the manufacturer (5 mg of crude peptide per ml of beads). The coupling reaction was quenched for 1 h at room temperature in 0.1 M ammonium acetate (pH 4.0), 0.5 M NaCl, 8.5 µM 2-mercaptoethanol, and beads equilibrated in PBS buffer. Peptide coupling efficiency was monitored by measuring the absorbance at 343 nm according to the manufacturer´s specifications.

Binding to the Atp24 cytosolic tail was performed as follows. 2.5 ml of *Arabidopsis* extract or rat liver cytosol (2 mg protein/ml) were diluted with 2.5 ml of 2x lysis buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.5 % Triton X-100 and a cocktail of protease inhibitors), incubated for 1 h at 4°C and centrifuged for 10 min at 2,500 x g. Supernatants were incubated twice 1 h with thiol-sepharose beads (precleaning) before incubation for 2 h at 4°C with 50 µl of beads with the coupled peptides. After the incubation, beads were washed five times with lysis buffer and then eluted by boiling in reducing SDS sample buffer. Proteins were separated by SDS-PAGE and analyzed by Western blotting.

2.5. Microscopical analysis

2.5.1 Confocal Laser Scanning Microscopy

2.5.1.1. Immunofluorescence Labeling

BY-2 cells 3 to 4 days after subculture were fixed with 1% glutaraldehyde in culture medium for 15 min at room temperature under gentle agitation. After two washes in culture medium, cells were treated with 0.1% (w/v) Pectolyase Y23 (Kikkoman Corp., Tokyo, Japan) and 1% (w/v) Cellulase RS (Onozuka; Yakult Honsha Corp., Tokyo, Japan) for 1 hr at 28 °C to partially digest cell walls, washed three times in PBS, and finally transferred to 10 ml of freshly prepared PBS containing 0.1% (w/v) $NaBH_4$. After overnight incubation at 4 °C to permeabilize cells and reduce autofluorescence, cells were allowed to settle onto poly-L-lysine–coated cover slips before the addition of blocking solution consisting of PBS, 5% (w/v) BSA, 5% (v/v) normal goat serum, and 0.1% (v/v) cold water fish skin gelatine (Aurion, Wageningen, The Netherlands) for 1 hr at room temperature. Cells then were incubated at 4C overnight in a 1:1000 dilution of the primary antibody in PBS plus 0.1% (v/v) acetylated BSA (Aurion, Wageningen, The Netherlands), washed four times in PBS, and incubated again in the dark for 4 hr at room temperature with Alexa-fluor 568 goat anti-rabbit immunoglobulin G (Molecular Probes Europe, Leiden, The Netherlands) diluted 1:300 in PBS plus 0.1% (v/v) acetylated BSA. Cells were washed again four times before observation. Control experiments in which primary antibodies were omitted were performed each time to verify the specificity of the labeling.

2.5.1.2. Microscopy

Before observation, fixed cells were mounted in a chamber containing PBS and 0.1% (w/v) Na ascorbate, pH 7.4, to reduce photobleaching. Observation of living cells was performed in a perfusion chamber with a continuous supply of fresh medium at a flow rate of 0.5 mL/min. Cells were observed with a Zeiss (Jena, Germany) LSM510 laser scanning confocal microscope equipped with an inverted Zeiss Axiovert 100M microscope and a x63, 1.2 numerical aperture water immersion objective. Laser scanning was performed using the multitrack mode to avoid "bleed-through". Unless stated otherwise, optical sections were 0.45 μm thick. Excitation and emission wavelengths were 488 and 505 to 545 nm for GFP and 543 and 560 nm for Alexa-fluor 568. Image processing occurred in LSM510 version 2.5 (Zeiss) and PhotoShop 7.0 (final image assembly; Adobe Systems, San Jose, CA).

2.5.2. Electron microscopy

2.5.2.1 Negative staining:

A 30-50μl sample was loaded on the paraffin or in a small vessel. Mica coated with a carbon film was cut to 3-4 mm^2 pieces. Hold a piece with forceps and push into the solution of protein at a 30-45 degree angle. Do not let the film detach completely from the mica. Let the film sit on the protein solution for 20-40 seconds. The mica was pulled back allowing the film to sit on the mica. Then slide the mica and film onto a solution of 1-2% uranyl acetate or uranyl sulphate. The film was picked up on copper grid coated with a holey formvar film, dried, and examined in the electron microscopy.

2.5.2.2. Immunogold Negative Staining

The membranes in a 10-mL aliquot were allowed to attach to the surface of a carbon-coated Formvar grid and were then stabilized by a 10-sec fixation in 2.5% glutaraldehyde in phosphate buffer, pH 7.0, before exposure to the primary antibody solution for 1 hr. After washing and blocking, the samples were exposed for 45 min to rabbit IgGs (diluted 1:100) coupled to 10-nm-diameter gold particles before staining for 5 sec in aqueous 3% uranyl acetate.

2.6. DNA and protein sequence computer analysis

The DNA and protein sequences analysis and alignment were done with "BioEdit" (Hall, T.A. 1999) or MacVector5 (Accelrys, Cambridge, UK), the integrated sequence analysis software for contig assembly, database searching and sequence manipulation. Primers were designed and analysed with "OLIGO" (Med Probe, Sweden). For molecular and atomic visualization a WebLab Viewer was used.

The of protein localization sites in cells and the proteins sorting signal were predicted by PSORT at http://psort.nibb.ac.jp/form2.html
For multiple sequence alignments "clustal-w" was used (available at
http://www2.ebi.ac.uk/clustalw/help.html
or at http://dot.imgen.bcm.tmc.edu:9331/multialign/multi-align.html).
The DNA and protein BLAST search were done at
http://www.ncbi.nlm.nih.gov/BLAST/
Other sequence analysis tools are available at the ExPASy site:
http://www.expasy.ch/;
or at the NPSA (network protein sequence analysis) site:
http://pbil.ibcp.fr/.
It is possible to retrieve sequences from different databases (such as GENEBANK, EMBL, TREMBL, SWISSPROT etc.) at the SRS (Sequence Retrieval System) site: http://www.embl-heidelberg.de/srs5/. For sequences database search, "WU-Blast2" or "Fasta3" were used, both available at the EBI (European Bioinformatics Institute) site: http://www2.ebi.ac.uk/. Other important database sites are:
MIPS (Munich Information Centre for Protein Sequences)
http://www.mips.biochem.mpg.de/;
SGD (*Saccharomyces* Genome Database)
http://genome-www.stanford.edu/Saccharomyces/;
and the proteome database
http://www.proteome.com/databases/index.html.

3. RESULTS

3.1. Searching for COPII related proteins in Arabidopsis

The COPII coat is supposed to be conserved in all eukaryotes and is involved in transport from the ER. The COPII coat is made of 5 proteins, and was first described in yeast (Antonny and Schekman, 2001). With the complete sequence of the Arabidopsis genome it is easy to identify the yeast COPII homologues in Arabidopsis (**Table 1.2 and 3.1 for sequence alignment see Appendix III**). Arabidopsis COPII subunits are encoded by multiple genes, though whether this indicates redundancy has to be investigated.

Table 3.1. Sequences similarity (and identity) of Arabidopsis COPII related proteins against Yeast and Human.

	Yeast (*S. cerevisiae*)	Human (*Homo sapiens*)
AtSec13 (Acc.# AAC16967)	63% (48%)	68% (54%)
AtSec22 (Acc.# AKK76469)	59% (40%)	65% (46%)
AtBet1 (Acc.# CAB61855)	44% (19%)	53% (29%)
AtSec12b (Acc.# BAB09140)	47% (24%)	43% (27%)

As shown in Table 3.1, all four proteins are highly homologous to their counterparts in yeast and human. Interestingly, AtSec13 contains some domains that are conserved in all eukaryotes. Searching the conserved domain database (CDD) on NCBI, we can find that AtSec13 contains 7 copies of WD40 repeats. The WD40 domain is found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. AtSec13 also contain two other domains: the KOG1332 domain (CD: KOG1332 in CCD) which involved in the formation of COPII complex, and the KOG2445 domain (CD: KOG2445 in CCD) which is involved in the formation of nuclear pore complex. AtSec22 and AtBet1 are putative ER/Golgi SNAREs. They all contain a coil-coiled region named the SNARE domain. Taken together, we can say that these proteins not only are homologous to their counterparts in yeast and human, but are also functionally conserved.

3.1.1 Cloning and expression fusion proteins

The sequences of AtSec13 and AtSec22 were cloned from EST clones. AtBet1 and AtSec12b were cloned from the CD4-7 cDNA library (The Arabidopsis Biological Resource Center (ABRC) at The Ohio State University, U.S.A). Sequences were confirmed by sequencing and sequence alignment. For protein expression in *E.coli*, all trans-membrane regions are removed and the cytoplasmic part was fused with a GST-tag, His-Tag or strep-tag. Trans-membrane domains were predicted by a web-base programme, PSORT Prediction (http://psort.nibb.ac.jp/form.html) and a stand-alone programme, Mac-Vector. The constructions were transformation into BL21 strains for protein expression. The fusions were purified as described above (see 2.4.6.2) and were further purified by electro-elution. The quantity and quality of the fusion proteins were check on gel with coomassie blue staining (**Fig. 3.1**.). GST-Bet1 was purified as a 37kDa fusion protein and used as an antigen for generating antibodies in rabbits. AtSec22-Strep-tag was purified as a 28 kDa protein. The protein was used for generating antibodies in guinea pig. GST-AtSec12b was purified as a 68kDa and AtSec12b-strep-tag as 45kDa fusion protein. AtSec12b-strep-tag was used for generating antibodies in rat. And GST-AtSec12b will be used for antibody purification.

AtSec13-Strp-tag was purified as a 35 kDa fusion protein, it was used as an antigen for generating antibodies in rat. Purified GST-AtSec13, a 58 kDa fusion protein will be used for antibody purification.

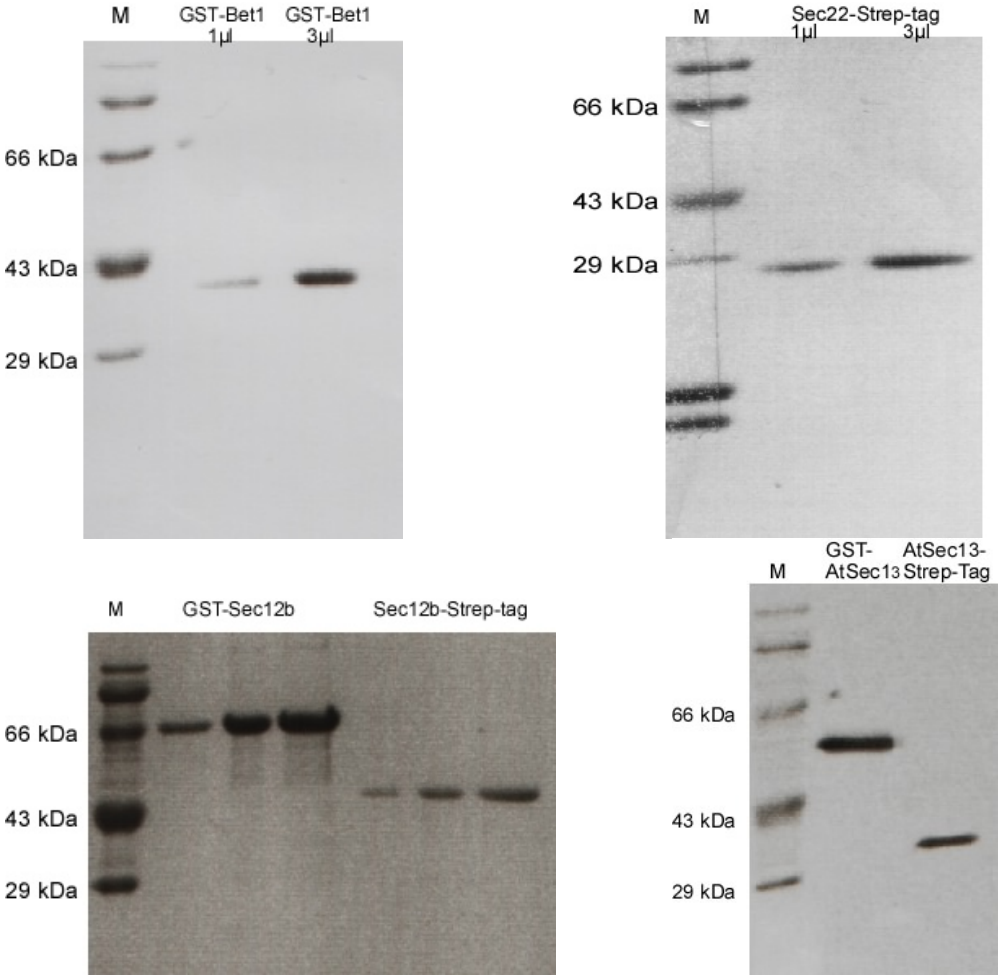

Fig. 3.1. Preparation of fusion proteins and the purification

Different amount of samples were loaded on the gel for checking the quantity of the proteins. The gels were stained with coomassie blue. (M= protein marker)

3.1.2. Antibodies

Antibodies against the antigens described above were prepared commercially. The antisera against AtBet1 AtSec12b and AtSec13 were purified as described in material and methods (see 2.4.7).

The specificity of the antibodies was tested on Arabidopsis microsomes and cytosol by western blotting. As show in **Fig. 3.2**, anti-AtBet1 recognized a 14kDa protein on microsomal membrane but not in the cytosol fraction. Anti-AtSec22 recognized a 25 kDa

protein only in the microsomal fraction. Anti-AtSec12b also recognized a protein in microsomal fraction at 42 kDa. Anti-AtSec13 recognized a 32 kDa on both microsomal membrane and in the cytosol, but was more intense in the cytosol fraction.

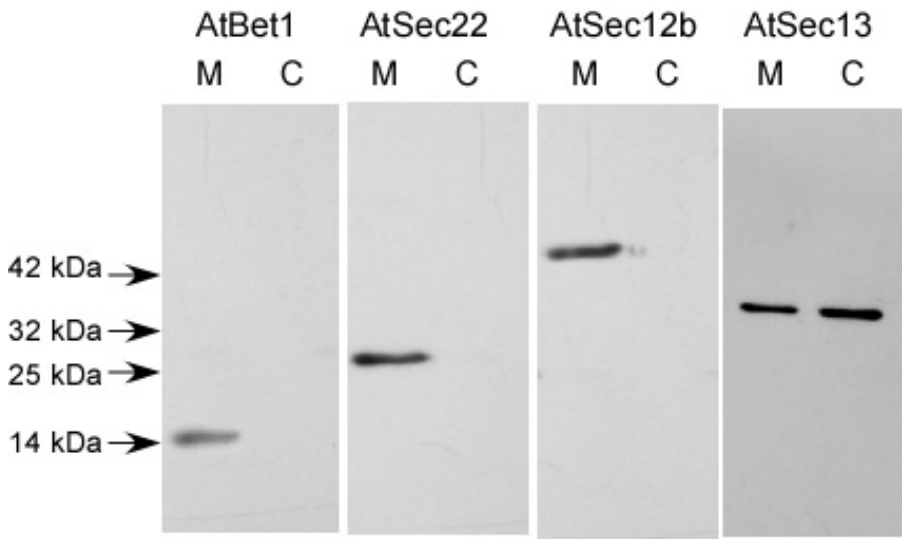

Fig. 3.2. Antisera generated against different fusion proteins were tested on microsome and cytosol fractions from Arabidopsis.

25 µg proteins per lane was loaded on the gel, the signal was detected by western blotting. Anti-AtBet1, anti-AtSec12b and anti-AtSec13 are antigen-purified antibodies. (M=microsomal fraction, C=cytosol fraction)

3.1.3. The principal subcellular location of the proteins

As show in Fig. 3.2 AtBet1 AtSec22 and AtSec12b are membrane proteims. To check the principal subcellular location of AtSec12b, AtBet1 and AtSec22, microsomal membranes were collected on a sucrose cushion and then subjected to a sucrose linear gradient centrifugation. Membrane fractions were prepared under low- (+EDTA) and high- (+$MgCl_2$) Mg^{2+} conditions. The antigen-profile of the gradient was resolve on the gel and detected with different antibodies. Calnexin, an integral protein of the ER, was used as a marker. As expected, the major calnexin profile showed a typical shift to a higher density when the ER was retained in its rough, ribosome-attached form. When Mg^{2+} was

removed by adding EDTA, the ribosomes were disociated and the majority of the ER was shifted to a lower density as indicated by the calnexin. When we look at the profile of AtSec12, AtBet1 and AtSec22, it is obvious that they are all shift with the calnexin (**Fig. 3.3**). This is a clear indication that the ER is the principal subcellular location for AtSec21b AtBet1 and AtSec22.

From this figure, we also can see that the majority of ER is present in the gradient between 36% and 46% of sucrose in high Mg^{2+} conditions (**Fig. 3.3**). Based on this observation, we can prepared ER-rich microsome from a step gradient and used this as a donor for the in vitro budding assay.

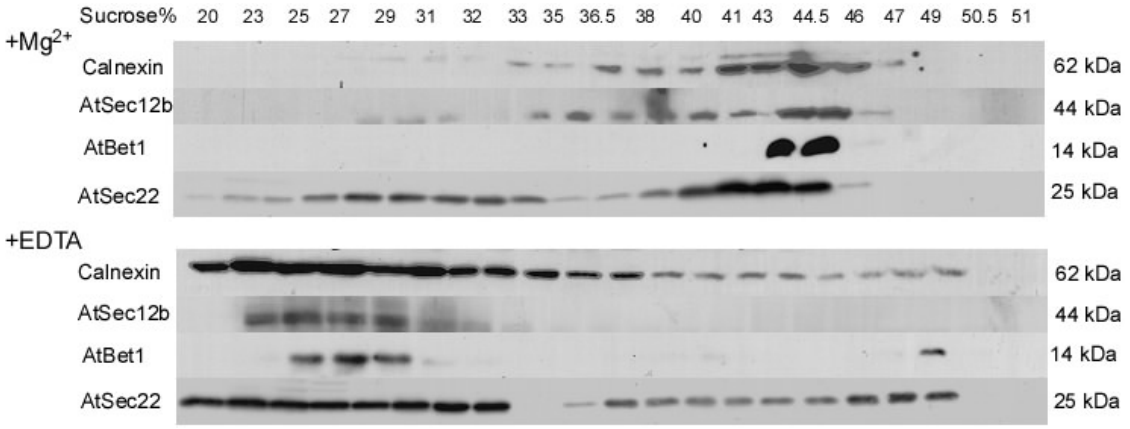

Fig. 3.3. Isopycnic sucrose density gradients of a microsomal membrane from Arabidopsis.

Homogenate were prepared under low (+EDTA) and high (+$MgCl_2$) Mg^{2+} conditions. The fractions were probed by western blotting with the antibodies indicated.

3.2. In vitro approach:

3.2.1. Yeast COPII vesicle budding

As a control and for testing the protocol, I performed a yeast COPII vesicle budding experiment using a kit supplied by Dr. K. Matsuoka (RIKEN, Japan). The experiment was carried out under the same conditions used in the Arabidopsis COPII vesicle budding

assay. The result is shown in the **Fig. 3.4.** Sec61 is a subunit of the translocon and was only detected on the MSP. This means that the donor microsomal membrane were completely removed in the HSP. Sec31 and Sec22 were detected in the HSP only in the present of ATP-RS and GMP-PNP. The vesicle budding is ATP-RS and GMP-PNP dependent as previously shown in the previous studies in yaest (Baker et al., 1988, Barlowe et al,. 1994, Matsuoka et al., 1998).

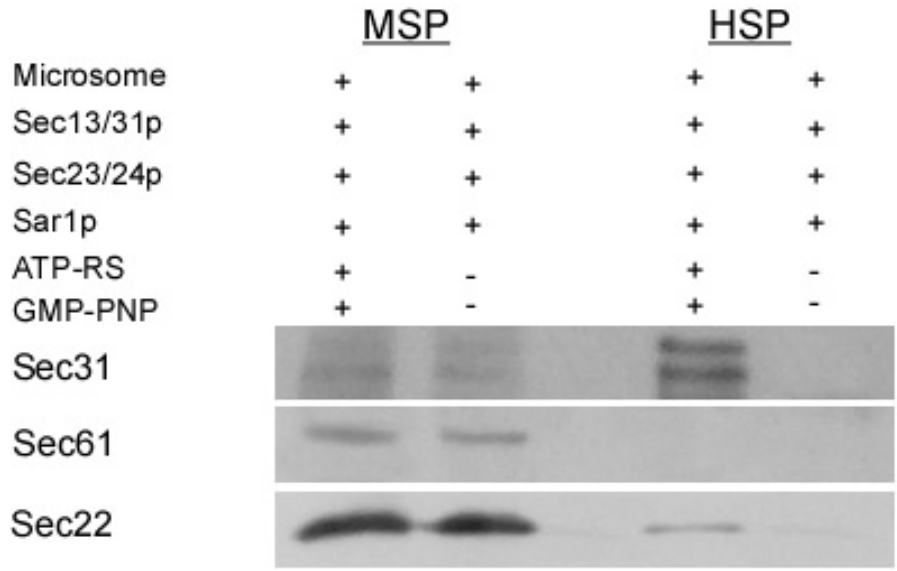

Fig. 3.4. COPII Budding assay using yeast microsomes as donor membrane and yeast cytosolic COPII components.

The budding was probed by different antibodies as show in the Figure. (MSP= medium speed pellet, HSP=high speed pellet)

3.2.2. COPII proteins salt out with 30% ammonium sulphate

Cytosol from Arabidopsis was subjected to sequential protein preciptitation with increasing concentrations of $(NH_4)_2SO_4$ and the fractions were probed with AtSec13, AtSar1 and AtSec23 antisera. It has been shown that the majority of coatomer (AtSec21) was found in the 40 % $(NH_4)_2SO_4$ fraction (Movafeghi et al., 1999). In contrast COPII components mainly salt out at 30% ammonium sulphate (**Fig.3.5**.). This allowed me to

concentrate COPII components in the cytosol and at the same remove the majority of coatomer. This 'cytosol' was used later in the in vitro budding assay.

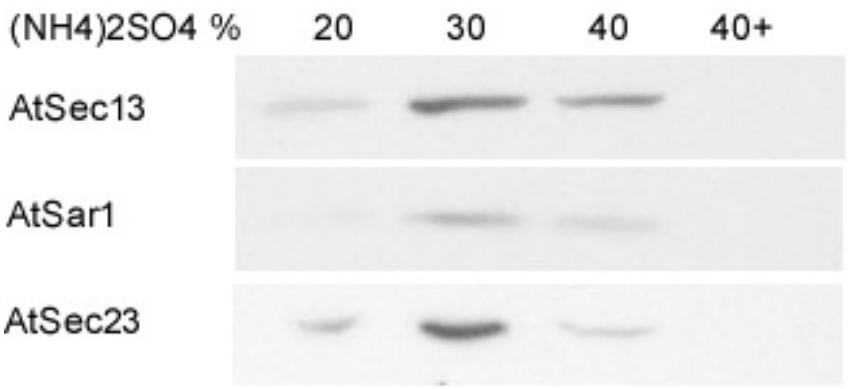

Fig. 3.5. COPII coats are precipitated from cytosol with respect to $(NH_4)_2SO_4$.
The majority of the COPII proteins were salt out at 30% $(NH_4)_2SO_4$ (25µg protein per lane).

3.2.3. The integrity of AtSec13 and AtSec23 complexes

Sec13 and Sec31 are always formed as a complex in the cytosol. Functional Sec13/31 complex is elutes from a gel filtration coloum as a 700 kDa protein complex. Sec23 and Sec24 are also formed a large complex in the cytosol. To check the integrity of these two complexes in Arabidopsis and BY-2 cytosol, we performed sucrose gradient analysis. Fresh prepared Arabidopsis and BY-2 cytosol were loaded on a 9 steps gradient. After centrifuging at 100,000 x g overnight 240 µl fractions were recovered. The distribution of the Sec13 and Sec23 complexes was detected by western blotting as show on **Fig. 3.6.** High molecular markers were used as a control as show in low part of the Figure 3.6., In both cases the Sec23 complex from Arabidopsis and BY-2 was distributed as a 200 kDa protein complex on the gradient. The distribution of the AtSec13 complex is however different from BY-2 cells. The AtSec13 complex shows a peak overlapping with the 669kDa molecular standard and the Arabidopsis coatomer as detected with anti-AtSec21. However the NtSec13 shows a shift to a lower molecular mass region. This was probably caused by degradation and/or disassociation of the complex.

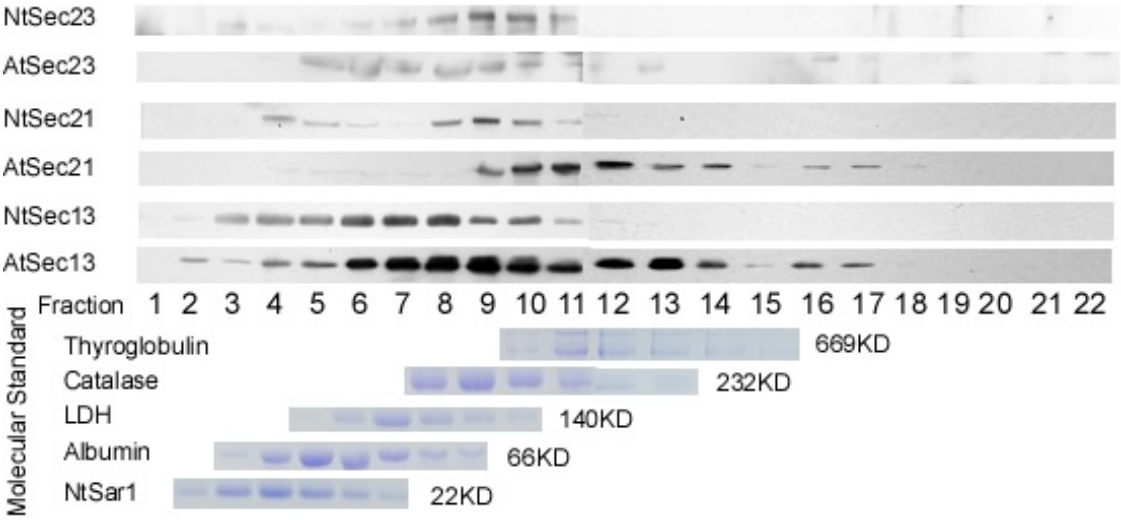

Fig. 3.6. Distribution of the Sec13/31 and Sec23/24 complexes on a linear sucrose gradient.

The proteins were detected by western blotting. High molecular markers were used as a molecular standard and stained with coomassie blue.

3.2.4. COPII vesicle budding with Arabidopsis

To test if COPII vesicle budding can induced in vitro, I performed a COPII budding assay using Arabidopsis microsomal membrane. ER-rich microsomes were collected from a step gradient at the 36% and 46% interface in the present of high Mg^{2+}. This allowed us to obtain ER-rich microsome and at the same time remove the majority of the Golgi. The source of AtSec13 and AtSec23 complex were from the 30% $(NH_4)_2SO_4$ 'cytosol' cut. MSP and HSP were collected and analyzed by western blotting using different antibodies (**Fig. 3.7**.). In this experiment, AtSec61 and AtSec12b were used as controls, since they are ER proteins and do not enter the COPII vesicle. We can see that those two proteins were only detected in the MSP and not in the HSP. It means that the donor ER-rich microsomes were completely removed from the budding mixture after centrifugation. Two ER/Golgi SNAREs, AtBet1 and AtSec22, were detected in the HSP when all components were included in the budding mixture. This is an indication that COPII

vesicles are formed during the incubation. However, AtSec13 was detected in the HSP even if membranes were absent from the cytosol the budding mixture. This is due to the protein aggregation and can be removed by centrifugation.

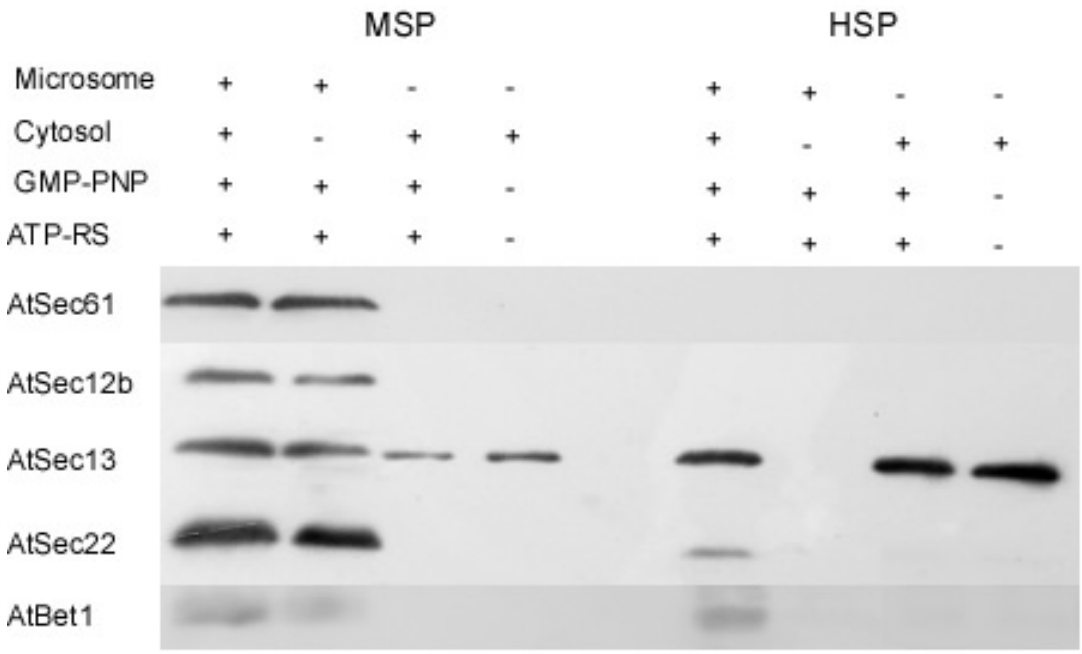

Fig. 3.7. COPII vesicle budding with Arabidopsis membrane.

Budding assay using Arabidopsis ER-rich microsomes as donor membranes and Arabidopsis 30% $(NH_4)_2SO_4$ 'cytosol' as a source for COPII components. The budding was monitored by different antibodies as show in the figure. AtSec61 and AtSec12b act as controls as they are only on the microsomes but not on the vesicles. AtSec13, a subunit of sec13 complex, is associated with microsomes and also on the vesicle. AtSec22 and AtBet1 are ER/Golgi SNAREs located at microsome and sorted into COPII vesicle. (MSP= medium speed pellet, HSP=high speed pellet)

3.2.5. Factors affecting budding

In vitro vesicle budding requires membrane, cytosol, GTP and ATP plus an ATP regeneration system (Rexach and Schekman, 1991; Wuestehube and Schekman, 1992). To test whether the all components in the budding mixture are essential, we performed experiments in which one component was missing from the budding mixture and the result was shown in the **Fig. 3.8.** As we can see in this Figure, vesicle budding does not

occur when microsomes (or cytosol, GMP-PNP, ATP-RS) were excluded from the budding mixture. Vesicle budding was increased when recombinant NtSar1A was added to the budding mixture.

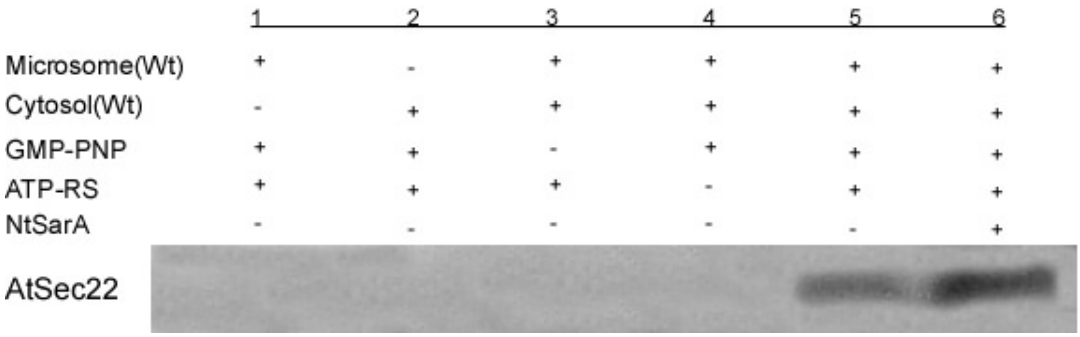

Fig. 3.8. Factors affecting COPII budding.

Budding experiment with exclusion of selected components in the budding mixture or addition of recombinant NtSar1A. The budding assay was probed by with western blotting using anti-AtSec22.

Since the addition of Sar1p can increase vesicle budding, in order to see to what extent vesicle budding can be enhanced by increasing the Sar1p in the budding mixture, we used recombinant NtSar1A as a source for Sar1p and different amount of NtSarA was added in the budding mixture. The result is shown in the **Fig. 3.9**. Vesicle budding (as monitored by anti-AtSec22) was increased by addition of 3µg NtSarA. When more NtSarA was added (6µg, 12µg), a further enhancement of the vesicle budding was not observed. This may due to the availability of Sec13 and Sec23 complexes in the budding mixture. However, an inhibition was observed when 24 µg of NtSarA was included in the budding mixture. The reason for this may be the presence of Triton 100 in the NtSarA solution, the more NtSarA was added, the more Triton 100 will be in the budding mixture and the increase of Triton100 probably has a negative effect on the budding assay.

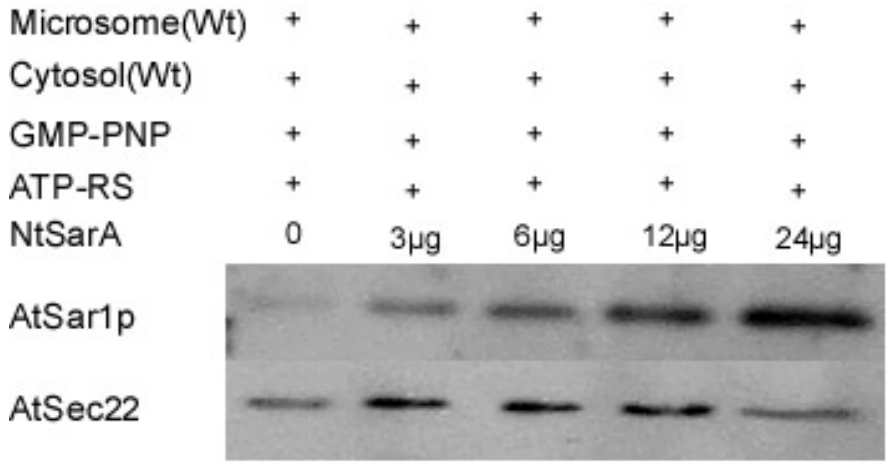

Fig. 3.9. COPII Budding assay in the present of different amounts of NtSarA.

Two transgenic Arabidopsis suspension culture (AtSec12 and AtSar1) over-producers were available (Bar-Peled and Raikhel, 1997). A comparison of some COPII-related proteins on microsome and cytosolfraction between the wild type and transgenic Arabidopsis is shown in the **Fig. 3.10.** The amount of Sar1p is significantly higher in the cytosol from the Sar1p over-producer. AtSec12 was significant increased in the microsome from the AtSec12 over-producer. Interestingly, AtSec13 has also increased in both cytosol and microsomes from the two over-producers. Whether this simply implies an up-regulation of the COPII machinery is unclear.

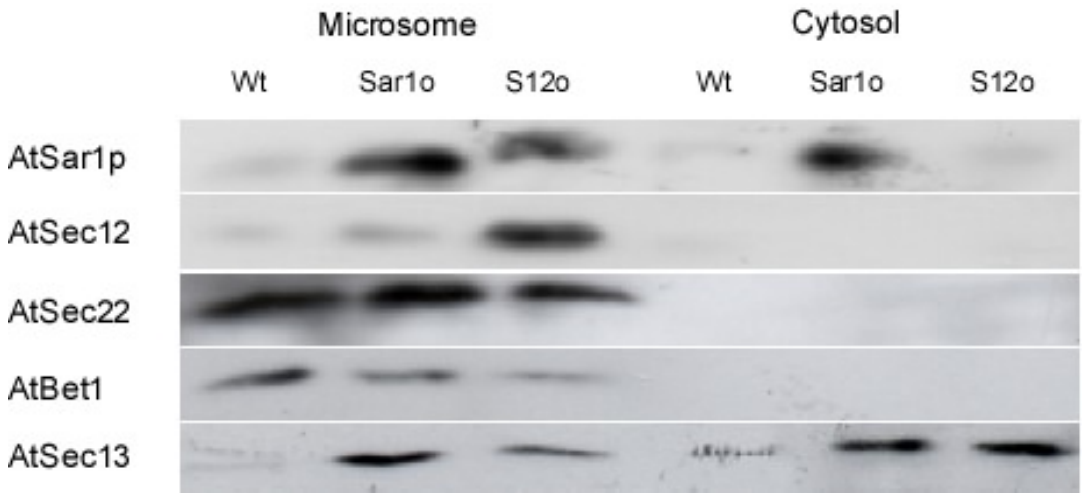

Fig. 3.10. A comparison of some COPII related proteins in microsomes and cytosol between the wild type and the transgenic Arabidopsis.

Equal amount (30 μg) of protein was loaded on the gel per lane.

To test the effect of AtSec12 on vesicle budding in vitro and the effect of the endogenous Sar1p from the over-producer in the budding assay, a budding experiment was performed in which different sources of microsome (from wild-type and AtSec12 over producer) were used as a donor and different cytosol (from wild-type and AtSar12 over producer) were used as a source for the COPII components for the budding assay. The result is shown in the **Fig. 3.11**. We can see that the use of AtSec12 over-producer microsome and the cytosol from AtSar1 over-producer significantly increases the in vitro vesicle budding efficiency.

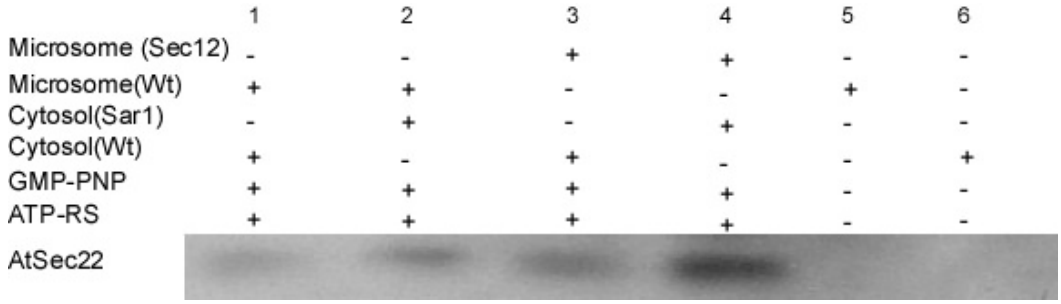

Fig. 3.11. COPII Budding assay using different source of microsome (from wild-type and AtSec12 over-producer) and cytosol (from wild-type and AtSar1p over-producer).

3.2.6. Purification of putative COPII vesicle

Our budding assay shows that putative COPII vesicles were formed during the in vitro incubation of microsome with cytosol. To purify these vesicles, a sucrose flotation gradient was used. The HSP collected on a sucrose cushion was placed at the bottom of the tube and the sucrose gradient was loaded on the sample. After centrifugation overnight, 9 fractions were recovered and the profile of the gradient was monitored with anti-AtSec13 by western blotting. As shown in **Fig. 3.12.** one peak corresponding to about 41 % sucrose on the gradient was observed. COPII-coated vesicles formed in vitro from yeast microsome equilibrate at around 42% sucrose in a gradient (Barlowe et al., 1994). To check the contents of the 41% sucrose fraction, the sample was

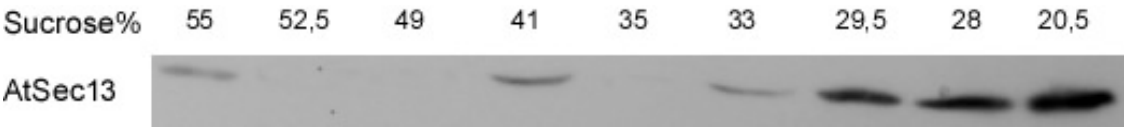

Fig. 3.12. Flotation gradient of HSP.

Observed in the EM by negative staining. Samples were also taken from the other fraction as control. A uniform population of vesicle was observed for fraction No. 6 (**Fig. 3.13**) with very few in the other fractions. The vesicles are about 50-55 nm in diameter and appeared coated on their surface. They are smaller than the COPII vesicle isolated from yeast (ca. 60/70 nm). According to Matsuoka AtSec31 is much smaller, and could be the reason for the reduced size of the vesicles.

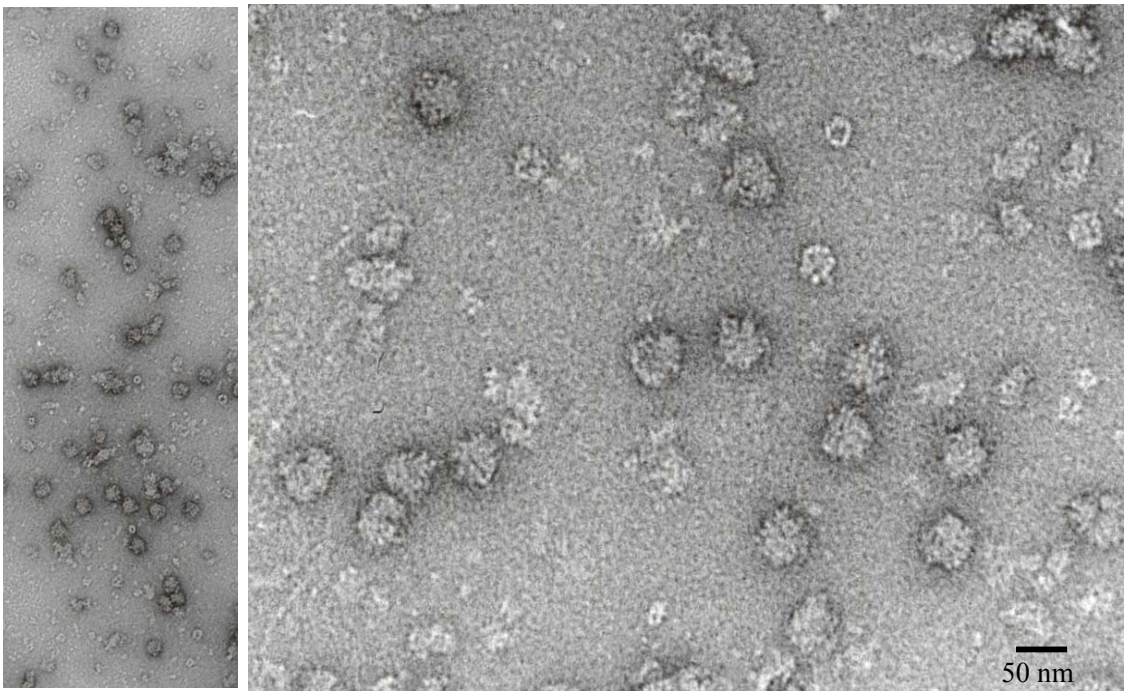

Fig. 3.13. EM picture of the fraction No. 6 (41% sucrose) with negative staining.
The picture on the left show the overview in low magnification. (Scale bar 50 nm)

3.2.7. Coat binding experiments:

Soluble cargos leave the ER by means of bulk flow and receptor-mediated export. Genetic and biochemical studies suggest that the membrane proteins ERGIC53, the p24 proteins and Erv 29p cycle between ER and Golgi compartments and act as a receptor for rexeptor-mediated export. A family of p24 homologous can be found from the Arabidopsis database. The Fig. 3.14 shows the alignment of the family. As shown in the figure, they all contain a KK motif at position -3,-4 from C-terminus and an YF or FF motif at the position -7,-8 from the C-terminus.

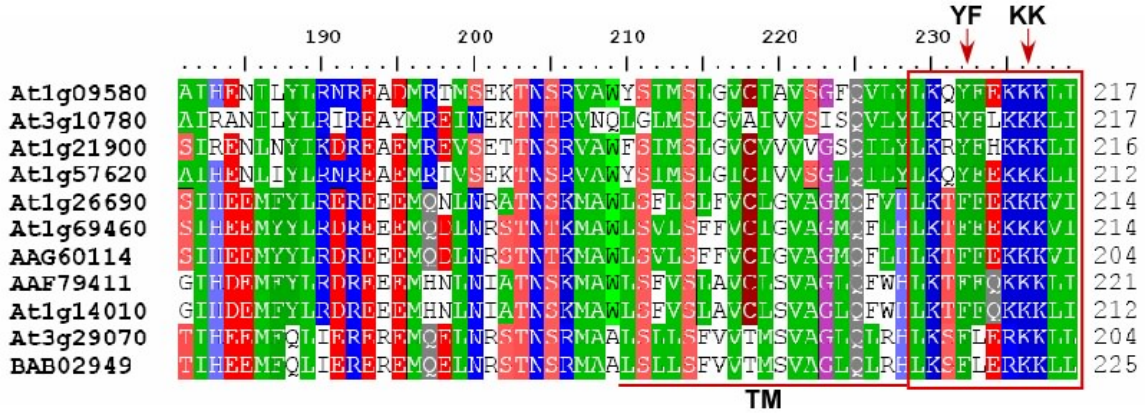

Fig. 3.14. Sequence alignment of p24 homologous from Arabidopsis.

Two conserved motif were shown at the C-terminus as indicated by arrow. (TM= transmembran domain)

To test whether COPII subunits could interact with sorting motifs in the cytosolic tail of p24 proteins, we have performed *in vitro* binding experiments, using Arabidopsis cytosol fractions as a source of COPII subunits and a synthetic peptide corresponding to the cytoplasmic carboxy-terminal domain of a member of the p24 family in *Arabidopsis thaliana*. This peptide, containing a dihydrophobic (YF) motif in the -7,-8 position, and a dilysine (KK) motif in the -3,-4 position (with respect to the carboxy-terminus) (Atp24 (YFKK)), was coupled via an amino terminal cysteine residue to activated thiol-sepharose. As a control, we used Atp24 peptides with lysines replaced by serines (Atp24-

YFSS, Atp24-AASS) or tyrosine/phenylalanine residues replaced by alanines (Atp24-AAKK, Atp24-AASS) (Figure 3.15). Bound proteins were eluted in sample buffer, separated on SDS-polyacrylamide gels and revealed by Western blotting with antisera to the COPII subunits AtSec13 and AtSec23.

Atp24 (YFKK)	- CKQ**YF**EK**KK**LI
Atp24-YFSS	- CKQ**YF**EK**SS**LI
Atp24-AAKK	- CKQ**AA**EK**KK**LI
Atp24-AASS	- CKQ**AA**EK**SS**LI

Fig. 3.15. Sequences of the peptides used in the present study.
Synthetic peptides were generated corresponding to the carboxy-terminal cytoplasmic tails of a member of the p24 family in Arabidopsis thaliana (Atp24), with an amino terminal cysteine residue (not shown) to allow binding to activated thiol sepharose. Atp24 peptides with lysines replaced by serines (Atp24-YFSS, Atp24-AASS) or tyrosine/phenylalanine residues replaced by alanines (Atp24-AAKK, Atp24-AASS) were also generated.

In agreement with the previous data (Contreras et al., 2004), the Atp24 (YFKK) cytosolic tail recruited COPI with a high efficiency. The variant without the dihydrophobic motif (AAKK) in the –7, -8 position also bound COPI although with lower efficiency (Fig. 3.16 C), suggesting that both motifs cooperate in COPI binding. Lack of binding with the AASS and YFSS peptides clearly showed that the dilysine (KK) motif is necessary for coatomer recruitment. On the other hand, neither of the two COPII subunits tested, AtSec13 or AtSec23, bound to the Atp24 (YFKK) cytosolic tail. However, when the two lysines in the -3,-4 position were replaced by serines (Atp24-YFSS), Sec23 was efficently recruited (Fig. 3.16A). In contrast, we could not detect significant binding of Sec13 to any of the peptides (Fig. 3.16B). Neither the Atp24-AAKK nor Atp24-AASS peptides bound Sec23, or Sec13. This indicates that the dihydrophobic (YF) motif may be responsible for COPII binding in plant cells, as it is in mammalian or yeast cells (Dominguez et al., 1998, Stamnes et al., 1995).

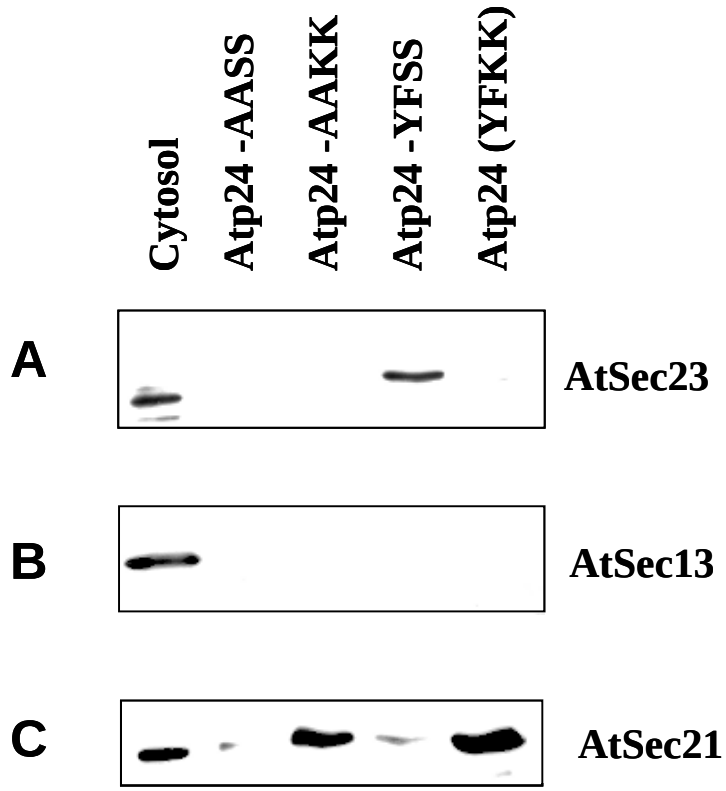

Fig. 3.16. Binding of COPI and COPII subunits from Arabidopsis to Atp24 cytosolic tails.

A cytosolic fraction from Arabidopsis callus cultures (2 mg protein/ml) was incubated in the presence of a synthetic peptide corresponding to the carboxy-terminal domain of a protein of the p24 family in Arabidopsis, Atp24 (YFKK), coupled via an amino terminal cysteine residue to activated thiol sepharose, as described in Material and Methods. In addition, we used Atp24 peptides where lysines were replaced by serines (Atp24-YFSS; Atp24-AASS) or phenylalanines were replaced by alanines (Atp24-AAKK; Atp24-AASS). Bound proteins were eluted by boiling in Laemmli´s sample buffer, separated by SDS-PAGE (8 % acrylamide) and revealed by Western blotting with antibodies against AtSec23 **(A)**, AtSec13 **(B)** or AtSec21 **(C)**. As a control, we also run an aliquot of the cytosol used for the assay (Cytosol).

The fact that the Atp24 (YFKK) peptide, which binds coatomer very efficiently, did not bind Sec23, while the Atp24-YFSS peptide, which does not bind coatomer, did bind Sec23 raised the possibility that COPI and COPII may compete in binding to the Atp24 (YFKK) peptide. To test for this possibility, we attempted to deplete the Arabidopsis cytosol of coatomer before analyzing COPII binding to the different peptides. As shown

in Fig. 3.17A, a combination of high speed centrifugation (200,000 xg, 2h; Contreras et al., 2004) and incubation with the Atp24-AAKK peptide (which binds COPI but not COPII) produced an efficient (roughly 80 %) removal of coatomer, as seen by Western blotting with the AtSec21 antibody. The coatomer-depleted fraction contained similar amounts of Sec23 or Sec13 as the control cytosol, indicating its suitability for monitoring COPII-binding to the different peptides. As shown in Fig. 3.17 B, we still observed some coatomer-binding to the Atp24-AAKK and the Atp24 (YFKK) peptides, but to a much lower extent than when using control cytosol. However, under these conditions, the Atp24 (YFKK) tail also bound Sec23, and binding was very similar to that obtained with the Atp24-YFSS peptide (Fig. 3.17B). These data indeed confirm that coatomer strongly competes with Sec23 binding and only after COPI depletion can we observe COPII binding.

As can be seen in Fig. 3.16 and Fig. 3.17 COPII and coatomer compete with Atp24 c-tail. The binding of coatomer inhibits the binding of COPII to the same motif. To check whether the increase of coatomer binding on the p24 c-tail is due to more coatomer available in Arabidopsis cytosol as compared with the rat cytosol, two experiments were performed. As can be seen in Fig 3.18. that precipitation of coatomer from rat liver cytosol requires 4 mM neomycin, while only 1 mM is required to completely deplete Arabidopsis cytosol. If maximal precipitation requires equal-molar amounts of neomycin and coatomer, this would be an indirect evidence that there is more coatomer in rat liver cytosol than in Arabidopsis. The experiment with purified proteins basically confirms the impression with the neomycin experiment. It can be calculated from Fig 3.18 C-D that 5 mg of rat liver cytosol (the amounts used for the in vitro binding experiments) contain roughly 2.3 micrograms of beta-COP, while the same amount of Arabidopsis cytosol contains only 0.8 micrograms of AtSec21. This means that rat liver cytosol contains almost three times the amount of COPI as is present in Arabidopsis cytosol.

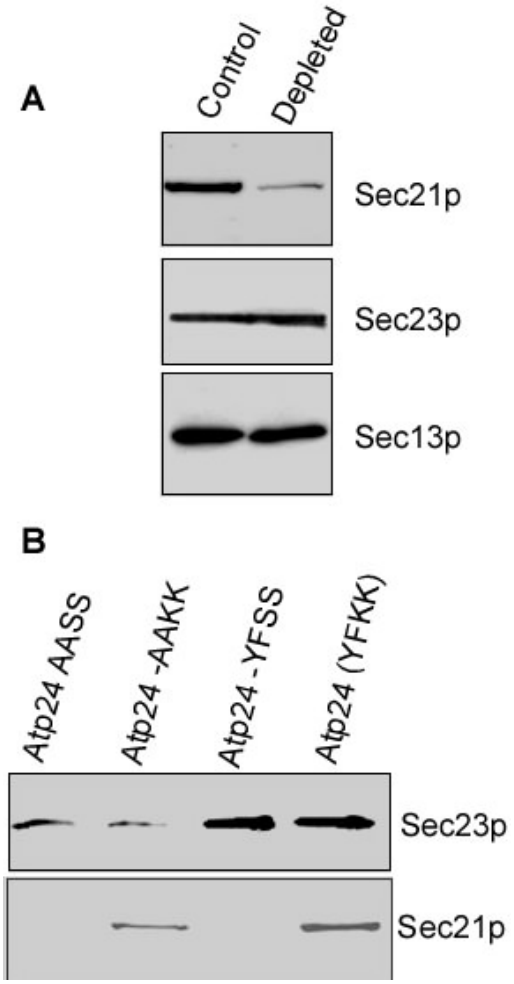

Fig. 3.17. Effect of coatomer depletion on binding of COPII subunits from Arabidopsis to Atp24 cytosolic tails.

(A) A cytosolic fraction from Arabidopsis callus cultures was subjected to high speed centrifugation (2h 30 min at 200,000 x g). The high speed supernatant was then incubated in the presence of AAKK beads for 2 h, to further remove COPI subunits. Cytosol (Control) and COPI-depleted fraction (Depleted) were analyzed by SDS-PAGE (8 % acrylamide) and revealed by Western blotting with antibodies against AtSec21, AtSec23 or AtSec13. **(B)** The COPI-depleted fraction was used to monitor COPI and COPII-binding to the different peptides, as in Figure 2. Bound proteins were eluted by boiling in Laemmli´s sample buffer, separated by SDS-PAGE (8 % acrylamide) and revealed by Western blotting with antibodies against AtSec23 or AtSec21

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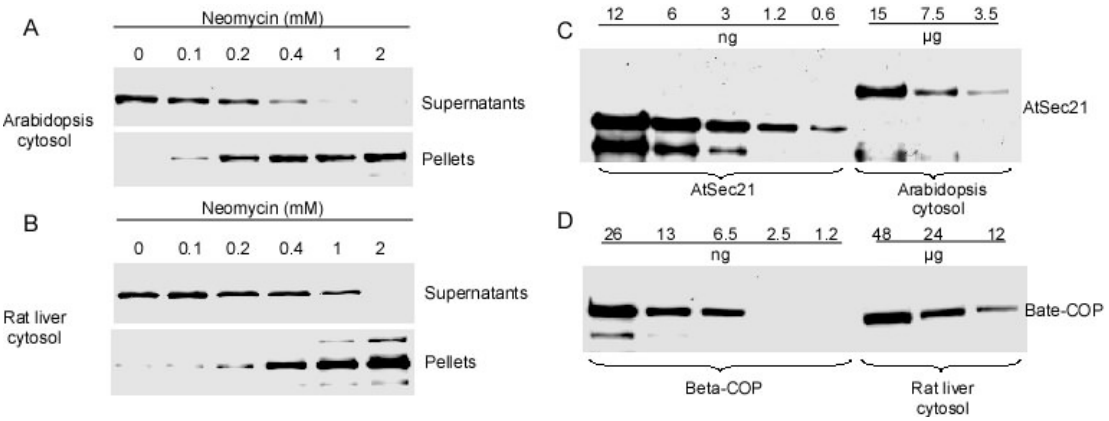

Fig. 3.18. Quantitation of coatomer in Arabidopsis and rat liver cytosol.

(A-B) Coatomer was precipitated with increasing the concentration of neomycin.
(C-D) Comparison of AtSec21 and beta-COP in cytosol with a concentration series of recombinant proteins.

Since a competition between COPI and COPII for binding to p24 family proteins has not previously been reported, we next investigated whether this effect was specific for plant cells. We therefore performed heterologous binding experiments using Atp24 peptides and rat liver cytosol as a source of COPI and COPII subunits. Before analyzing COPII binding to these peptides, we first tested whether the antibodies raised against plant Sec23 and Sec13 could recognize their orthologs in rat liver. As shown in Fig. 3.19A, the AtSec23 antibody recognized a major band of the expected molecular weight in rat liver cytosol. In the case of AtSec13, the antibody recognized several proteins in rat liver cytosol, including two major bands at 41 and 17 kDa, but also a band around 34 kDa, probably corresponding to Sec13 (Fig. 3.19 C). Therefore, we felt justified to use these antibodies to identify the proteins recruited by the Atp24 peptides. As shown in Figure 3.19A, the Atp24 (YFKK) cytosolic tail bound Sec23 with a similar efficiency as the Atp24-YFSS peptide, in contrast to what we had observed in Arabidopsis. We also used an antibody raised against mammalian Sec24, and found that both Atp24 (YFKK) and Atp24-YFSS peptides bound Sec24 with similar efficiency (Fig. 3.19B). Both peptides were also able to recruit Sec13, in contrast to what we had observed in Arabidopsis. Neither the Atp24-AAKK nor the Atp24-AASS peptides bound Sec23, Sec24 or Sec13, confirming again that the dihydrophobic motif is responsible for COPII binding. We also analyzed coatomer binding to the four peptides, using an antibody against mammalian α-COP. As shown in Figure 3.19D, the Atp24 cytosolic tail bound COPI very efficiently, but the same was true for the Atp24-AAKK peptide. In contrast, the Atp24-YFSS peptide was unable to recruit significant amounts of COPI.

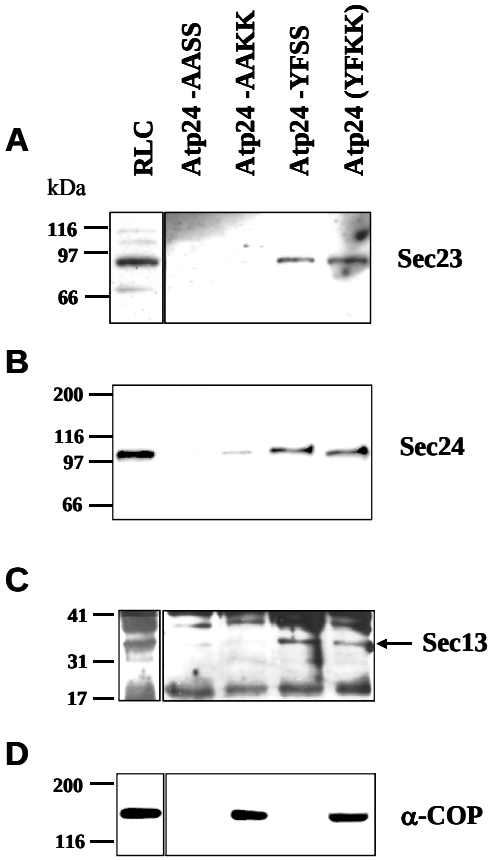

Fig. 3.19. Binding of COPI and COPII subunits from rat liver cytosol to Atp24 cytosolic tails.

Rat liver cytosol (2 mg protein/ml) was incubated in the presence of a synthetic peptide corresponding to the carboxy-terminal domain of Atp24 (YFKK), or to Atp24 peptides where lysines were replaced by serines (Atp24-YFSS; Atp24-AASS) or phenylalanines were replaced by alanines (Atp24-AAKK; Atp24-AASS). Bound proteins were eluted by boiling in Laemmli´s sample buffer, separated by SDS-PAGE (8 % acrylamide) and revealed by Western blotting with antibodies against AtSec23 **(A)**, mammalian Sec24 **(B)**, AtSec13 **(C)** or mammalian α-COP **(D)**. As a control, we also run an aliquot of the rat liver cytosol used for the assay (RLC).

3.3. In vivo approach:

3.3.1. AtSec13 and AtSar1p are presnet on the ER of Arabidopsis

We previously generated antibodies against an AtSec23 fragment (Movafeghi et al., 1999). In order to increase the certainty of valid identification of ERESs by antibody labelling we expressed fusion-proteins and subsequently prepared and purified antibodies against two other COPII-components: the GTPase AtSar1, and the coat protein AtSec13. Similarly, we prepared an antibody against AtSec12, the GEF required for Sar1 recruitment. We tested in protein gel blots all of these antibodies on membrane and cytosol fractions obtained from suspension cultured Arabidopsis and tobacco BY-2 cells (Fig.3.20. A). With the exception of AtSec12, each antiserum recognized a single polypeptide in both subcellular fractions. This polypeptide corresponded to the expected molecular mass for the protein in question: 87 kDa for AtSec23, 33 kDa for AtSec13, 22 kDa for AtSar1. For AtSec12 a polypeptide of around 42 kDa was detected only in the membrane fraction, as expected for a type I integral memnbrane protein (Bar-Peled and Raikhel, 1997). Because our microscopical investigations were to be performed on BY-2 cells expressing various fluorescent ER and Golgi (X) FP constructs, we also prepared protein gel blots from fractions isolated from BY-2 cells. The same polypeptides were identified in both fractions, however based on relative concentrations of applied proteins the signals were estimated to be 40-50% weaker in the case of the BY-2 antigens. Interestingly, the AtSec12 antiserum consisently failed to cross react with membrane preparations from BY-2 cells. Since a complete sequence for this protein from tobacco is not available we cannot provide a reason for this.

We also tested the COPII antisera on fractions collected from a linear sucrose density gradient of Arabidopsis membranes to verify their association with the ER. As seen in Fig.3.20. B AtSec12, AtSec13, and AtSar1 have distribution profiles very similar to the ER marker calnexin, and are clearly different to that of the Golgi marker reversibly glycosylated polypeptide (RGP). These results are consistent with our previous demonstration that the behaviour of AtSec23-bearing membranes from cauliflower inflorescence in response to Mg^{2+}-ions is typical for ER in sucrose gradients.

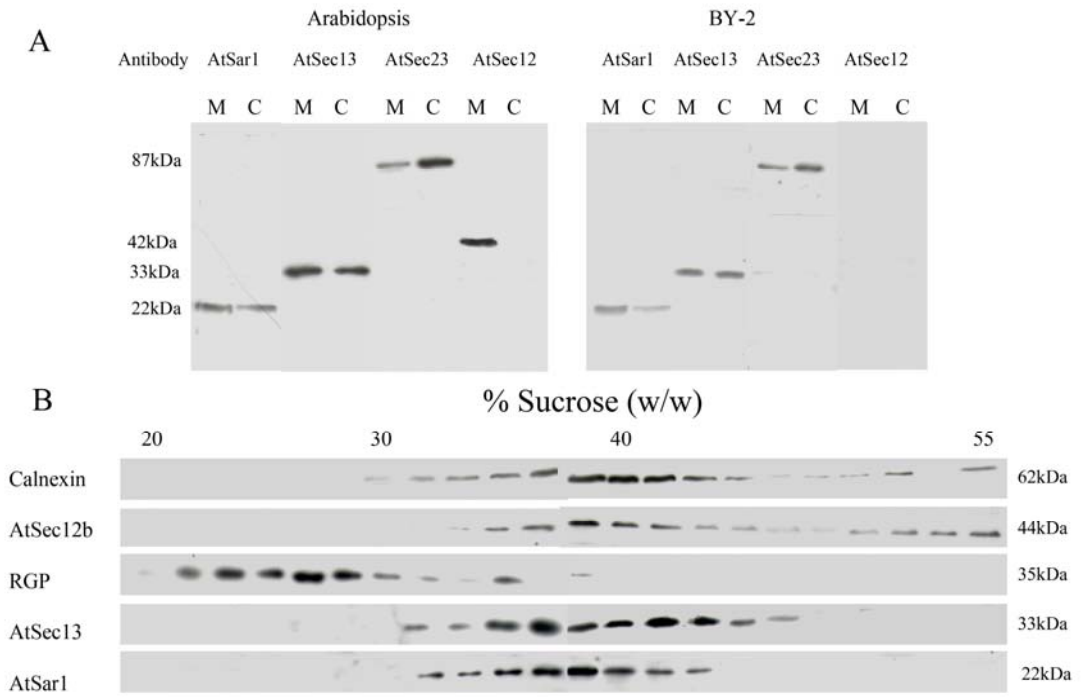

Fig. 3.20. Cross-Reactivities of Antisera raised against Recombinant Arabidopsis COPII Proteins.

(A) Cytosolic proteins and total membranes were isolated from the suspension cultures of Arabidopsis and tobacco BY-2 cells as described in Methods, and probed with the antisera indicated. Equal amounts of protein were applied to the lanes in each gel blot (20 µg per lane for the Arabidopsis gel; 30 µg per lane for the BY-2 gel). M, membrane; C, cytosol.

(B) Arabidopsis total cell membranes separated on a linear isopycnic sucrose density gradient (as described in Methods). Individual fractions were probed with COPII antisera and with standard antisera for ER (calnexin), and Golgi (RGP) marker proteins.

3.3.2. COPII immunostaining in BY-2 cell lines expressing fluorescent ER and Golgi markers

Immunofluorescence labeling of wild type and transgenic BY-2 cells with COPII antisera gave rise in all cases to a punctate pattern (Fig. 3.21.). A similar picture was produced with all three antisera: anti-AtSec23 (Fig. 3.21. A, K), anti-AtSec13 (Fig. 3.21.B), and anti-AtSar1 (Fig. 3.21. E, H, M). The signal density was lower in median sections (Fig. 3.21. A-C) than in optical sections through the cell cortex parallel to the cell surface (Fig.

3.21.D-M), presumably due to the higher incidence of ER in surface view. Colocalization of labeling when using any two of the three COPII antisera lay regularly between 50-60%, but not higher. This may be due to the fact that ERESs are continually being formed, and that at any one moment a number will have recruited only Sar1, some the Sec23/24 dimer in addition, and others the Sec13/31 dimer as well. Also, we cannot exclude the possibility that, due to the existence of multiple isoforms of COPII coat proteins which can be found in the Arabidopsis data base, not all ERESs in BY-2 cells can be equally well monitored with our antisera.

In BY-2 cells expressing the Golgi marker GmMan1-GFP, ERESs - as seen by immunofluorescence labeling with AtSar1- greatly outnumber Golgi stacks, especially in cortical optical sections (Fig. 3.21. D-F). Thus, using the same threshold settings, ERES density was found to be approximately 3.9, 4.4 and 3.5 ERESs/µm2 in Figure 3.21. F, I and L, respectively. By comparison, Golgi stacks had a density of 1.5 /µm2 in Figure 3.21F. ERESs sized 540 ± 87; 558 ± 115 and 512 ± 83 (average diameter in nm, n>100) are also significantly smaller than Golgi stacks (average diameter = 880 ± 82 nm, n=77). Visualized against a background of the ER in BY-2 cells expressing GFP-HDEL, ERESs do not appear to be preferentially associated with cisternal as against tubular portions of the cortical ER network (Fig. 3.21. G-L). At high magnifications, individual punctate fluorescent signals are often found sitting directly on strands of ER (see arrows in Fig. 3.21 M). Using the same parameters for the estimations obtained from Figure 3.21.F, the average diameter of these signals is 456 ± 9 nm (n=43). Interestingly, and in agreement with the depiction of ERESs in mammalian cells (Rust et al., 2001), the colocalization (yellow) of green (ER) and red (Sar1) signals, is restricted to narrow semi-circular profiles between the ER and the ERESs. Punctate fluorescent signals of a similar size were also seen lying adjacent to the ER strands. These could represent either ERESs out of the plane of section, or individual released COPII vesicles, as suggested by Rust et al., (2001)

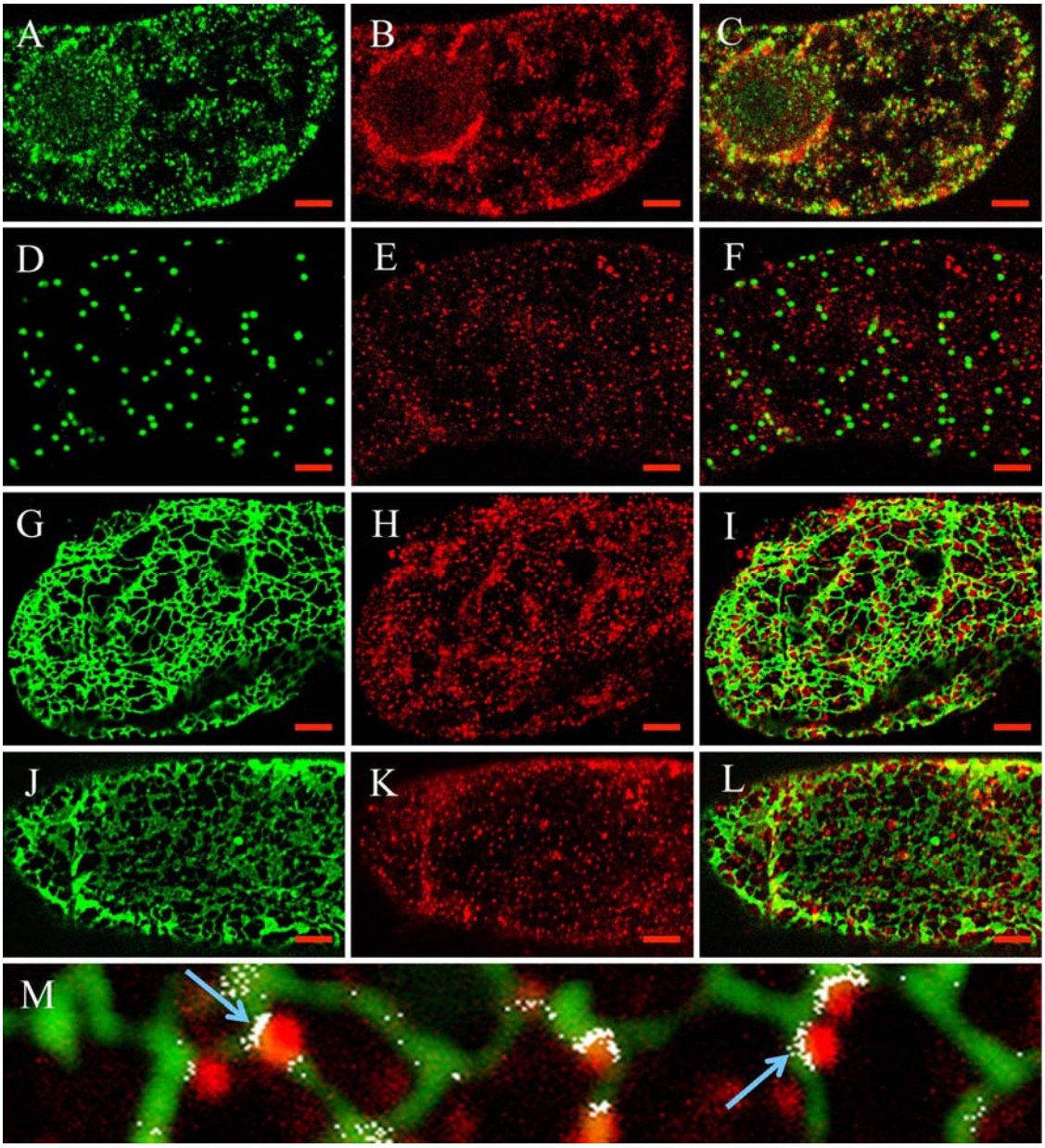

Fig. 3.21. Confocal immunofluorescence images of BY-2 cells labeled with AtCOPII antisera.

(A) Sar1 staining, (B) Sec13 staining, (C) Merge image of A and B; median optical section, wild type cell. (D) Golgi stacks visualized in GmManI-GFP transformed cell; cortical section, (E) Sar1 staining of cell in D, (F) Merge image of D and E. (G) Cortical ER visualized in GFP-HDEL transformed cell, (H) Sar1 staining of cell in G, (I)Merge image of G and H. (J) Cortical ER in GFP-HDEL transformed cell, (K) Sec13 staining of cell in J, (L) Merge image of J and K. (M) High magnification of Cortical ER (green: GFP-HDEL) with ERESs as visualized with anti-Sar1 (red; colocalisation indicated in white). (Scale bar: 5µm).

3.3.3 Establishment of a BY-2 cell line expressing Sec13-GFP

In order to visualize and study the dynamics of ERESs in living cells we transformed BY-2 cells with LeSec13-GFP under the control of an inducible promoter. Beginning roughly 24 h after exposure to dexamethasone the BY-2 cells were expressing the fusion construct in suffiecient quantities for confocal microscopy. In optical sections taken through the cortex (Fig. 3.22 A) a dense punctate image was obtained, not unsimilar to the antibody staining with COPII antisera (Fig. 3.21). When cells expressing the GFP-fusion construct were fixed and immunostained with AtSec13 antibodies, almost a perfect colocalization was obtained (Fig. 3.22 E-G), confirming the Sec13 identity of the protein carrying the GFP signal. In contrast to mammalian cells where ERESs are no longer formed on the ER during mitosis and the Golgi apparatus fragments (Lowe et al., 1998; Roth, 1999), the Sec13-GFP signal in mitotic BY-2 cells is clearly seen to aggregate in and around the nuclear spindle (Fig. 3.22C), whereas Golgi stacks remain dispersed throughout the cytoplasm (Fig. 3.22 D).

Median optical sections from BY-2 cells expressing LeSec13-GFP reveal, in addition to punctate fluorescence throughout the cytoplasm, an intense staining of the nuclear envelope and a diffuse staining of the nuclear matrix except for the nucleoli (Fig. 3.22 B). This, for us initially unexpected result, is fully in keeping with the known behavior of Sec13 in other eukaryotic cells. In yeast it has been shown that, in addition to being a COPII coat protein, Sec13p is also incorporated into the nuclear pore complex Nup84p (Siniossoglou et al., 2000). Moreover, in mammalian cells Sec13 has recently been shown to shuttle between the cytosol and nuclear matrix (Enninga et al., 2003). We have performed on the Sec13-GFP BY-2 cell line the same kind of FRAP experiments that were carried out by the latter authors, and have obtained identical results (Fig. 3.23). After photobleaching, the Sec13-GFP signal in the cytoplasm recovers within 1-2 minutes (Fig. 3.23 C), whereas the nuclear envelope-associated signal is first seen after roughly 1 h (Fig. 3.23 D-F). In contrast, the recovery of the nuclear matrix Sec13 signal takes about 10 min (Fig. 3.23 H-J). These results yet again underline the credibility of the fluorescence signal we obtained with the LeSec13-GFP construct.

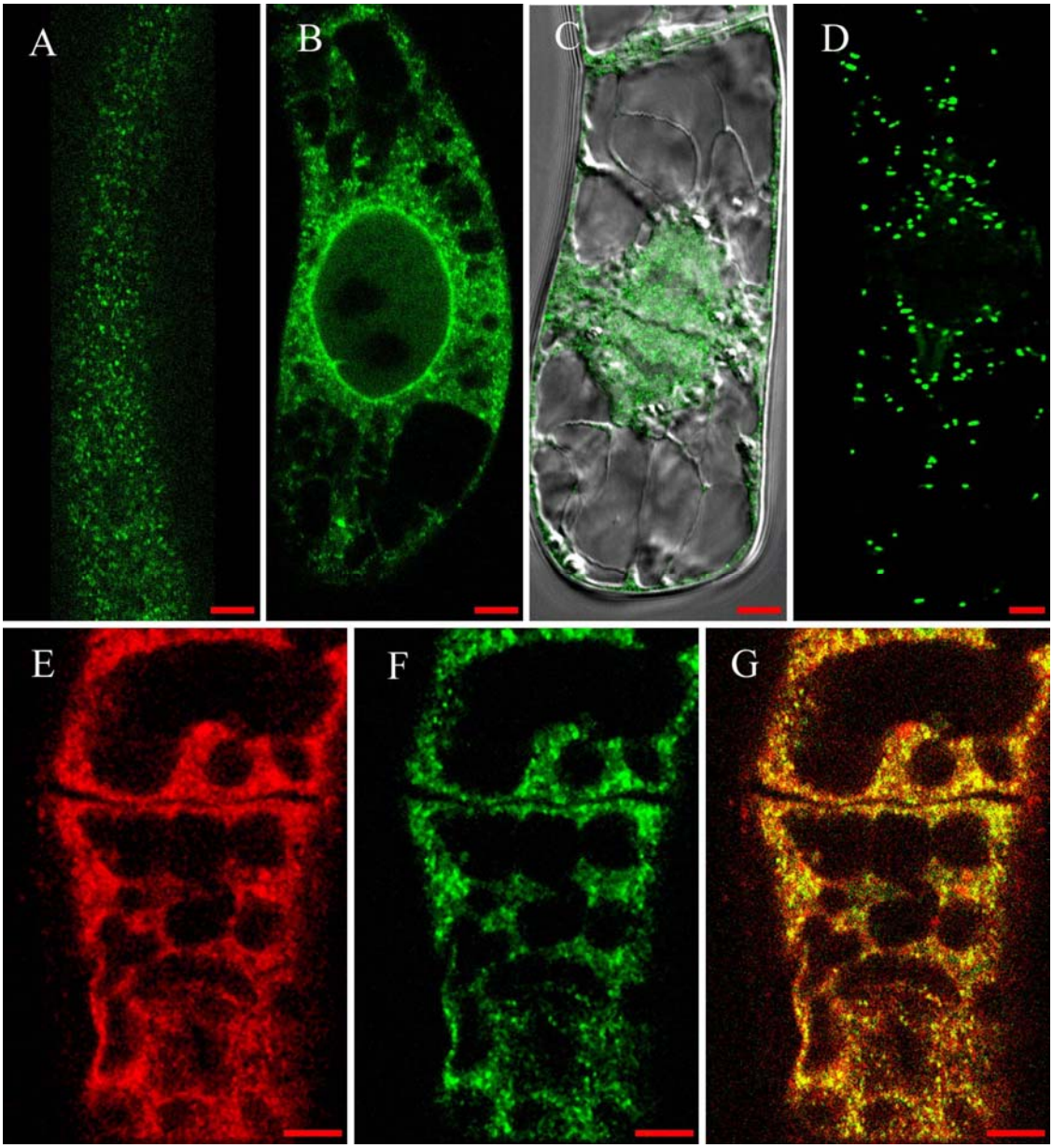

Fig. 3.22. Visualization of ERESs in BY-2 cells expressing LeSec13-GFP.

(A) Punctate GFP signal in cell cortex. (B) Median optical section revealing, a disctinct staining of the nuclear envelope and nuclear matrix, in addition to punctate signals in the cytoplasm. (C) LeSec13-GFP fluorescence pattern in a mitotic cell (fluorescence image superimposed on DIC image). (D) Distribution of Golgi stacks (GmManI-GFP) in a mitotic BY-2 cell. (E-G) Antibody staining of LeSec13 in LeSec13-GFP cell line, E- GFP signal, F- immunofluorescence labeling, G- merged image. (Scale bar: 5µm).

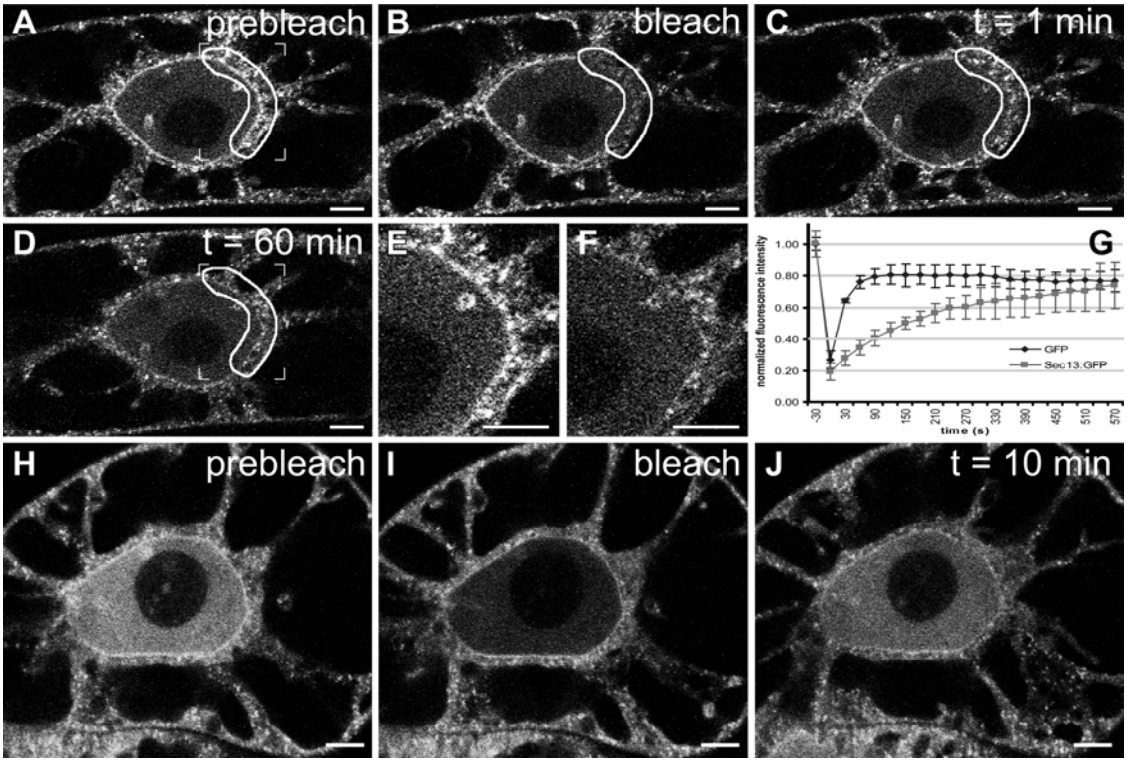

Fig. 3.23. FRAP experiments on BY-2 cells expressing LeSec13-GFP

(A-F) FRAP analysis of BY-2 cell expressing LeSec13-GFP where the nuclear envelope, ER, and cytosol were bleached and fluorescence recovery was followed. (E and F) High magnifications of two regions in A and D respectively. (G) Plots show fluorescence recovery of nuclear Sec13-GFP in the bleached area. (H-J) FRAP analysis pf BY-2 cell expressing LeSec13-GFP. The nucleus was bleached, and the fluorescence recovery in the nucleus was monitored. (Scale bar: 5µm).

3.3.4. ERESs in relation to the ER and Golgi in living BY-2 cells

Since we monitor ERESs in living BY-2 cells with LeSec13-GFP, simultaneous fluorescence imaging of the ER and Golgi apparatus can only be performed with YFP- or RFP-constructs. We chose the latter, because of their better spectral separation, and prepared BiP-RFP and GmManI-RFP as ER and Golgi markers respectively. The validity of BiP-RFP as an ER marker was confirmed by bombarding transgenic BY-2 cells expressing GFP-HDEL. The typical cortical ER network was revealed by both fluorescent markers and showed complete colocalization (Fig. 3.24 A-C). When the inducible LeSec13-GFP cell line was bombarded with BiP-RFP the green ERESs are highlighted against the tubular ER network, however the density of the punctate Sec13-GFP signals is such that an extensive yellow merge image was obtained over much of the ER (Fig. 3.24 D-F).

In order to confirm the validity of GmManI-RFP as a Golgi marker, we bombarded transgenic BY-2 cells expressing GmManI-GFP with GmManI-RFP. Again a perfect colcalization with both Golgi marrkers was obtained (Fig. 3.24 G-I). The LeSec13-GFP cell line was then bombarded with GmManI-RFP in order to visualize ERESs and Golgi in living cells. Optical sections in the cortical region of a cell are given in Fig. 3.24 J, K). As with the antibody staining data presented above, ERESs greatly outnumbered Golgi stacks (Fig. 3.24. L). Some of the Golgi stacks were densely surrounded at their periphery with ERESs giving rise to a kind of halo (see the group of Golgi stacks in the middle of Fig. 3.24 L, and also Fig. 3.24 N), whereas others were relatively free (Fig. 3.24. M).

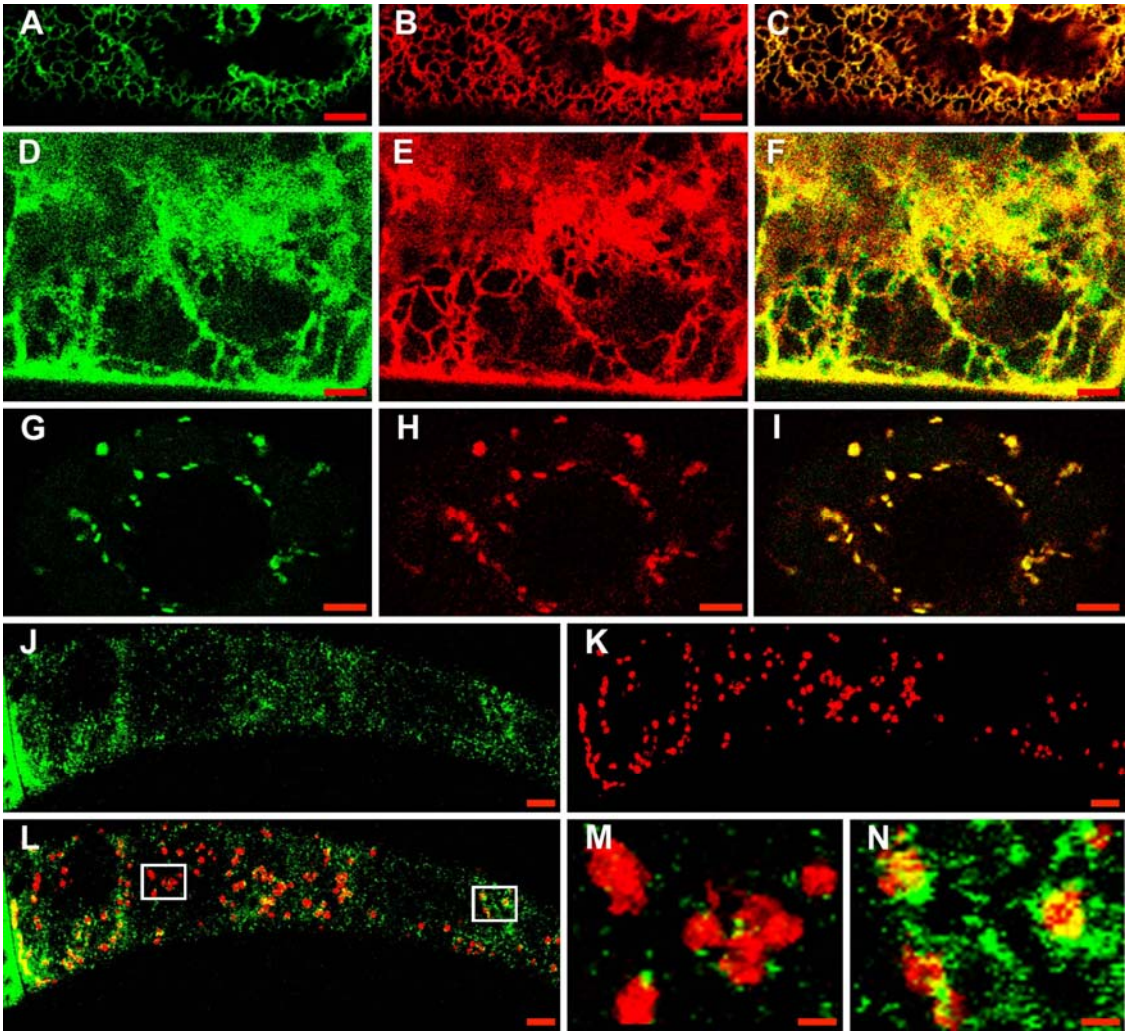

Fig. 3.24. Labeling of ER and Golgi in BY-2 cells expressing AtSec13-GFP.

(A) Cortical ER in a cell expressing HDEL-GFP which was bombarded with BiP-FRP; channel selected for green fluorescence (B) The same cell as in A, but channel selected for red fluorescence. (C) Merge image for the cell depicted in A and B. (D) An AtSec13-GFP expressing cell bombarded with BiP-FRP; channel selected for red fluorescence. (E) The same cell as in D, but channel selected for green fluorescence. (F) Merge image for cell depicted in A and B. (G) Golgi stacks in a cell expressing GmManI-GFP which was bombarded with GmManI-RFP; channel selected for green fluorescence. (H) The same cell as in G, but channel selected for red fluorescence. (I) Merge image for cell depicted in G and H. (J) ERESs in a cell expressing AtSec13-GFP which was bombarded with GmManI-RFP, channel selected for green fluorescence. (K) The same cell as in J, but channel selected for red fluorescence. (L) Merge image for cell depicted in J and K. (M, N) High magnifications of two regions in L showing high and low density associatiion of ERESs with Golgi stacks. (Scale bar: 5µm).

3.3.5. Mobile Golgi stacks collect ERESs at their rims

In order to analyze the relationship between ERESs and Golgi stacks at greater resolution we have observed live BY-2 cells expressing LeSec13-GFP and GmManI-RFP by dual wavelength microscopy in a confocal microscope equipped with Nipkov spinning disc optics. The results are given in the form of a movie Fig.3.25 (B) of 183 sec duration, from which three frames (0 sec, 61 sec and 183 sec) are presented in Fig. 3.25 (A). Three features are immediately apparent from these sequences. First, and in confirmation of data already presented, ERESs greatly exceeded Golgi stacks in number. Second, in confirmation of the observations of Nebenführ et al. (2001), not all Golgi stacks were simultaneously in movement. Thirdly, Golgi stacks were more often encountered with peripherally associated ERESs than not. Nevertheless, there are clear examples where a single Golgi stack moves into the plane of vision without associated ERESs, but then a few seconds later seen to be completely surrounded by them (compare frames in the third row of Fig. 3.25 B-C). We have also seen examples where a single Golgi stack, immobile for a period of many seconds, was observed with and without its associated ERESs (Figs. 3.25 B –C).

In surface view (i.e. looking at a stack from above or below) Golgi-associated ERESs appeared in the form of a partial or complete corona (see Fig. 3.25. B-C). Rarely did we find images where the GFP and RFP signals were superimposed. Golgi stacks were also frequently seen in side view where the RFP signal took the form of a cigar. Very often however, the signal was only partially visible as red, and more often was yellow: the merge color. This observation indicates that ERESs-Golgi interactions take place at the rim(s) of the Golgi cisternae rather than at their faces. These results also show that Golgi stacks do not have a fixed orientation with respect to the ER: as already reported by Nebenführ et al. (2001) they tumble as they move.

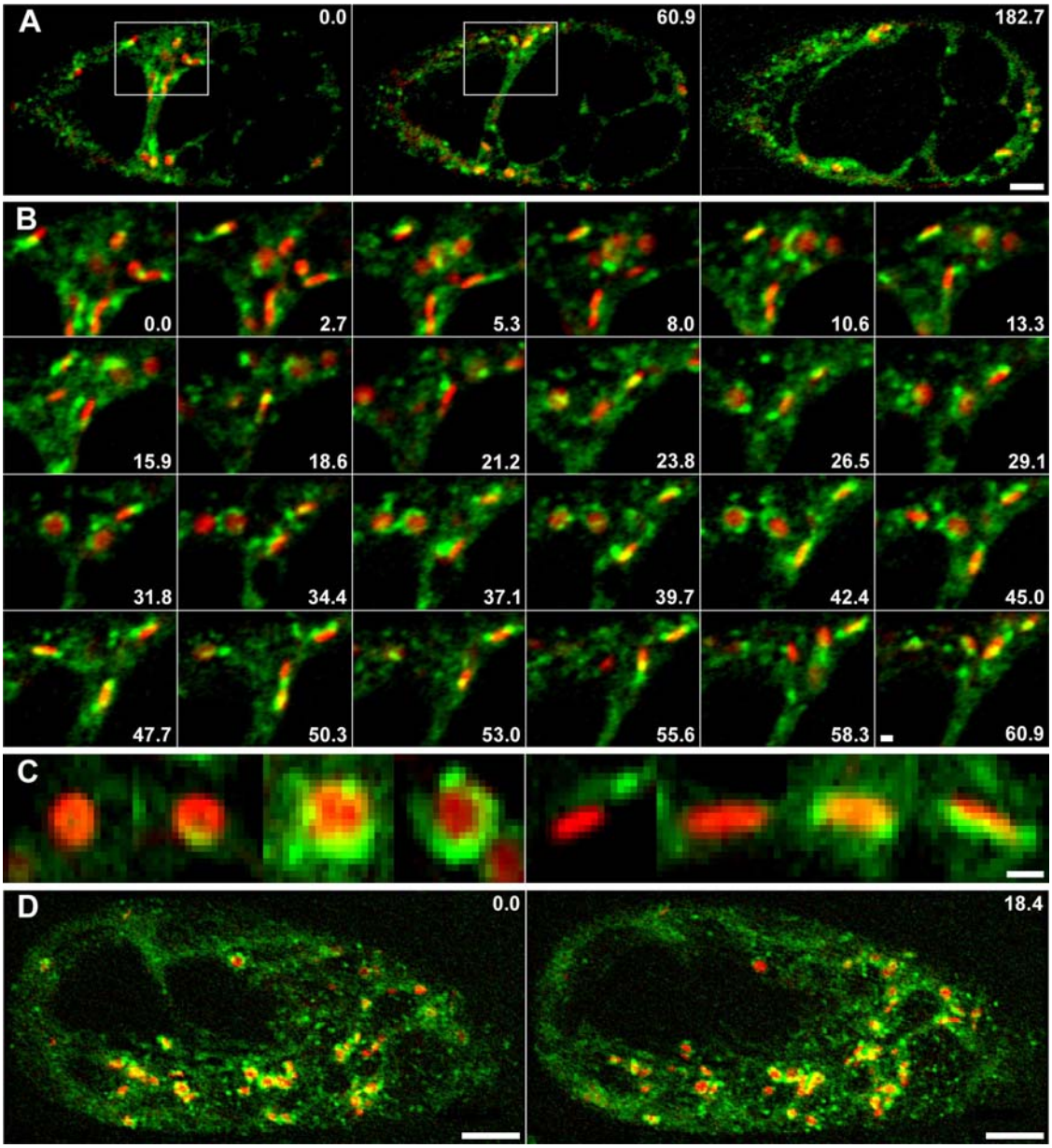

Fig. 3.25. Mobile Golgi stacks collect ERESs at their rims.

(A) Dual wavelength microscopy movie of the BY-2 cell line expressing LeSec13 and ManI-RFP, three frames (0 sec, 61 sec and 183 sec) are presented. (B) The detail sequences of the movie of the regions which was indicated with a square in the Fig. 3.25 A. (C) Gallery of the different status and position of the Golgi with respect to the LeSec13-GFP (Top views left, side views right). (D) In depth (250µm) projection a BY-2 cell follow at two time points. Note that the Golgi stack located centre in D) has not moved, but is visualized with and without associated ERESs.

3.3.6. Preliminary Characterization of ERESs

Perturbation of the early secretory pathway can be achieved by treating cells with the drug brefeldin A, which rapidly diminishes COPI from membranes by preventing their rebinding once released (Scheel et al., 1997; Peyroche et al., 1999). As a consequence, membrane fusion between the Golgi and the ER becomes indiscriminate and leads to the redistribution of the greater part of the Golgi apparatus into the ER (Nebenführ et al., 2002). Because it has been claimed that, in addition to its effect on the Golgi apparatus, BFA may also act at the level of ER export in plants (see Brandizzi et al., 2002; Hawes, 2004), we decided to see what happens to ERESs in BY-2 cells after the addition of BFA. As shown in Fig. 3.26 (compare A, B, C with D, E. F) BFA induced the redistribution of the Golgi apparatus in cells expressing LeSec-13-GFP and GmMani-RFP into the ER. However, no change in Sec13 fluorescence was observed. Visualization of ERESs through AtSec13 antibody staining produced the same results (Fig. 3.26 G-I). In this case we looked at BY-2 cells after 30 min of BFA treatment, at which time ER-Golgi hybrid structures can be observed (Ritzenthaler et al., 2002). Again, the typical punctate antiSe13 staining was recorded, indicating that ERESs are still present at a time after considerable BFA-induced morphological changes in the Golgi apparatus have taken place.

It has been established that the molecular target for this drug is a complex formed between the GTPase ARF (ADP ribosylation factor) and its guanidine exchange factor (GEF) (Peyroche et al., 1999). Although there are several ARFs, and some ARF-GEF complexes are resistant to BFA and can make up for multiple sites of action within any given cell and differences between cell types (Geldner 2004), it is generally recognized that the immediate consequence of BFA action is an inhibition of vesicle budding by preventing Arf-mediated coat protein recruitment.

Actin filaments play an important role in directing the movement of the Golgi bodies (Boevink et al., 1998; Nebenführ et al., 1999). Golgi stops moving upon the addition of actin depolymerizing agents latrunculin. To see the effect of this drug on the ERESs, we treated the LeSec13-GFP cell line expressing ManI-RFP with 25µM Latrunculin B for 60 min (Fig. 3.26 J-O). The results show that the ERESs were not significant affected when Golgi movement was halted.

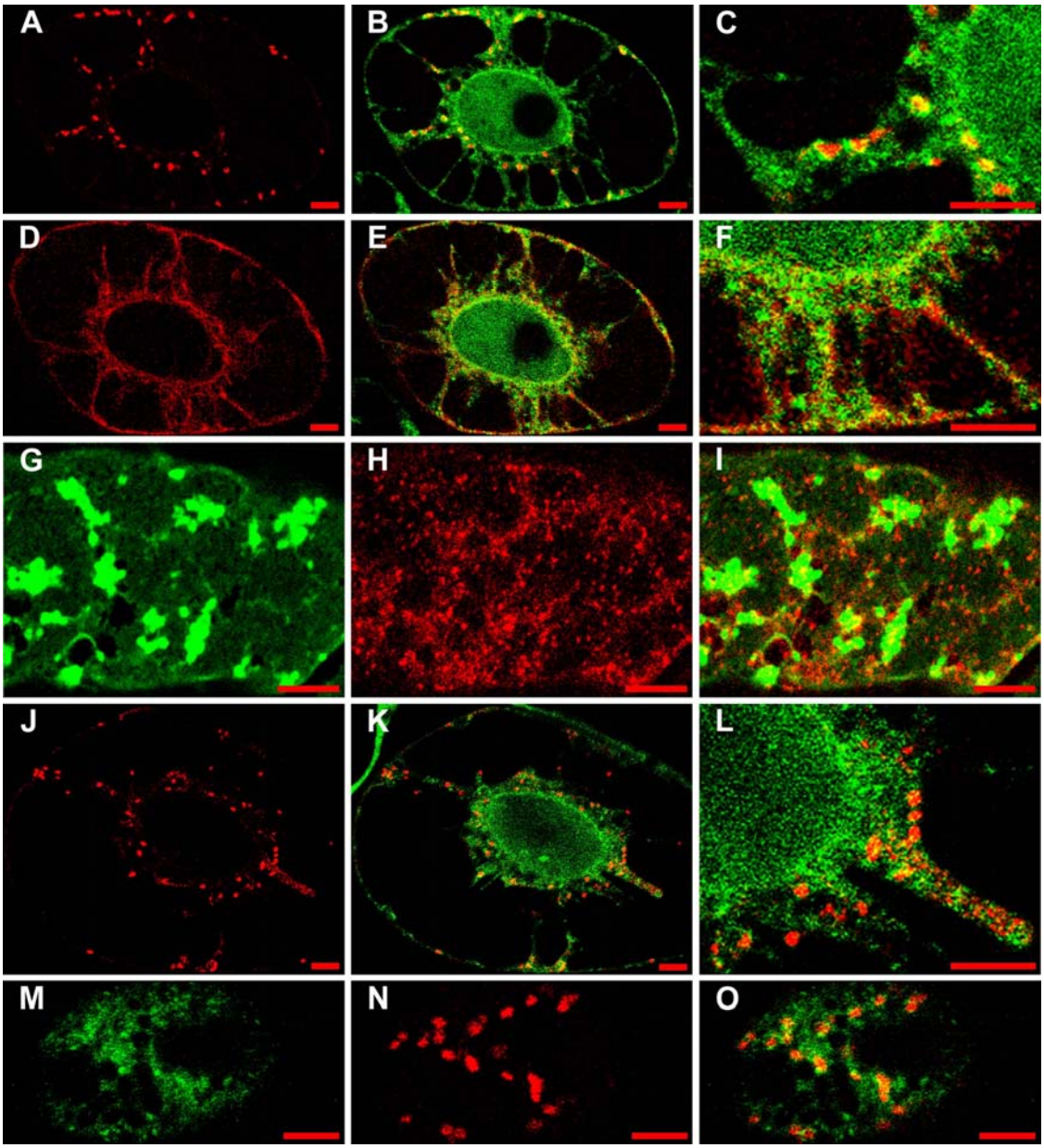

Fig. 3.26. The effect of BFA and Latrunculin on the ERESs.

(A-C) LeSec13-GFP cell line expressing ManI-RFP without BFA treatment. (D-F) LeSec13-GFP cell line expressing ManI-RFP with 10 µm BFA treatment for 30 min, ManI-RFP is redistributed to the ER. (G-I) Anti-Sec13 immunolabeling of GFP-HDEL cell line after 30 min treatment with 10 µm BFA. (J-L) LeSec13-GFP cell line expressing ManI-RFP with 25µM Latrunculin B for 60 min. (M-O) Cortical view of J-L.

ER to Golgi transport can be blocked by Sar1 (T34N) GDP form mutant (Andreeva et al., 2000). Here we used Sp-RFP and ManI-RFP to monitor the effect of the Sar1 (T34N) in the LeSec13-GFP cell line. Fig. 3.27. A-C shown that Sp-RFPs are mainly accumulated in the vacuole in LeSec13-GFP BY-2 cell line without the present of the Sar1 (T34N). When the cells were co-bombarded with NtSar1 the ER to Golgi transports were blocked. The Sp-RFP (Fig.3.27 D-F) and ManI-RFP (Fig. 3.27 G-J) were accumulated in the ER. As a consequence of the blockage of the Sar1-GDP mutant, the ERESs were partial loss (Fig. 3.27 D and G compare with Fig. 3.22. A and B).

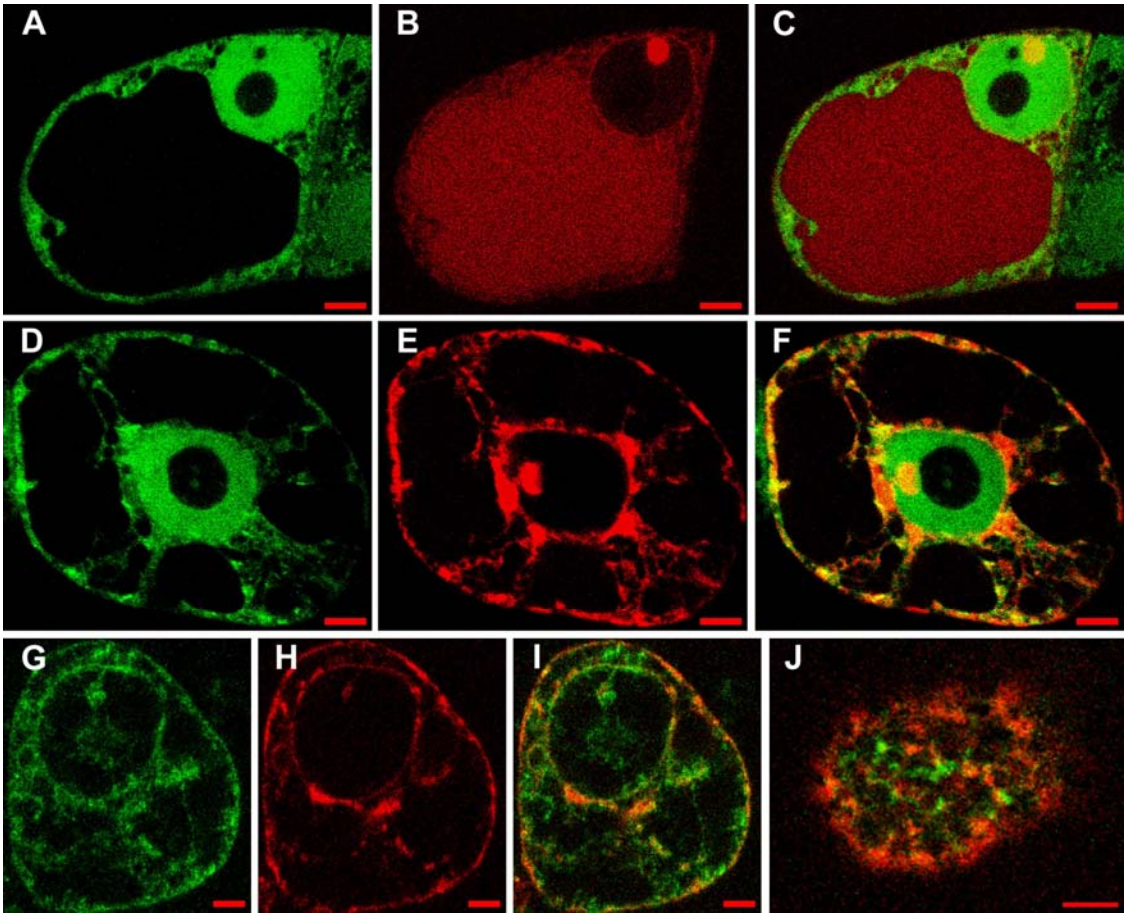

Fig. 3.27. The effect of Sar1 mutant on the ERESs.

(A-C) LeSec13-GFP BY-2 cell line expressing Sp-RFP. RFPs are mainly accumulated in the vacuole. (D-E) LeSec13-GFP BY-2 cell line expressing Sp-RFP co-bombarded with NtSar1-GDP mutant. (G-I) LeSec13-GFP BY-2 cell line expressing ManI-RFP co-bombarded with NtSar1-GDP mutant and (J) is the cortical view.

4. DISCUSSION

4.1. Induction and isolation of plant COPII vesicles

The secretory apparatus of eukaryotic cells comprises a dynamic membrane system with capabilities for bi-directional transport. The movement of newly synthesized proteins through the cell's secretory system involves several specific cycles of membrane vesicle budding and fusion (Rothman and Wieland, 1996; Schekman and Orci, 1996; Jahn and Südhof, 1999). Intracellular transport between early compartments of the secretory pathway therefore relies on a series of protein-sorting events that are accomplished by coat protein complexes. Three major classes of coated vesicles (COPII COPI and CCV) have been identified and characterized from yeast and mammalian cells and have been shown to act as carriers mediating uni- or bi-directional transport between adjacent membranes in the secretory pathway (Rothman and Wieland, 1996; Nickel and Wieland, 1997; Lippincott-Schwartz et al., 1998; Pelham and Rothman, 2000; Kirchhausen, 2000).

COPII components and COPII-coated vesicles were originally discovered in baker's yeast (*Saccharomyces cerevisiae*) using genetic approaches coupled with a cell-free assay monitoring the transfer of a marker protein from the ER to the Golgi (Novick et al., 1980; Baker et al., 1988; Salama et al., 1993; Barlowe et al., 1994). Vesicular transport was reconstituted in vitro using five cytosolic proteins: the small GTPase Sar1p, the Sec23p/24p complex, and Sec13p/31p complex (Salama et al., 1993; Barlowe et al., 1994). This vesicle budding assay has been widely used to monitor the transport from ER to Golgi (Baker et al., 1988), to purify the vesicles (Barlowe et al., 1994; Matsuoka et al., 1998a; Otte et al., 2001), and to characterize the protein-sorting mechanism into the vesicle (Matsuoka et al., 1998b; Otte et al., 2001; Sato and Nakano, 2004). However, these tools were previously not available for the plant field.

How to monitor the budding event in vitro is the central question for setting up such an assay using plant cell as a experimental system. Basically, three groups of proteins may be used to monitor the budding event in vitro: (1) COPII coat components (AtSar1,

AtSec13/31 complex and AtSec23/24 complex), these are cytosolic proteins and also associate with ER. However, these proteins can form aggregations during the incubation. As can be seen in Figure 3.7 AtSec13 is also detected in the HSP when only cytosol is present in the budding mixture. Thus, vesicle budding and protein aggregation can not be directly distinguished from one another by high speed centrifugation. (2) Cargo molecules like the α-factor precursor in yeast and vesicular stomatitis virus glycoprotein (VSV-G) in mammalian cells are widely used for monitoring in vitro vesicle budding and the reconstitution of the ER to Golgi transport (Baker et al., 1988; Rexach and Schekman 1991; Aridor et al., 1998). However, comparable cargo molecules have not yet been used in plant cell research. Moreover the mechanism of cargo and COPII interaction in plant cell is still unclear. (3) The ER/Golgi SNAREs (Bet1 Bos1 and Sec22) are membrane proteins and are sorted into COPII vesicles during the COPII nucleation process (Mossessova et al., 2003; Springer and Schekman 1998; Miller et al., 2003; Matsuoka et al., 1998b). In particular two ER to Golgi v-SNAREs, Bet1p and Bos1p which also exist in plants, have been shown to interact specifically with the COPII components Sar1p, Sec23p, and Sec24p, in a guanine nucleotide-dependent fashion (Springer and Schekman 1998). Analysis of fusion proteins suggests that the cytoplasmic domain of the v-SNARE protein Sec22p is required for its packaging into ER-derived COPII vesicles (Campbell and Schekman 1997). Sec22 is therefore concentrated into synthetic COPII vesicles during the in vitro vesicle budding (Matsuoka et al., 1998b). The data from this study show that ER is the principle subcellular location for both AtBet1 and AtSec22 (Figure3.2). Moreover, the presence of these two proteins, but not the translocon protein AtSec61, in the HSP is strongly supportive of their incorporation into a putative plant COPII vesicle.

In agreement with previous studies on vitro vesicle budding in yeast (Baker et al., 1988; Rexach and Scheman 1991; Barlowe et al., 1994), our results also show that GTP or its analogs together with an ATP regeneration system (Rexach and Scheman 1991) are required to promote vesicle budding in reactions that contain crude cytosol as a source of COPII proteins. The production of transport vesicles being monitored by the presence of AtSec22 (figure 3.8). The ATP requirement seen when a crude cytosolic protein fraction

is used to drive vesicle budding may be explained by the replenishment of micromolar levels of GTP that are consumed by abundant nucleotide hydrolases in cytosol (Barlowe et al., 1994).

Sar1p is a 21 kDa GTPase that is activated by GDP/GTP exchange on the cytosolic face of the ER by Sec12p, an integral membrane glycoprotein (Nakano et al., 1988; d'Enfert et al., 1991; Barlowe and Schekman 1993). Sar1p activation is the first and essential step for COPII vesicle formation, but how Sar1 binds to the ER and subsequently recruits the Sec23/24p and Sec13/31p complexes is not yet fully clear. Although protein-protein interactions between Sar1 and Sec23/24 complex play a important in this process, a protein-lipid interaction may also play a key step according to the recent observation that the activation of phosholipase D (PLD) by the small GTPase Sar1p is required to support COPII assembly and ER export (Pathre et al., 2003). PLD, which catalyzes the formation of phosphatidic acid (PA), may provide the required elevation in acidic lipid composition in the ER sub-domain. It is also known that PLD may play a role in enhancing PI(4,5)P2 production by generating PA, a reported activator of PI(4)P5-kinases (Brown et al., 1993; West et al., 1997; Arneson et al., 1999). Arabidopsis AtSar1p has been shown to be functionally similar to yeast Sar1p (Bar-Peled and Raikhel 1997) and Sar1 mutants also block the ER to Golgi transport (Takeuchi et al., 2000). Figure 3.5 shows that AtSar1p is present in the cytosol at a low level and becomes a limiting factor for in vitro vesicle budding, because the addition of recombinant NtSar1A enhances vesicle budding (Figure 3.9). In addition, exogenous AtSar1p from an AtSar1p overproducer cytosol can also enhance the in vitro vesicle budding (Figure 3.11).

Sec12 is a GEF for Sar1p. It contains an N-terminal, cytoplasmically exposed domain that facilitates nucleotide exchange on Sar1p (Nakano et al., 1988; d'Enfert et al., 1991; Barlowe and Schekman 1993). Sec12 is an ER membrane protein, and does not enter the COPII vesicle during vesicle budding (Matsuoka et al., 1998a). It may therefore be used as a control marker for the in vitro budding assay. Figure 3.7 shows that when donor membranes are removed by centrifugation AtSec12 is not detected in the HSP, and this was confirmed by another marker Sec61, the translocon (Figure 3.7). In yeast,

overexpression of Sec12p reduces ER export, presumably via the titration of Sar1p (d'Enfert et al., 1991) and the transient overexpression of AtSec12 in tobacco protoplasts resulted in the recruitment of the GTPase to the ER (Phillipson et al., 2001). However, the stable overexpression of AtSec12 in transgenic plants does not affect cell viability (Bar-Peled and Raikhel 1997). One likely explanation would be that plant cells contain regulatory mechanism to respond to an imbalance in the AtSec12/AtSar1p ratio (Phillipson et al., 2001). Under these conditions, AtSec12 overexpression temporarily inhibits ER to Golgi transport by preventing the COPII vesicle formation through the depletion of AtSar1p. Our in vitro data show that AtSec12 has a positive effect for in vitro vesicle budding when more AtSar1p is available (Figure 3.11). Actually, in the AtSec12 stable transformed cell line, more AtSar1p was recruited on the ER membrane. This indicates that some Sar1p is indeed bound by AtSec12 (Figure 3.10). Another interesting observation is that the COPII component AtSec13 is also increased in both cytosol and microsome fractions from the Sar1p and Sec12 stable overexpressing Arabidopsis cell lines (Figure 3.10). Whether this indicates an up-regulation of the ER to Gogli transport system is not clear. Further experiments are needed to clarify this point.

The functional complex for Sec13/31p is about 700 kDa and 200 kDa for Sec23/24p. The availability of these two complexes in the cytosol is another factor that affects the in vitro budding assay. When using cytosol from BY-2 cell for the in vitro budding assay, no vesicle budding was observed (preliminary data Takeuchi and Yang). The reason for this is that the Sec13/31p complex was dissociated or degraded (Figure 3.6). In contrast, using Arabidopsis cytosol prepared in the identical way to BY-2 cytosol does lead to successful vesicle formation (Figure 3.7) presumably because it contains both functional complexes (Figure 3.6).

The COPII vesicle has been characterized as a 60 nm vesicle in yeast (Barlowe et al., 1994). However, the vesicle we have isolated is about 50 nm in diameter. A database search reveals that there are two Sec31 homologues in Arabidopsis and their predicated molecular weights predication indicate that they are one third smaller than yeast Sec31. This may probably the reason why COPII vesicles from Arabidopsis are smaller.

4.2. COPII binding studies

The family of p24 proteins have been shown to localize to the early secretory pathway in mammalian cells, i.e. the ER, the intermediate compartment and the Golgi complex (Dominguez et al., 1998, Stamnes et al., 1995, Sohn et al., 1996, Rojo et al., 1997, Füllerkrug et al., 1999, Gommel et al., 1999, Emery et al., 2000) and to cycle constitutively between these membranes (Füllerkrug et al., 1999, Gommel et al., 1999, Nickel et al., 1997, Rojo et al., 2000). P24 proteins have also been shown to be major constituents of COPI- (Stamnes et al., 1995, Sohn et al., 1996, Gommel et al., 1999) and COPII- (Belden and Barlowe 1996, Schimmöller et al., 1995) coated vesicles, and their cytoplasmic tails to contain signals for anterograde and retrograde transport, to facilitate their bidirectional movement in the early secretory pathway (Fiedler et al., 1996, Dominguez et al., 1998, Belden and Barlowe 2001, Füllerkrug et al., 1999, Nickel et al., 1997, Goldberg 2000). Some members of this family have been proposed to act in the donor membrane as cargo receptors in their lumenal side and coatomer and/or ARF receptors in their cytoplasmic side (Aniento et al., 2003, Belden and Barlowe 1996, Schimmöller et al., 1995). The p24 composition of COPI-coated vesicles reflects also the ability of p24 proteins to form heterooligomeric complexes and may confer upon them the ability to cycle through the early secretory pathway (Marzioch et al., 1999, Jenne et al., 2002). 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.

p24 proteins have hardly been investigated in plant cells. However, several members of this family are characterized by the presence of two hydrophobic residues (mostly phenylalanine or tyrosine) in the -7,-8 position and a dilysine motif in the -3,-4 position (with respect to the cytosolic C-terminus), which have been previously shown to cooperate in binding both ARF1 and coatomer (Contreras et al., 2004). Belden and Barlowe (2001) showed binding of purified COPI and COPII subunits to the cytoplasmic

tail of two p24 proteins in yeast, Emp24p and Erv25p. While binding of COPII subunits depended on a pair of aromatic residues found in the -7,-8 position of both tail sequences, COPI binding also required the presence of a dilysine motif found in the Erv25 cytosolic tail (but not in the Emp24p tail). In those experiments, both the Sec23/24p complex and the Sec13/31p complex bound to the peptides, either when the COPII subunits were added individually or in a mixture. However, when using individual subunits at varying concentrations, the Sec13/31p complex displayed the highest binding affinity, followed by Sar1p and the Sec23/24p dimer. Under our assay conditions, and using a cytosolic fraction as a source of COPII subunits, we found that only Sec23 (presumably as part of the Sec23/24 dimer, Movafeghi et al., 1999) bound efficiently to the peptides. This would be in agreement with other reports suggesting that the Sec23/24 dimer (but not the Sec13/31 complex) is the one interacting with sorting signals and is thus involved in cargo recognition (Springer and Schekman 1998, Peng et al., 1999). In contrast to what happens with AtSec13, Sec13 from rat liver cytosol was found to bind to the Atp24 (YFKK) and Atp24-YFSS peptides. However, the assay can not discriminate between direct binding to the peptides or binding to previously bound Sec23/24 complex.

A striking difference in the binding of plant versus animal COPII subunits to p24 cytosolic tails is the strong competition by COPI, which was not observed when using rat liver cytosol. This has not been previously reported. In contrast to previous observations (Sohn et al., 1996), we could hardly detect binding of COPI from rat liver to the dihydrophobic (YF) motif in the -7,-8 position. In addition, the YF motif did not show any cooperativity with the dilysine motif in the –3,-4 position (the Atp24 -YFKK and Atp24-AAKK peptides bound coatomer from rat liver cytosol with a very similar efficiency), which is also different to what happens with plant cytosol (Contreras et al., 2004, and Figures 3.16C and 3.17B). This suggests that coatomer from rat liver binds specifically to the dilysine motif and not to the dihydrophobic motif. Different COPI subunits have been proposed to bind to distinct cytosolic signals to mediate sorting in different directions. Cosson and Letourneur (1994) suggested an interaction with the dilysine motif of a coatomer subcomplex composed of α-, β′- and ε-COP, whereas Fiedler et al. (1996) found that only a subset of COPI subunits (α, β′ and ε) bound to

dilysine motifs and consequently proposed this subcomplex to be involved in retrograde Golgi to ER transport. In contrast, the other subunits (β, γ and ζ) bound to diphenylalanine motifs, and were proposed to be involved in anterograde ER to Golgi transport (Fiedler et al., 1996). However, cross-linking studies have now established that γ-COP is indeed the COPI subunit responsible for interacting with KKXX motifs (Harter and Wieland 1998). A binding site for dibasic/diphenylalanine motifs has been shown to exist within the γ-COP subunit (Zhao et al., 1999).

In plant cells there is as yet no evidence for the existence of coatomer subcomplexes, and experiments have not been done to ascertain which COPI subunits might be responsible for binding to distinct sorting signals. However, the strong cooperativity between dilysine and dihydrophobic motifs, as shown here, could be indicative of different binding sites in plant as opposed to animal coatomer. In the absence of a dihydrophobic motif, COPI might therefore bind with lower affinity to the dilysine motif in the –3,-4 position. However, the presence of a dihydrophobic signal in the -7,-8 position may provide plant cells with additional COPI binding sites, and therefore a stronger interaction. High affinity binding of coatomer to a p24 cytosolic tail containing both dihydrophobic and dilysine motifs would then prevent COPII binding to the dihydrophobic motif *in vitro*. In rat liver there is no such high affinity COPI binding, and the dihydrophobic motif binds COPII even in the presence of COPI. In plants, a C-terminal dilysine motif has been shown to recruit both ARF1 and coatomer *in vitro* (Contreras et al., 2004) and to confer ER localization to type I membrane proteins *in vivo* (Benghezal et al., 2000). However, since all plant p24 proteins found in the databases so far have both dilysine and dihydrophobic motifs, how can they support COPII binding and ER export in the presence of COPI subunits *in vivo*? It may be that the conformation of these proteins in the ER or the Golgi membranes or their oligomerization status will modulate their affinity towards the respective COP coat proteins. In addition, Sar1p, which is also required for Sec23/24p binding to microsomal and liposomal membranes, may also play an important role. The homogeneity in the presence of sorting signals in plant p24 proteins is in clear contrast with the presence of different subfamilies of p24 proteins in mammalian cells, which seem to localize preferentially to different sets of membranes within the early

secretory pathway (Emery et al., 2000, Jenne et al., 2002), probably through their ability to interact with either COPI and COPII coat proteins. 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 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. Future experiments should be aimed at addressing the steady state distribution of p24 proteins in plant cells and their putative role(s) in both ER export and/or cis-Golgi to ER retrograde transport.

4.3. Visualization of ER exit sites in BY-2 cells

4.3.1. ERESs in higher plant cells are not organized in transitional ER and do not disappear during mitosis

In mammalian gland cells engaged in regulated secretion, ERESs collect at specialized domains of the ER known as transitional ER (tER). Such domains are characterized by a high density of vesicle/tubule budding profiles in thin sections (see for example Krinsje-Locker et al., 1994; Sasso et al., 1994; Bannykh et al., 1996). The presence of COPII-coat proteins at these sites has been confirmed by immunogold labelling (Orci et al., 1991; Tang et al., 2000, 2001; Horstmann et al., 2002). tER is also often recognized in microrganisms. A well-known case is that of the fission yeast *Pichia pastoris*, which in contrast to *Saccharomyces cerevisiae* possesses a stacked Golgi apparatus (Mogelsvang et al., 2003), has discrete several tER domains each lying juxtaposed to a Golgi stack (Rossanese et al., 1999). Another clear example is that of the model alga *Chlamydomonas reinhardtii* where tER and adjacent Golgi stacks are held in an ER amplexus attached to the nuclear envelope (Zhang and Robinson 1986). The reason for such aggregations of COPII budding sites were thought to lie in the oligomerization status of Sec12, which in Pichia pastoris has been shown to have large lumenal tails allowing for the interaction of adjacent molecules (Bevis et al., 2002). However, other „scaffolding proteins“ e.g. Sec16p (Supek et al., 2002), now seem to be required for this event since COPII budding

sites in *P. pastoris* still form when the localization of Sec12 to the tER is disrupted (Soderholm et al., 2004).

In contrast, ERESs in cultured cells and those mammalian cells exhibiting constitutive secretion are randomly distributed on the surface of the ER and have primarily 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), or through the expression of (X)FP-fusion constructs with Sec 13 (e.g. Hammond and Glick, 2000; Ward et al., 2001), and Sec24 (e.g. Stephens et al., 2000; Stephens, 2003).

Vesiculation/tubulation profiles at the ER in thin sections of higher plant cells have only rarely been recorded in the literature (e.g. Craig and Staehelin, 1988; Staehelin, 1997; Ritzenthaler et al. 2002), suggesting that ERESs in this cell type are also randomly distributed. This has been amply confirmed in the present study on BY-2 cells, through antibody staining with three different affinity-purified plant COPII antisera (Sar1, Sec23 and Sec13), and on the basis of the fluorescence pattern produced when a Sec13-GFP construct is expressed.

It is well-known that during mitosis the Golgi apparatus in mammalian cells breaks down into vesicles (Shorter and Warren, 2002). It has been claimed that these vesicles, together with Golgi matrix proteins that are required as a scaffold for the reconstitution of the Golgi apparatus at the onset of the subsequent interphase, lie in close proximity to ERESs whose function is arrested during mitosis (Prescott et al., 2001; Seemann et al., 2002). However, the recently published data of Stephens (2003) indicates that the ERESs visualized in mitotic cells by immunostaining in previous publications are artifactual: live cell imaging with YFP-Sec23 clearly showed a displacement of COPII into the cytosol during mitosis. In plants the Golgi apparatus does not fragment during mitosis, and in BY-2 cells many Golgi stacks appear to be immobilized in the immediate vicinity of the mitotic spindle (Nebenführ et al., 2000). In agreement with this, our results, also obtained by live cell imaging (with Sec13-GFP), demonstrate that ERESs do not disappear during mitosis, and are presumably functionally intact.

4.3.2. Preliminary characterization of higher plant ERESs

The COPII coat on ERESs in mammalian cells are known to develop sequentially: firstly by recruitment of Sar1-GTP to Sec12, then the sequestration of Sec23/24 followed by the binding of Sec13/31 (Barlowe, 2003; Bonifacino and Glick, 2004). Membrane-bound Sec13/31 therefore represents fully-assembled ERESs, and is the reason why the bulk of our observations have been made with Sec13 antibodies or with a transiently expressed Sec13-GFP construct. Although the interaction between Sec12 and Sar-GTP is pivotal to the formation of ERESs, Sec12 is excluded from COPII vesicles induced in vivo (Barlowe et al., 1994; Barlowe, 2002) nor is it concentrated in ERESs Saccharomyces cerevisiae (Rossanese et al., 1999) or in mammalian cells (Weissman et al., 2001). Unfortunately, due to the lack of cross-reactivity between our AtSec12 antibodies and BY-2 membrane proteins we have not been able to determine the in situ distribution of Sec12 in BY-2 cells. However, in vesicle budding experiments performed with Arabidopsis ER membranes we could show that Sec12 (and the translocon protein Sec61) are not incorporated into COPII vesicles (see Fig. 3.7).

Sar1 recruitment, and subsequent formation of ERESs in mammalian cells can be prevented by overexpression of a mutant form of Sar1 locked in the GDP form (Sar1T39N), which prevents COPII-dependent ER export (Kuge et al., 1994; Ward et al., 2001). This mutant has been tested on plants and also inhibits ER-to-Golgi transport (Takeuchi et al., 2000). This effect has been confirmed in the present study on BY-2 cells in which the expression of this inactive Sar1 protein led to retention of a secretory form of RFP in the ER and to the redistribution of a Golgi-localized mannosidaseI-RFP into the ER. Under these conditions, Sec13-GFP positive ERESs became fewer in number and were difficult to visualize as punctate light sources.

It has been reported that dephosphorylation of Sec13/31 prevents its recruitment to membranes in vitro (Salama et al., 1997). Corresponding to this, the protein kinase A inhibitor H-89 has been shown to be an effective inhibitor of ERESs formation in mammalian cells (Aridor and Balch, 2000; Lee and Linstedt, 2000, Puri and Lindstedt, 2003).

BFA has been a most useful tool in investigations into the secretory and endocytic pathways (reviewed by Nebenführ et al., 2002). Research on mammalian and fungal cells

has established that this drug interacts with a complex formed between the GTPase ARF (ADP ribosylation factor) and its guanidine exchange factor (GEF) (Peyroche et al., 1999). The discovery that the Arabidopsis protein GNOM, which is important for the correct targeting of the auxin efflux carrier PIN1 to the plasma membrane, is a BFA-sensitive ARF-GEF (Geldner et al., 2003, 2004), now makes it very likely that the molecular target for BFA is the same for all eukaryotic cells.

ARF-GEFs have so far not been reported at the ER in any cell type, so claims that BFA can act at the level of ERESs (Brandizzi et al., 2002a; Hawes, 2004) must be regarded with caution. Our results showing that BFA has little effect on the ability to recognize ERESs are in agreement with anti-Sec31 staining data obtained on NRK cells (Puri and Lindstedt, 2003). But, as such these results say nothing about the export competence of the ERESs so visualized. Ward et al. (2001) previously showed that COPII components still cycle at ERESs after addition of BFA. More recent FRET measurements performed on Vero cells indicate that BFA interferes with the kinetics of the interaction between Sec23 and Sec31, whereas the interaction between Sar1 and Sec23 remained unaltered (Forster, Stephens and Pepperkok, unpublished results). However, treatment with BFA for short periods, during which time COPI assembly was inhibited, did not alter the steady state distribution of any COPII component. Thus, it seems likely that any effect of BFA on ER-export is an indirect one resulting from a perturbation in the fine-tuning of the interdependent COPI and COPII machineries (Stephens et al., 2000; Ward et al., 2001) upon whose maintenance successful ER-Golgi transport depends.

3.3.3. ERESs and the Golgi apparatus: models and data

Randomly distributed ERESs in mammalian cells are relatively immobile (displacement time of 5-15 $\mu m\ h^{-1}$; Stephens, 2003) in comparison to the rate of cargo transport between the cortical ER and the perinuclear Golgi apparatus (0.5-1 $\mu m\ s^{-1}$; Stephens et al., 2000). As previously mentioned, ERGIC/VTCs are responsible for this long range transport and these are generally considered to arise from the homotypic fusion of COPII vesicles (Stephens and Pepperkok, 2001; Duden, 2003). Recent data indicates that each ERGIC/VTC is formed from a single ERES (Pepperkok, unpublished observations). Upon completion of mitosis in mammalian cells, ERESs form de novo (frequency: 2 h^{-1}.

100 μm^{-2}) and continue to do so during interphase and remain visible for several minutes (Stephens, 2003). During this time COPII proteins continually cycle on and off the membrane, but with different kinetics for each of the three major components (Sar1, Sec23/24, Sec13/31; Forster, Stephens & Pepperkok, unpublished observations). According to Stephens (2003) ERESs can also fuse with one another and divide. In common with mammalian cells ERESs in BY-2 cells are quite stationary, and may also be seen to agregate and divide (see Fig. 3.25 and accompanying video). However, our data indicates that ERESs in BY-2 cells is much higher and that they appear to be more dynamic structures than their mammalian counterparts: individual ERESs were rarely visible for periods longer than 8-10 s.

These data have immediate consequences for one of the three models which have been postulated to explain ER-to-Golgi transport in plants (Neumann et al., 2003). According to the "secretory unit"concept, each individual Golgi stack has its own ERES and both traverse across the surface of the ER. This means that ERESs and Golgi stacks must be equal in number, that both be motile and that ERESs should be long-lived entities. None of these features are shown by BY-2 cells. Common to the other two models, is that ERESs greatly outnumber Golgi stacks, and by implication are relatively stationary. In the one case, Golgi stacks were considered to sweep up export vesicles as they moved over the surface of the ER (the "vacuum cleaner" model; Boevink et al., 1998). By contrast, the "stop-and-go" model of Nebenführ et al. (1999) foresees cargo collection by the Golgi stacks restricted only to those stacks which have temporarily come to a halt over an ERES (Fig. 4.1). In the sense that ERESs are seen to continually associate and disappear from the rims of moving Golgi stacks in BY-2 cells, our data is not conform with the latter model. On the other hand, we have also presented data that a stationary Golgi stack can be visualized over a 20 s period with and without associated peripheral ERESs. Thus, Golgi motility per se does not seem to be a precondition for successful ER-to-Golgi transport, and is conform with FRAP measurements dealing with the recovery of photobleached Golgi marker proteins on immobilized (Brandizzi et al., 2002b) and moving (Brandizzi and Hawes, 2004) Golgi stacks.

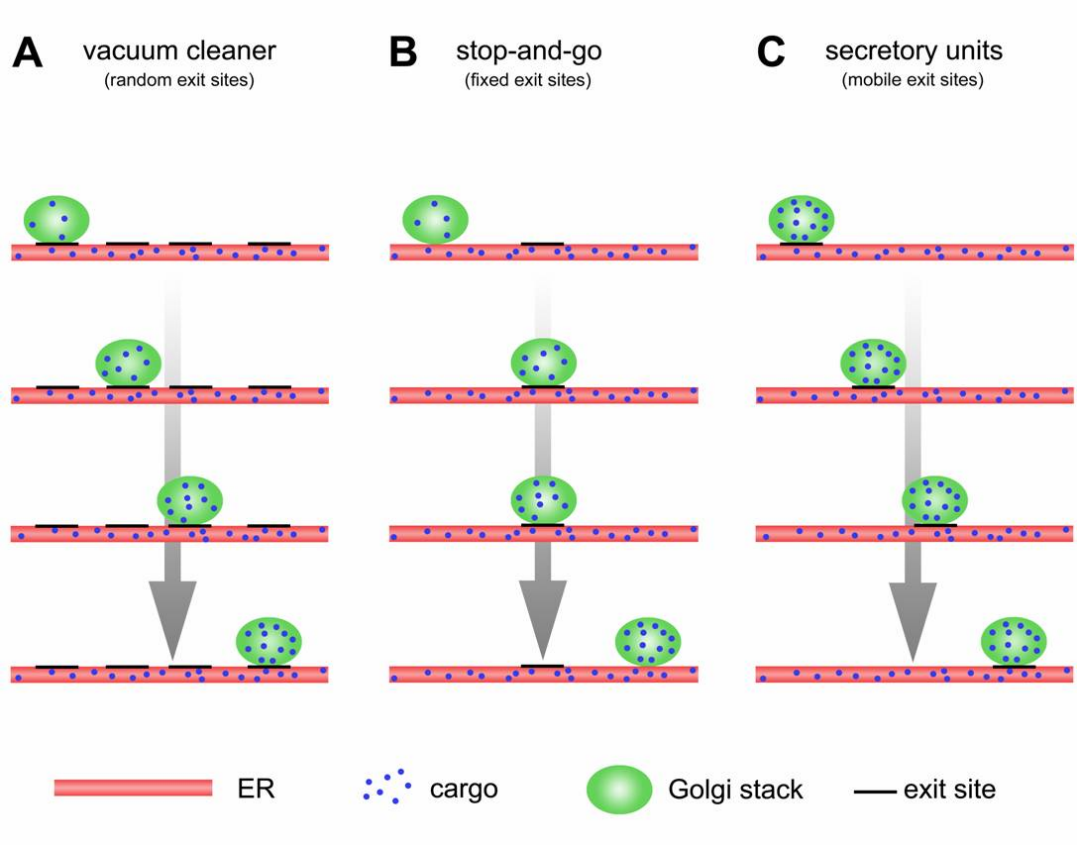

Fig. 4.1. Models of ER-to-Golgi protein transport (From Neumann et al., 2003)

Since ERESs in BY-2 cells greatly outnumber Golgi stacks, and seem to form and disappear from the surface of the ER quicker than they can associate with the stacks, means that only ERESs which interact with a passing Golgi can deliver cargo. The rest must obviously constitute a potential which is not realized: for these, COPII coats must dissociate from the surface of the ER without a vesicle having been formed. The continual formation and release of COPII vesicles with no short-range control over their fusion target would not appear to be a realistic alternative. On the other hand, we are not yet able to provide a plausible explanation for how the presence of an overlying Golgi stack can trigger the culmination of COPII-mediated cargo release.

3.3.4. ER-to-Golgi transport: vesicles versus tubules

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 Barlowe, 2003; Bonifacino and Glick, 2004 for reviews), the actual existence of COPII vesicles in vivo has never really been established beyond doubt. Budding, COPII-coated profiles at tER have been recorded on numerous occasions in thin sections from conventional chemically fixed tissues (see above for references), as well as in high pressure frozen-freeze substituted P. pastoris cells (Mogelsvang et al., 2003). However, as occasionally demonstrated (Martinez-Menarguez et al., 1999), many of these budding profiles may instead represent tubular outgrowths. 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 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 very difficult to evaluate the above results in the context of our findings on ERESs in BY-2 cells. However, 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 assuming that bidirectional vesicle trafficking between these compartments does not occur.

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6. APPENDIX

I. Abbreviation

2, 4-D	2, 4-Dichloro-phenoxyacetic acid
aa	amino acid
ALP	alkaline phosphatase
APS	ammoniumpersulfate
ARF	ADP-ribosylation factor
BSA	bovine serum albumin
BY-2	Tobacco Bright Yellow 2
COPI/II	coatamer protein complex I/II
dH_2O	deionized H_2O
DIC	differential interference contrast
dsDNA	double strand DNA
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
FM 4-64	N-(3-triethylammmoniumpropyl)-4-(p-diethylaminophenylhexatrienyl) pyridinium dibromide
5-FOA	5-fluoroorotic acid
FPLC	fast protein liquid chromatography
FRAP	Fluorescence recovery after photobleaching
g	gram
g	gravity
GAP	GTPase activating protein
Gap1	general amino acid permease
Gas1p	glycophospholipid-anchored surface protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GMP	guanosine monophosphate
GMP-PNP	5'-guanylyl imidodiphosphate
GPI	glycosyl phosphatidyl inositol
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour(s)
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Ig	immunoglobulin
IPTG	isopropylthio-ß-D-galactoside
kDa	kilo Dalton
KOAc	potassium acetate
LB	Luria Bertani
l	litre

Lst1	lethal with sec-thirteen
m	meter
M	molar (mol x l^{-1})
mA	milli Amper
2-ME	β–mercaptoethanol
mg	milligram (10^{-3}g)
µg	microrgram (10^{-6}g)
MgOAc	magnesium acetate
min	minute(s)
ml	millilitre (10^{-3} l)
µl	microlitre (10^{-6} l)
µm	micrometer (10^{-6} m)
mM	millimolar (10^{-3} M)
µM	micromolar (10^{-6} M)
NaOAc	sodium acetate
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometer (10^{-9}m)
NSF	N-ethylmaleimide sensitive fusion
OD	optical density
ON	overnight
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
Pma1	plasma membrane ATPase
PMSF	phenyl methyl sulfonyl fluoride
Rnase	ribonuclease
Rpm	rotation per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
ssDNA	single strand DNA
tab.	tablet
TBS	tris buffered saline
TCA	thrichloroacetic acid
TEMED	1,2-bis-(dimethylamino)-ethane
tER	transitional ER
TGN	*trans*-Golgi network
TRAPP	transport protein particle
Tris	tris(hydroxymethyl)aminomethane
VAMP	vesicle associated membrane protein
VSV	vesicular stomatitis virus glycoprotein epitope
VTCS	vesicular tubular clusters
v/v	volume/volume
w/o	without
wt	wild type
w/v	weight/volume
Ø	diameter

Amino acids abbreviations:

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Species abbreviations:

A. thaliana	*Arabidopsis thaliana*
C. elegans	*Caenorhabditis elegans*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
H. sapiens	*Homo sapiens*
L. esculentum	*Lycopersicon esculentum*
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*

II. Frequently used buffers and solutions

10X Stock Phosphate-buffered Saline (PBS)/Liter

NaCl	80 g
KCl	2 g
Na_2HPO_4	26.8 g
KH_2PO_4	2.4
pH	7.4

50X TAE Buffer/Liter

Tris base	242 g
Acetic acid	57.1 g
0.5 EDTA, pH 8.0	100ml
pH	8.5

10X TBS/Liter

Tris	121.2 g
NaCl	180 g
pH	7.4

Buffer88

Hepes	25mM
Sorbitol	250mM
KOAc	150mM
$Mg(OAc)_2$	5mM

10X ATP RS (ATP Regeneration Buffer)

10 mM ATP-Na2
400mM Creatine phosphate-Na2
2 mg/ml creatine phosphor-kinase (type I from rabbit muscle)

10 mM GMP-PNP

TAE 50X

242 g/l Tris-base
57.1 ml/l Glacial acetic acid
18.612 g/l EDTA

DNA loading buffer 10X

30% (w/v) Ficoll
0.25% (w/v) Xylene Cyanol FF
0.25% (w/v) Bromophenol Blue
0.5 M EDTA, pH 8.0

30% Acrylamide-stock solution

29.2%(w/v) Acrylamide

0.8% (w/v) Bisacrylamide

APS 10% (w/v)

Laemmli loading buffer 2X

0.1M Tris-HCl, pH 6.8
2% (w/v) SDS
2% (v/v) ß-Mercaptoethanol
20% (v/v) Glycerol
0.002%(w/v) Bromophenol Blue

SDS electrophoresis buffer

0.19 M Glycine
25 mM Tris-base
0.1% (w/v) SDS

Coomassie fixing solution

25% (v/v) Isopropanol
10% (v/v) Glacial acetic acid

Coomassie staining solution

10% (v/v) Glacial acetic acid
60 mg/l Coomassie brilliant blue R250

Western blot transfer buffer

20 mM Tris-base
150 mM Glycine
20% (v/v) Methanol

Ponceau S solution

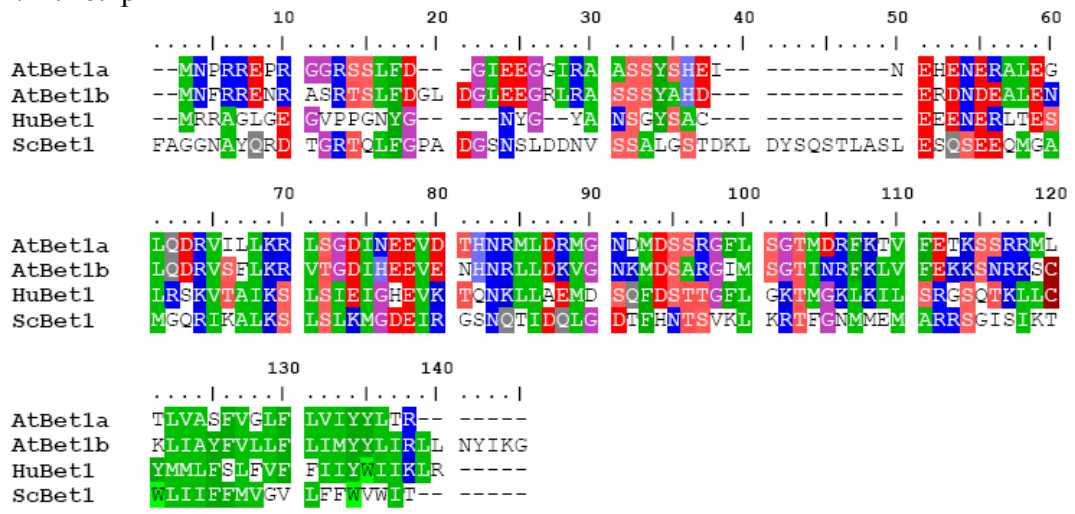
2.5 g/l Ponceau S
15% (v/v) Glacial acetic acid
40% (v/v) Methanol

TE

10 mM Tris-HCl, pH 7.4-8.0
1 mM EDTA, pH 8.

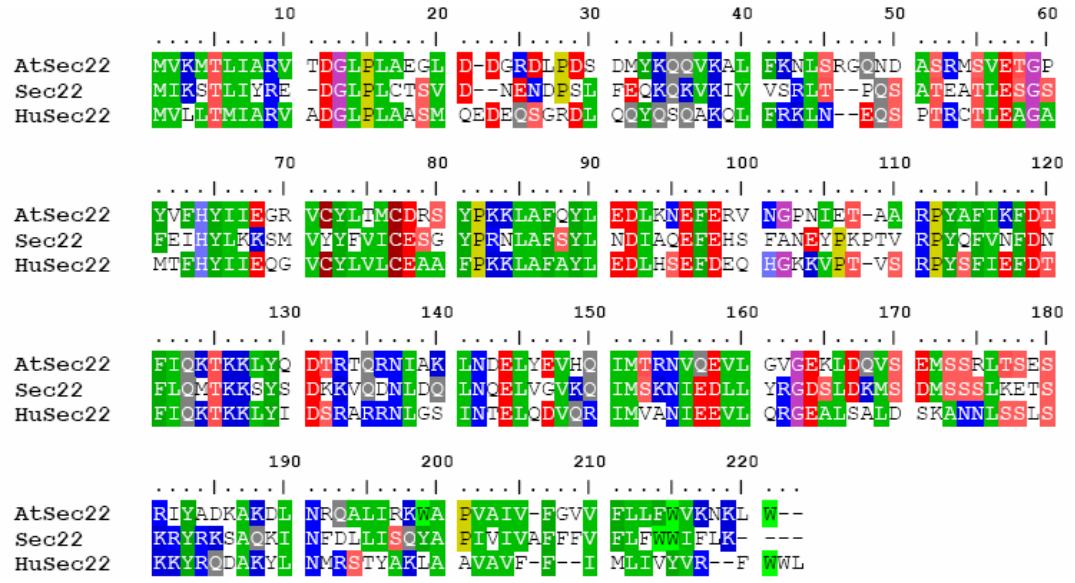
III. Sequence alignment

1. AtBet1p



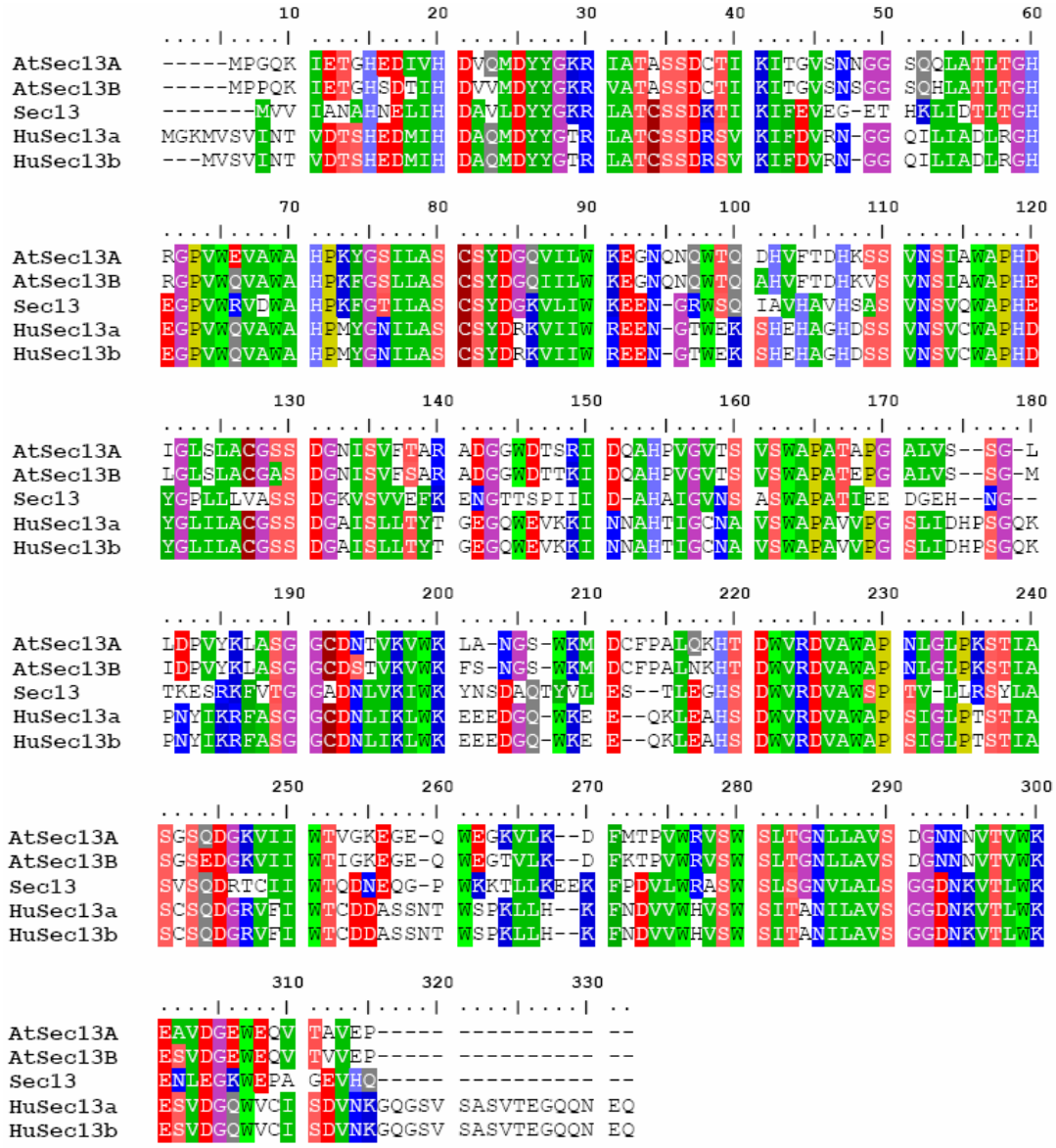
AtBet1a (Genbank Acc. # CAB61855) AtBet1b (Genbank Acc. # BAC42093)
HuBet1 (Genbank Acc. # AAB62941) ScBet1 (Genbank Acc. # CAB38096)

2. AtSec22



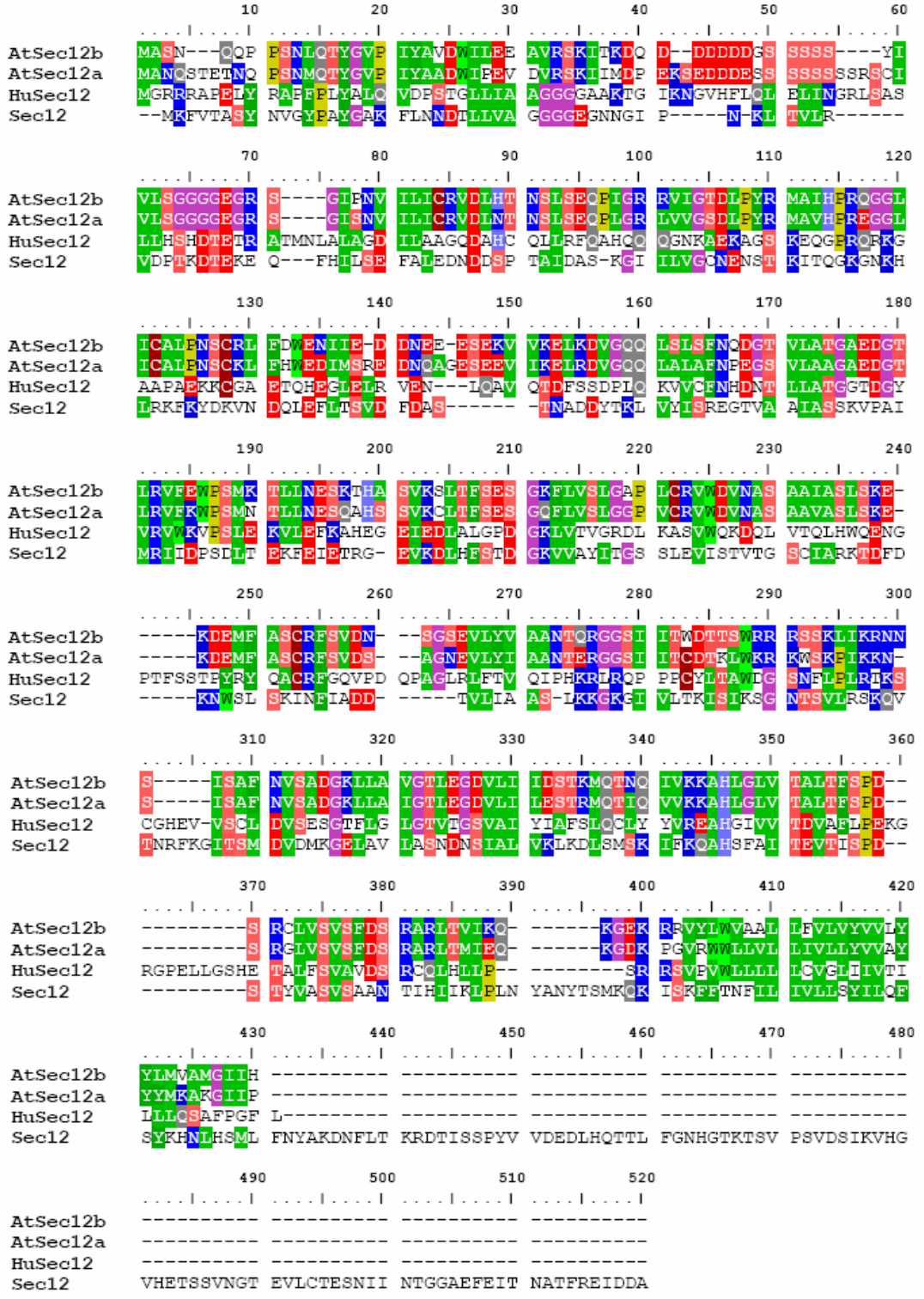
AtSec22 (Genbank Acc. # AKK76469) Sec22 (Genbank Acc. # AAB67373)
HuSec22 (Genbank Acc. # AAC39893)

3. AtSec13



AtSec13A (Genbank Acc. # AAC16967) AtSec13B (Genbank Acc. # AAF03492) Sec13 (Genbank Acc. # AAB67426) HuSec13a (Genbank Acc. # NP_109598) HuSec13b (Genbank Acc. # AAH02634)

4. AtSec12



AtSec12b (Genbank Acc. # BAB09140) AtSec12a (Genbank Acc. # AAC67323) HuSec12 (Genbank Acc. # AAF19192) Sec12 (Genbank Acc. # NP 014423)

IV. List of figures

V. List of tables