Cis-Golgi Cisternal Assembly and Biosynthetic Activation Occur Sequentially in Plants and Algae

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Traffic 2013; 14: 551–567

Traffic 2013; 14: 551–567

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doi:10.1111/tra.12052

Golgi stacks are assembled from lipids and proteins produced in the endoplasmic reticulum (ER) and transported in COPII vesicles to the cis-side of the Golgi (1–4). COPII vesicles (5) also deliver ER-synthesized cargo molecules to the Golgi, where they are modified by different sets of enzymes as they pass through the cis- to the trans-side of the stacks (6,7). In addition, in plants the biosynthesis of complex polysaccharides has been shown to occur exclusively in Golgi cisternae (8). Upon completion of these biosynthetic reactions, the mature products are sorted and packaged into different vesicles in the trans-Golgi network (TGN) from where they are transported to their final destinations, the cell surface and extracellular space, and the multivesicular bodies and vacuoles/lysosomes (9–12).

Common to all Golgi are three types of cisternae – cis, medial and trans – and a Golgi-associated TGN. However, the spatial organization of Golgi cisternae varies greatly among organisms (13). In Saccharomyces cerevisiae both the ER export sites (also known as ERES) and the individual cisternae are dispersed, and the individual cisternae have been shown to undergo maturational changes over time (14,15). In contrast, in Pichia pastoris each ER export site is coupled to a single Golgi stack by means of a ribosome-excluding scaffold system that encompasses the entire Golgi stack (16,17). A similar close spatial relationship between ER export sites and Golgi stacks has been observed in the flagellate algae Scherffelia dubia (18) and Chlamydomonas reinhardtii (19), the green alga Scenedesmus acutus (20) as well as in protozoa such as Trypanosoma brucei (21).

In higher plants, the spatial relationship between ER export sites and Golgi stacks is affected by three factors, the transient nature of the ER export sites (22), the dispersed organization of the Golgi stack-TGN units (23) and the rapid (up to 4 μm s⁻¹) movement of Golgi stacks along actin filaments that are often anchored to ER membranes (24,25). In plants, two distinct models of ER-to-Golgi trafficking have been proposed. The ‘ER-Golgi secretory unit’ model (19,26–28), which is based on fluorescent microscopy data, postulates that each Golgi stack is permanently coupled to an ER export site and that both move together along actin filaments. However, in Arabidopsis columella cells only 15% of the Golgi stacks are docked to an ER export site and in root meristem cells only ~70% are ER export site bound (29). As calculated by Yang et al. (22), the speed of the ER-Golgi units documented by daSilva et al. (26) lies between 0.1 and 0.3 μm s⁻¹, which corresponds to the wiggling but not to the fast (4 μm s⁻¹) traveling Golgi stacks reported by Nebenführ et al. (25). This suggests that Golgi stacks that are not docked to an ER export site can travel up to 10 times faster than those that are coupled to such a site.

The cisternal progression/maturation model of Golgi trafficking predicts that cis-Golgi cisternae are formed de novo on the cis-side of the Golgi. Here we describe structural and functional intermediates of the cis cisterna assembly process in high-pressure frozen algae (Scherffelia dubia, Chlamydomonas reinhardtii) and plants (Arabidopsis thaliana, Dionaea muscipula; Venus flytrap) as determined by electron microscopy, electron tomography and immuno-electron microscopy techniques. Our findings are as follows: (i) The cis-most (C1) Golgi cisternae are generated de novo from cisterna initiators produced by the fusion of 3–5 COPII vesicles in contact with a C2 cis cisterna. (ii) COPII vesicles fuel the growth of the initiators, which then merge into a coherent C1 cisterna. (iii) When a C1 cisterna nucleates its first cisterna initiator it becomes a C2 cisterna. (iv) C2-Cn cis cisternae grow through COPII vesicle fusion. (v) ER-resident proteins are recycled from cis cisternae to the ER via COPII-type vesicles. (vi) In S. dubia the C2 cisternae are capable of mediating the self-assembly of scale protein complexes. (vii) In plants, ~90% of native α-mannosidase I localizes to medial Golgi cisternae. (viii) Biochemical activation of cis cisternae appears to coincide with their conversion to medial cisternae via recycling of medial cisterna enzymes. We propose how the different cis cisterna assembly intermediates of plants and algae may actually be related to those present in the ERGIC and in the pre-cis Golgi cisterna layer in mammalian cells.

Key words: Arabidopsis, cisternal assembly, COPI, COPII, electron tomography, ER export sites, ERGIC, ER-to-Golgi transport, Golgi apparatus, p115 scaffold

Received 1 June 2012, revised and accepted for publication 28 January 2013, uncorrected manuscript published online 31 January 2013, published online 25 February 2013

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The alternative ‘dock, pluck and go’ model (30) postulates that the coupling of Golgi stacks to ER export sites in plant cells is transient, and occurs only when an ER export site is actively producing COPII buds and vesicles for export to the Golgi. To this end, budding COPII vesicles are born within a 40 nm thick scaffold layer that contains Atp115 (Arabidopsis ortholog of p115/Usos) and appears to have an affinity for the cis-side of the Golgi-encompassing Golgi scaffold/matrix (29–31). This COPII bud-associated scaffold system enables active ER export sites to capture passing Golgi stacks and to maintain a stable association for COPII vesicle-mediated cargo transfer until the local supply of exportable cargo molecules has been exhausted and COPII bud formation stops. In support of this latter hypothesis, our electron tomography analysis of >50 plant Golgi has demonstrated that all of the ER-associated Golgi were docked to an ER export site via a ribosome-excluding scaffold system (29). Furthermore, only 10% of the visualized budding COPII profiles were not associated with a docked Golgi. Based on these results, we postulate that the ‘ER-Golgi secretory units’ of the daSilva et al. (26) model correspond to the Golgi stacks that are attached to an active ER export site in the ‘docked’ stage of the ‘dock, pluck and go’ model of Staehelin and Kang (30). These docked Golgi can move around with their attached ER export sites, but the movements are less linear and typically 10 times slower than the maximum Golgi speeds reported (25). In the docked state, the ER-Golgi relationship in plants would be equivalent to the more stable ER-Golgi complexes seen in P. pastoris, S. dubia, C. reinhardtii, S. acutus and T. brucei.

Trafficking between ER export sites and the Golgi of mammalian cells is complicated by the fact that the ER export sites are dispersed, whereas the interconnected Golgi stacks are clustered around the centrosomes (32). To enable efficient cargo trafficking between these two membrane systems, mammalian cells have evolved an intermediate membrane compartment, the ER-Golgi intermediate compartment (ERGIC), within which Golgi cisterna elements are pre-assembled prior to their transport to the Golgi stacks (33). ER to ERGIC transport involves COPII vesicles, but the nature of the vesicular carriers between ERGIC and Golgi has yet to be determined.

The mechanisms of cis-Golgi cisterna assembly and trafficking of molecules through Golgi stacks have been debated for the past 40 years (1,13,34–37). The cisternal progression/maturation model of Golgi trafficking primarily in the form of high resolution, 3D morphological information on the Golgi apparatus and associated membrane systems of mammalian cells (41,43,44), P. pastoris cells (16), algae and plants (10,30,45). In particular, electron tomography has enabled researchers to produce quantitative, nano-scale data on ER, Golgi and TGN membrane and scaffolding systems as well as associated vesicles in micron-scale volumes of cytoplasm. In turn, these data have provided increasingly tight morphological constraints for trafficking models based on light microscopic, biochemical and physiological studies, particularly when combined with information derived from immuno-electron microscopy studies of cryofixed cells. For example, electron tomography analyses of plant and algal Golgi have demonstrated (i) that retrograde vesicle trafficking between cis-Golgi cisternae and ER cisternae is mediated by COPIIa-type vesicles, whereas COPIIb-type vesicles recycle membrane between Golgi-associated TGN, trans-and medial-Golgi cisternae as well as late stage cis cisternae, but not between cis and ER cisternae (45), thereby refining previous biochemical and immunolabeling studies (46,47); (ii) that ~35% of the trans-Golgi cisternal membrane is recycled during the conversion of trans-Golgi cisternae into TGN cisternae (10) and (iii) that TGN cisternae release their secretory and clathrin-coated vesicles by means of cisternal fragmentation, while leaving behind cisternal membrane fragments (10).

The current investigation reports on the process of COPII-mediated assembly of cis-Golgi cisternae in the scale-forming alga S. dubia, which has been called the ‘reality check’ system of the cisternal progression/maturation model (13), in the alga C. reinhardtii, as well as in Arabidopsis and Dionaea muscipula (Venus flytrap). Our data support a mechanism by which cis-Golgi cisternae originate from cis cisterna initiators generated by the fusion of 3–5 COPII vesicles in contact with the surface of a C2-type cis-Golgi cisterna. Expansion of the initiators gives rise to a coherent C1 cis cisterna, which becomes a C2-type cis cisterna when a new cisterna initiator nucleates on it. The assembly of protein complexes is observed in C2 cisternae. COPIIa-type vesicles bud from all cis cisternae, but cis cisternae appear to remain biosynthetically inactive until they are transformed into medial cisternae via COPIIb-type vesicle recycling.

Results

The data presented in this report were collected from cells preserved by high-pressure freezing/freeze-substitution methods. The samples used for the thin section and
electron tomography studies of membrane structure were embedded in Epon, whereas those employed for the immunolabeling experiments were embedded in Lowicryl HM20. The data sets produced included 1500 electron micrographs (∼600 S. dubia, ∼600 Arabidopsis, ∼150 C. reinhardtii, ∼150 D. muscipula) of hundreds of thin sectioned cells, and tomograms of 54 cells (18 S. dubia, 14 Arabidopsis, 9 C reinhardtii, 13 D. muscipula) with a total of ∼10,000 slice images. Each micrograph and slice image was analyzed individually. Interpretation of the data was aided by the results of previous tomographic analyses of ∼50 Golgi in diverse types of plant cells (48,49,29,10) produced in the Staehelin laboratory over 10 years.

Scherffelia dubia Golgi contain five morphologically distinct cis-cisternae, but de novo cisterna formation occurs only in the cis-most (C1) cisterna position

The alga S. dubia was chosen for this study, because scale-forming algae have been utilized as model systems for investigation and validation of the cisternal progression/maturation model of Golgi-mediated membrane trafficking (50−52). Most notably, during flagellar regeneration each S. dubia Golgi stack produces a new cisterna every 15 s (18,53), which greatly enhances the possibility of observing assembly intermediates of forming cisternae. Thus, by determining the architecture of forming cis-Golgi cisternae in this alga, we hoped to identify characteristic assembly intermediates of de novo formed cis cisternae, thereby providing a means for identifying the mechanism of Golgi trafficking in nonscale forming algae and in plants.

Interphase S. dubia cells contain two very large Golgi stacks, which measure ∼1.5 μm in diameter, consist of 16–20 individual cisternae (Figures 1A, S1, Movie S1). Several important ultrastructural studies of these Golgi in chemically fixed cells have been reported (18,50,53,54). Our micrographs of high-pressure frozen/freeze-substituted S. dubia cells have confirmed many of the observations reported in those studies, while also capturing critical intermediate stages of cis cisterna assembly and enabling us to define functionally important differences in the luminal staining properties of cis-, medial- and trans-Golgi cisternae.

Based on the position of the cisternae in a stack, the staining of their luminal contents, and the thickness of the cisternal lumen, it is possible to distinguish cis-, medial- and trans-types of Golgi cisternae based on structural criteria alone (Figure 1A,B). The cis cisternae of each Golgi stack are positioned adjacent to a large and very active ER export site that produces all of the COPII-type vesicles needed for the assembly of the stack (Figures 2, Movie S2; (45)). In addition, cis cisternae can be distinguished from the following medial and trans cisternae based on their highly variable diameter (Figures 1A, 2D,E and 3), and the absence of staining of their luminal contents (Figures 1A,B). Interestingly, the COPII vesicles that occupy the narrow space between the transitional ER membrane and the forming cis-Golgi cisternae exist in two configurations, randomly dispersed, single vesicles, and 3−5 vesicle clusters connected via 65-nm-long linker molecules (Figures 2E and S2). In the absence of any genomic information about this alga, we are unable to speculate on the nature of these linkers.

S. dubia Golgi stacks typically contain five cis-type cisternae (Figure 1), which are numbered C1−C5 in Figures 2D,E and 3. Figure 3A−C illustrates cis-side, face-on views of three electron tomography-based Golgi models. Arguably the most striking difference between these models is the huge variability in size of the orange colored, cis-most (C1) cisterna initiators. In Figure 3A, the

Figure 1: Electron micrographs (A,B) of a Golgi stack (A) and Golgi cisternae (B) of high-pressure frozen and freeze-substituted cells of the scale-forming alga Scherffelia dubia. The interphase Golgi stack (A) measures ∼1.5 μm in diameter and consists of 20 individual cisternae. The five cis-cisternae are positioned adjacent to an ER export site and can be identified based on their position within the stack, their small and variable size, and their luminal staining characteristics. In contrast to medial- and trans-Golgi cisternae and contractile vacuole (CV) tubes, developing scales (arrows) that are readily detected using osmium staining were not observed in the cis-cisternae. B) Higher magnification view of cis- and medial-Golgi cisternae. Note the absence of staining of the luminal contents of the cis cisternae and the sharp transition to the densely stained medial/trans-types of cisternae. Scale bars (A) 500 nm; (B) 100 nm.
Figure 2: Spatial relationship between ER export sites generating COPII vesicles, COPIa and COPIb vesicles, and forming cis-type Golgi cisternae in \textit{S. dubia}. Tomographic slice image views of a budding COPII vesicle (A), free COPII (∼70 nm) and COPIa (55 nm) vesicles (B), and a COPII vesicle in the process of fusing with a C1 cis cisterna initiator (C). D) Electron tomography-based model of the cis-side of a \textit{S. dubia} Golgi stack (C1–C5 cisternae, multiple colors) together with associated ER membranes (yellow) and COPII (beige) and COPIa (green) vesicles. The medial- and trans-type cisternae are shown in white. E) 3D view of cis-Golgi cisternae and associated COPII-type vesicles. Note that while 16 of the 23 visible COPII vesicles appear single and randomly dispersed, seven are organized into two small clusters (dashed circles) held together by 65-nm-long linker molecules (Figure S2). F–H) More detailed 3D views of an ER export site with budding COPII vesicles alone (F), together with COPII (beige) and COPIa (green) vesicles and C1 cis-cisterna initiators (orange) (G), and together with C1–C5 cis cisternae and COPIb vesicles (purple) (H). Scale bars = 100 nm.

Single C1 cisterna initiator, which is being assembled on the surface of the underlying C2 cisterna (green), is very small, its surface area being equivalent to the surface area of ∼5 COPII vesicles. In contrast, the Golgi stacks illustrated in Figures 3B,C possess multiple C1 cisterna initiators that vary in size from very small (Figure 3C, white arrowheads) to medium size with one appearing partly fenestrated. Growth of these C1 cisterna initiators involves the fusion of COPII vesicles with the cisternal membranes (Figures 2A–C and 3A–C). The presence of multiple C1 initiators of different sizes growing on the surface of C2 cisternae suggests that the nucleation of a new C1 cisterna initiator can occur spontaneously when 3–5 COPII vesicles become tethered together adjacent to the surface of a C2 cisterna. Conversion of a C1 cisterna into a C2 cisterna capable of nucleating a new set of cisterna initiators appears to be preceded by all of the C1 initiators fusing together into a coherent single cisterna.

\textit{COPII vesicles mediate the growth of C2-Cn cis cisternae, while COPIa vesicles bud from their rims}

The coalescence of the \textit{de novo} formed cisterna initiators into a coherent \textit{cis} cisterna marks the end of the first phase of \textit{cis} cisterna assembly, which is confined to the \textit{cis}-most region of the Golgi stack. The second phase of the \textit{cis} cisterna assembly process starts when the C1 cisterna becomes a C2 cisterna with the formation of a new C1 cisternal initiator on its surface. From this point on, it appears that all of the growth of the C2–C5 cisternae occurs through the fusion of individual COPII vesicles with the cisternae. This continued growth is evidenced by the progressive increase in size of the C2–C5 \textit{cis} cisternae depicted in Figure 3D. In contrast, the surface area of the medial- and trans-Golgi cisternae remains fairly constant.

Assembly of \textit{cis-Golgi} cisternae requires both input from COPII-type vesicles, and the recycling of displaced ER proteins back to the ER via COPI-type vesicles (13). As reported previously (45), both \textit{S. dubia} and \textit{Arabidopsis} generate two types of COPI vesicles, termed, COPIa and COPIb. In electron tomographic images of high-pressure frozen/freeze-substituted cells, the two types of COPI vesicles have the same average diameter (∼55 nm in \textit{S. dubia} and ∼45 nm in \textit{Arabidopsis}), but can be distinguished based on coat architecture, coat thickness, cargo staining, apparent cisternal origin and spatial distribution around...
Figure 3: Face-on 3D tomographic model views of *S. dubia* Golgi cisternae in which the cis-most (C1) cisternae are colored orange and the underlying C2 cisternae are colored green. The models provide examples of C1-type cis cisterna assembly intermediates as well as a stepwise increase in size of the C2–C5 cis-type cisternae. The C1 cisterna initiators vary in size from a single small blob (A) to two intermediate sized compartments with branched tubules (B), and to fenestrated cisternal elements with tubular extensions (C). The spherical geometry and size of the ends of many of the tubules suggests that they are preferential sites of vesicle fusion. D) Face-on views of all of the cisternae (17) of the Golgi stack shown in (A). The five cis-type cisternae are displayed in the top row, and the stepwise increase in size of the C1–C5 cisternae is clearly seen. In contrast, the size of the medial and trans-type (C6–C16) cisternae is fairly constant. Scale bars (A–C) = 200 nm; (D) = 500 nm.

The lack of staining of the luminal contents of growing cis-Golgi cisternae of *S. dubia* suggests that they are biosynthetically inactive

One of the most striking differences in appearance of thin sectioned plant and algal Golgi stacks prepared by traditional chemical fixation versus high-pressure freezing/freeze-substitution methods relates to the differential staining of the luminal contents of the cis-, medial- and trans-types of Golgi cisternae when the latter methods are used (55). The differences in staining of the cis and medial cisternae is particularly evident in the Golgi stacks of *S. dubia*, where no luminal staining is evident in the cis cisternae, and pronounced staining of luminal materials is a characteristic feature of both medial and trans cisternae (Figures 1 and 4A–D).

The functional importance of this observation becomes intriguing when one compares the staining of the cell wall and flagellar scales that can be resolved in the tomographic slice images of these samples (Figure 4A–D). Scale protein assemblies are never seen in the cis-most (C1) cisternae, but distinct, faintly stained scale structures are regularly observed in the C2–C5 cis cisternae (Figure 4A,B). Importantly, the staining of these scale protein assemblies did not change between the C2 and the C5 cisternae. However, as soon as the scales reached the first (C6) medial cisterna (Figure 4C,D), the staining of their surface subunits increased dramatically. Note, in particular, the stepwise increase in staining of the scales in the C6 and C7 cisternae (Figure 4C). Within the C8 medial cisterna (Figure 4D) the density of staining of the scales is similar to the staining of the presumably fully assembled scales seen in the TGN and the contractile
Figure 4: Tomogram slice images of S. dubia Golgi cisternae illustrating differences in architecture and staining of the proteoglycan scales seen in cis-, medial- and trans-Golgi and TGN cisternae, as well as in contractile vacuoles. Most striking is the difference in staining between the wispy scale complexes seen in the C2–C5 cis cisternae (A,B), and the sudden appearance of more darkly stained and distinct scale complexes in the C6 and C7 medial (and trans cisternae – not shown) cisternae (C,D). The sudden change in scale staining coincides with the change in luminal staining of the cisternae, with the lumina of the cis cisternae being essentially devoid of stainable materials, and the lumina of the medial cisternae being filled with stainable molecules (see also Figure 1B). E,F) Late stage trans-Golgi network (TGN) cisternae and contractile vacuoles (CV; see also Figure 1A) also appear to be devoid of uniformly dispersed and stained luminal molecules. In all micrographs, the arrows point to individual proteoglycan scale complexes. Scale bar = 100 nm.

vacuoles (Figure 4E,F). Interestingly, the background luminal staining characteristic of the medial and trans cisternae disappears as the TGN cisternae are converted to contractile vacuoles (Figures 1A and 4E,F). Based on these observations, we propose that in S. dubia the cis-Golgi cisternae serve exclusively as Golgi membrane assembly compartments. The transformation of a cis- to a medial-Golgi cisterna marks both the end of the membrane assembly phase and the activation of the biosynthetic functions. Below we test whether these observations and hypotheses also apply to the Golgi of higher plants.

**De novo assembly of cis cisternae in Arabidopsis occurs within the cis-most, Atp115-containing region of the Golgi scaffold, and the assembly intermediates resemble those seen in S. dubia**

In high-pressure frozen/freeze-substituted cells of Arabidopsis each Golgi stack and associated TGN cisterna is encompassed by a ribosome-excluding Golgi scaffold/matrix (Figure 5A,B, Movie S3). Within this scaffold, cis-, medial- and trans-Golgi cisternae can be distinguished based on their position within a stack, their geometry and the staining of their luminal contents (Figure 5A,B; 55). The Arabidopsis Golgi typically possesses two cis cisternae that are highly variable in size, associated with COPII vesicles (Figure S3) and lack luminal staining (Figure 5B).

The variability in cisternal size and structure is most evident in the C1 level cisternae (orange) of the tomographic models (Figure 6A–D, Movie S4), where the cisterna initiators are seen to change from small cisternal blobs to branched, tubular structures and then finally into flat cisternae with increasingly smoother rims. The C1 cisternae are always smaller than the C2 cisternae suggesting that the C2 cisternae may serve as templates for the expanding initiators. The surface area of the smallest cisterna initiators observed is close to combined surface areas of 3–5 COPII vesicles (Figure 6A,E). The tubular shape of the small initiators and of the marginal domains of growing C1 cisternae suggest that early expansion of the initiators is achieved by fusion of COPII vesicles with the tip regions of the initiators rather than with their sides (Figure 6B,C). De novo formation of cisterna initiators occurs only in the C1 region of the stack, which is embedded in the Atp115-containing, cis-most region of the Golgi scaffold (Figure 5C).

Following the maturation of the C1 cisterna into a coherent, smooth rimmed, disk-shaped membrane structure (Figure 6D), the C1 cisterna develops the capacity to serve as a nucleating surface for new cisternal initiators, thereby transforming itself into a C2 cisterna. The C2 cisternae in Arabidopsis then grow about 50% in size before they
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Figure 5: Tomographic slice image of an Arabidopsis root meristem Golgi stack and associated TGN cisternae, and an anti-Atp115 labeled Golgi scaffold. A) The stack measures ∼0.7 μm in diameter and contains six cisternae. The cis-Golgi cisternae can be distinguished from the medial and trans cisternae based on their location in the stack, the small and variable size of the cisternae, and the lack of staining of their luminal contents. In contrast, the medial and trans cisternae are more uniform in size, and both types of cisternae possess stained luminal materials. Note that in the higher magnification image (B) of the cis cisternae shown in (A) the C3 cisterna exhibits a combination of cis and medial morphological traits suggesting that it was being transformed from a cis to a medial cisterna via retrograde COP Ib-type vesicle transport at the time of fixation. A ribosome-excluding Golgi matrix/scaffold (A) encompasses both the Golgi stack and the TGN cisternae. C) Immunolabeled Golgi stack and associated ER cisterna in a tobacco BY2 cell expressing the scaffold protein Atp115-GFP. Scale bars = 300 nm.

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In *Arabidopsis*, the cis-Golgi cisternae function as membrane assembly and sorting compartments, whereas the biosynthetic activities are localized in the medial and trans cisternae.

As mentioned above, we postulate that the lack of luminal staining of cis-Golgi cisternae in algae and plants is due to their undetectably low biosynthetic activities, and that the conversion of a late cis cisterna into a medial cisterna involves activation of biosynthetic machinery brought on by the recycling of proteins from the first medial cisterna to the adjacent, late stage cis cisterna. To test this hypothesis, we have analyzed the functional properties of cis, medial, and trans cisternae of the *Arabidopsis* Golgi using immunolocalization methods. In particular, we have examined the distribution of ER resident proteins as well as several Golgi marker proteins and polysaccharide products in cells preserved by high-pressure freezing/freeze-substitution methods. To determine which Golgi cisternae recycle ER proteins back to the ER, we immunolabeled root tip cells with anti-native BiP antibodies. The specificity of the antibodies is shown in Figure S4, and the immunolabeled thin section electron micrographs in Figure 7A,B. Although most of the gold labels are seen over ER cisternae, some are also associated with cis- (but not medial- or trans-) Golgi cisternae, consistent with the hypothesis that BiP is recycled to the ER from growing cis-Golgi cisternae.

α-1,2-Mannosidase I (ManI) is the enzyme that mediates the first reaction of the N-linked glycan-processing pathway in the Golgi. To determine the localization of this enzyme in the *Arabidopsis* Golgi, we have employed an antibody (56) that detects both of the native ManI isotypes (65 and 63.5 kDa). As shown in Figure 8A the specificity of this antibody was confirmed as documented by the immunoblots of wild type and the At1g51590 and At3g21160 mannosidase I knockout mutant plants. The anti-mannosidase antibody was found to predominantly label the medial Golgi cisternae (Figure 8B,C). To confirm this finding, immunolabeled serial thin sections of an entire Golgi stack were used to generate an electron tomography model of the Golgi stack and associated anti-mannosidase/anti-rabbit-gold particle labeled antibodies (Figure 8D). Quantitative analysis of the distribution of the anti-ManI immunogold labeling over several Golgi stacks, demonstrated that ∼90% of the gold particles were located over the C3 and C4 cisternae (Figure 8E). These results demonstrated that mannosidase I is predominantly associated with the C3 and C4 medial cisternae.

To further test the functional differentiation of cis and medial cisternae, we carried out double labeling of *Arabidopsis* root tip cells overexpressing GFP-HDEL with the anti-Man I antibody and an anti-GFP antibody. GFP-HDEL localizes mostly to the ER and to the cis-Golgi. The
Golgi-associated GFP-immunogold particles are confined to the cis cisternae and they never overlap with anti-Man I immunogold particles in the medial cisternae (Figure S5). This segregation of immunogold particles provides further support for the hypothesis that in plants the cis-Golgi cisternae serve as cisterna assembly and ER protein recycling sites and that activation of the biosynthetic functions coincides with the cis to medial cisterna transformation.

Antibodies raised against two complex cell wall matrix polysaccharides, xyloglucan (XG) and polygalacturonic acid/rhamnogalacturonan I (PGA/RGI) were previously used to localize their synthesis and transport within the Golgi stacks of sycamore maple suspension cultured cells. The antibodies bind to backbone regions of the two polysaccharides (8). As shown in Figure S6A,B, the anti-XG antibodies localized exclusively to trans-Golgi and TGN cisternae, whereas and the anti-PGA/RGI antibodies labeled the medial-, trans-Golgi and TGN cisternae.

The defining structural characteristics of cis-Golgi cisternae of S. dubia and Arabidopsis are also seen in C. reinhardtii and in Venus flytrap cells

To determine if the structural features of cis- and medial/trans-Golgi cisternae of S. dubia and Arabidopsis are characteristic of algal and plant Golgi in general, we have produced tomograms and models of Golgi stacks of the green alga C. reinhardtii and the plant D. muscipula (Venus flytrap) preserved by high-pressure freezing/freeze-substitution techniques. Figure 9A illustrates a tomographic slice image of a large ER cisterna and an adjacent Chlamydomonas Golgi stack. The slice image shows three cis-type cisternae, which are variable in size and possess unstained lumen in contrast to the medial and trans cisternae where the luminal contents appear stained. The tomographic models (Figure 9B) depict cis-side, face-on views of two Golgi stacks. The cis-most cisternae demonstrate the same type of highly variable architecture as documented for S. dubia (Figure 3A–C) and Arabidopsis (Figure 6A–C).

The Venus flytrap Golgi stack (Figure 9C) exhibits two cis-type Golgi cisternae with unstained lumen and a sharp transition to the medial/trans cisternae with their stained luminal contents. In the tomographic Golgi model (Figure 9D) the cis-most cisterna appears highly fenestrated and exhibits evidence of vesicle fusion sites being located preferentially at the ends of peripheral tubules.

Discussion

Cis-Golgi cisterna assembly occurs in discrete steps that are spatially and temporally separated

The central findings of this study are that in algae and plants cis-Golgi cisternae are formed de novo as postulated by the cisternal progression/maturation model, and that the assembly process appears divided into discrete steps that are both spatially and temporally separated. The initial assembly steps are confined to the cis-most region of the Golgi stack, the C1 cisterna region, and include the formation of cis cisterna initiators that expand and merge into a coherent cis cisterna. Completion of this initial stage renders the newly formed cis-type cisterna competent to nucleate the formation of a new set of cis cisterna initiators leading to the transformation of the C1 cisterna into a C2-type cis cisterna.

C2 cisternae of S. dubia also possess the ability to mediate the self-assembly of scale proteins into complexes
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Figure 7: Immuno-electron micrographs of ER and Golgi cisternae of Arabidopsis meristem cells labeled with anti-native BiP – gold antibodies. The binding immunoglobulin protein (BiP), an ER chaperone, is localized mostly to the ER, but some labeling is seen over cis-Golgi cisternae (arrowheads) from where it is recycled back to the ER. No gold particles are present over medial- and trans-Golgi cisternae. Scale bars = 300 nm.

visible by electron microscopy (Figure 4A,B). Similarly, in Arabidopsis embryo cells, the C2 cis cisternae possess the capacity to induce storage proteins to form tight aggregates in bud-like protrusions of the cisternal rims for transport though the Golgi stack (49). One hypothesis is that there is a triggering mechanism (e.g. acidification of the C2 cis-cisternae, relative to the cis-cisternal initiators and ER (57,58) that promotes the assembly of the protein complexes in S. dubia and for storage protein aggregate formation in Arabidopsis. In support of this idea, the formation of heteromeric complexes of Golgi enzymes in mammalian cells has been shown to be dependent on Golgi acidity (59).

The presence of multiple cis Golgi cisternae may allow cells to increase the rate of cisterna production by providing more time for the recognition and recycling of ER resident proteins back to the ER. Transformation of the fully formed cis cisternae into medial cisternae appears to involve the retrograde transport of medial cisternae enzymes via COPIb-type vesicles from the preceding medial cisternae (Figures 2H, 8 and S5; (45)). The advantage of having a spatial and temporal separation of the different cisternal assembly steps would be that it provides a means for introducing quality control points and for regulating the rate of cisterna production.

De novo formation of cis-Golgi cisternae from COPII vesicles via cisterna initiators is confined to the cis-most (C1) cisterna region of Golgi stacks

Our electron tomography data suggest that the initiator formation of cis-Golgi cisternae in S. dubia, Chlamydomonas and plants involves the same types of structural assembly intermediates, and that based on structural criteria, the assembly process can be subdivided into different steps as outlined above.

In S. dubia, the cis cisterna initiators appear to be generated de novo from the fusion of 3–5 COPII vesicles upon coming into contact with the underlying C2 cisterna. The number of COPII vesicles involved in the formation of a cisternal initiator is based on two measurements, the surface area of the smallest cisterna initiators in terms of COPII vesicle surface area equivalents (Figure 3C), and the number of COPII vesicles joined together into clusters by 65 nm long linker molecules with shorter molecules forming links to the surface of the C2 cisterna (Figures 2E and S2). In contrast to S. dubia, we have not observed any linkers between COPII vesicles in C. reinhardtii, Arabidopsis (Figure 5; (45,29)), Venus flytrap, or the yeast P. pastoris (16), despite the fact that the surface area of the smallest cisterna initiators of Arabidopsis is also equivalent to the surface area of 3–5 COPII vesicles (Figure 6A). We do not have any information on the nature of the linker molecules seen in S. dubia nor do we know why such linkers were only observed in S. dubia. However, the formation of pre-fusion COPII vesicle clusters in S. dubia might reflect a mechanism for speeding up the formation of cis cisterna initiators via homotypic vesicle fusion.

The need for speed in the assembly of the cisterna initiators is suggested by the following simple calculations: S. dubia cells possess two Golgi stacks (Figure S1), which have an average diameter of ~1.5 μm and consist of 16–20 cisternae. During flagella regeneration each Golgi produces a new cisterna every 15 s (18,53). Assuming a COPII vesicle size of ~70 nm and a cisternal assembly rate of 1 cisterna per 15 s (while neglecting the effects of COPIa-mediated membrane retrograde transport from the Golgi to the ER), one can calculate that each ER export site has to produce ~230 COPII vesicles in 15 s, or ~15 COPII vesicles per second to provide the needed membrane components for cisternal assembly. Because the assembly and maturation of cis-Golgi cisternae in interphase cells is spread out over five cisternae (Figures 1B, 2D–H and 3D), the formation of each new, C1 cisterna requires approximately ~50 COPII vesicles in 15 s. For comparison, in plant cells, where most Golgi stacks have two cis-type cisternae (Figure 5), and where a new cisterna is generated...
approximately every 2–4 min (60), the assembly of a cis-most cisterna requires the processing of ∼25 COPII vesicles in 120–240 s. Furthermore, the surface area of the cis-most cisterna assembly area is much smaller in Arabidopsis than in S. dubia, which greatly increases the probability of 3–5 COPII vesicles being able to form a cluster capable of efficiently nucleating a cis cisterna initiator within the required time period. COPII vesicle-mediated expansion of the smallest cisterna initiators leads to branched, tubular cisterna assembly intermediates.
(Figures 3B and 5B) probably due to the preferential fusion of the COPII vesicles with the tip regions of the tubular domains. Over time the branched tubular initiators expand and then fuse into one coherent C1 cisterna, which would then be ready to function as a C2 cisterna (Figures 3 and 6).

**The C2 to Cn cis cisternae expand in surface area via fusion with COPII vesicles and produce COPIa-type Golgi-to-ER recycling vesicles**

The Golgi stacks of plants and algae contain from 2 to 5 cis-type cisternae (Figures 1, 5 and 9). As discussed above, the de novo assembly of cis cisternae via cisterna initiators appears to be limited to the C1 cisterna region of the stacks. Cisternal initiators are not observed to form in the C2 to Cn cis cisterna regions, but these latter cisternae do undergo expansion and release COPla-type vesicles with unstained cores (Figure 2B) that can recycle ER molecules from cis cisternae to the ER (Figures 3, 6 and 9; (45)). As reported previously and documented in Figure 2D,H, there is virtually no overlap in the distribution of the COPla vesicles and the dark core COPlb-type vesicles, which recycle enzymes between, medial- and Golgi-associated TGN cisternae (10,45).

Typically, one third of the cisternae within an algal, plant or yeast Golgi stack can be identified as cis-type cisternae. In *P. pastoris* (16) the stacks contain 1 cis-, 1 medial- and 1 trans-Golgi cisterna; in plant Golgi, two out of six cisternae are cis-type cisternae (Figures 5 and 9C; (55)); in *Chlamydomonas* three out of nine (Figure 9A), and in *S. dubia* 5 out of 16–20 cisternae (Figure 1). We do not know what determines the size and the number of cisternae in Golgi stacks in different organisms, but a faster rate of cisterna production and larger cisternae provide a means for increasing the output of a given Golgi stack. In this context, the biosynthetic capability of the two giant Golgi stacks of *S. dubia* is truly amazing, considering that during cytokinesis they can produce $\sim 1.2$ million cell wall scales for a daughter cell in $\sim 3$ h (53)!

**Transformation of cis-Golgi cisternae into medial cisternae and concomitant activation of biosynthesis appear to be coupled to enzyme recycling via COPlb-type vesicles**

The lack of staining of the luminal contents of cis-Golgi cisternae, and the sudden appearance of stained luminal contents in medial cisternae in electron micrographs of high-pressure frozen/freeze-substituted plant and algal
cells (Figures 1, 4, 5 and 9) has remained an enigma for the past 20 years (30,55). Evidence presented in this study suggests that the lack of staining of cis-Golgi cisternae is due to the undetectably low biosynthetic activities. This finding lends further support to the hypothesis that cis-Golgi cisterna assembly occurs in a stepwise manner, and that a given Golgi cisterna does not progress to the next developmental stage until it has completed the assembly steps of the preceding stage. In this view, the overriding function of the cis-Golgi cisternal compartments in plant and algae is to produce new, full size Golgi cisternae before they become engaged in the biosynthetic activity of the Golgi as medial cisternae.

The flagellar scales of S. dubia are composed of glycoproteins with acidic polysaccharide side chains (61). As documented in Figure 4A,B, self-assembly of the glycoproteins into scale complexes occurs in the C2 cis cisternae. However, once assembled, no changes in structure and staining of the scales are seen until they reach the first medial (C6) cisterna (Figure 4A–D). The lack of changes in scale staining during cis cisterna growth and maturation is consistent with the hypothesis that the acidic polysaccharide chains are only added after the cis cisternae have been transformed into medial-Golgi cisternae. The appearance of more heavily stained scales coincides with the appearance of cisternae with stained luminal contents, a characteristic feature of medial cisternae in algae, plants, and P. pastoris (16). Since the COPII-type recycling vesicles with dense cores that bud from medial- and trans-Golgi cisternae do not extend beyond the cis/medial cisterna transition region towards the cis side of the stack (Figure 2H; (45)), we postulate that the conversion of a biosynthetically inactive cis cisterna into a biosynthetically active medial cisterna involves the recycling of biosynthetic enzymes from a medial cisterna back to a late stage cis cisterna. In this context, the general background staining of the luminal contents of the medial and trans-Golgi cisternae is likely due to the presence of nucleotide sugars needed for glycoprotein and polysaccharide synthesis (62).

To test this hypothesis in Arabidopsis, we have employed antibodies raised against native BiP, a soluble ER resident protein that is postulated to be recycled from cis-Golgi cisternae to the ER, and antibodies against native Manl, the first enzyme of the N-linked glycan-processing pathway in the Golgi (63), to determine via immuno-electron microscopy localization methods from which Golgi cisternae displaced ER proteins are recycled back to the ER, and where the processing of N-linked glycans starts. Figure 7 demonstrates that BiP is recycled from cis-Golgi cisternae, and that 90% of the native Manl molecules are located in medial Golgi cisternae (Figure 8B–E). These results provide evidence in support of our hypothesis that cis-Golgi cisternae serve primarily as sites of cisternal membrane assembly, and that the biosynthetic functions of plant and algal (and probably yeast) Golgi are located in medial and trans-Golgi cisternae.

Consistent with this hypothesis, we have localized a GFP-HDEL fusion protein expressed in Arabidopsis root tip cells to ER and cis-Golgi cisternae (Figure S5), and demonstrated that nascent complex polysaccharides immunolocalize to medial and trans-Golgi cisternae (Figure S6). Previous immunolabeling studies of high-pressure frozen sycamore maple suspension culture cells have reported that biosynthesis and methylesterification of the homogalacturonan backbone domains of pectic polysaccharides occurs in medial cisternae, and that the two plant-specific sugar groups of N-linked glycans, β1, 2-xylene and α1, and 3-fucose are added specifically in medial and trans-Golgi cisternae, respectively (8,64).

During the past decade, a number of studies have employed Golgi enzyme-GFP fusion protein constructs to determine the in situ localization of integral Golgi membrane proteins, including xyloglucan synthesizing enzymes (65) N-glycan-processing enzymes (66) and, most notably, α-mannosidase I (25,67). In these latter studies, α-mannosidase I-GFP was reported to localize to the ER as well as to cis, medial and trans-Golgi cisternae. In contrast, our immunolabeling data show that native α-mannosidase I is only present in medial-Golgi cisternae.

Similar reservations also apply to the Chevalier et al. (65) investigation in which xyloglucan synthesis enzymes were localized via GFP-enzyme fusion proteins to cis-, medial- and trans-Golgi cisternae. If these enzymes were active, then their polysaccharide products should co-localize with the corresponding enzymes. However, three immunolocalization studies using both anti-xyloglucan backbone and sidechain antibodies have shown that xyloglucan molecules can only be detected in trans-Golgi and TGN cisternae (8,68,69) and never in cis or medial cisternae. A second problem of the Chevalier et al. (65) paper relates to the fact that the cells were treated with toxic concentrations (20%) of the penetrating cryoprotectant glycerol (70) prior to high-pressure freezing. In conclusion, these different studies demonstrate that GFP reporters of membrane proteins of the secretory pathway can produce inaccurate localization data as postulated by Moore and Murphy (71).

In mammalian cells the first half of the cis cisterna assembly process occurs in the ERGIC and the second half on the surface of the cis-most Golgi cisterna

A fundamental difference between the ER-Golgi secretory apparatus of plant, algal, P. pastoris and protozoan cells and the ER-Golgi secretory apparatus of mammalian cells is that the latter includes an intermediate compartment between ER export sites and Golgi, the ER-Golgi intermediate compartment [ERGIC; (72)], also known as a vesicular tubular cluster (VTC; (73)]. Functionally, the ERGIC has been defined as the first post-ER sorting compartment for anterograde and retrograde protein traffic (33). Its main structural features include a COPII vesicle-budding ER export site, a closely associated cluster of vesicles and small, pleomorphic...
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vesicular-tubular elements formed by homotypic fusion of COPII vesicles, COPI vesicles budding from the tubulovesicular element, and an encompassing, ribosome-excluding scaffold system that physically couples the ER export site to the different types of tubulovesicular membrane compartments (72). The signature marker protein of the ERGIC is the membrane protein ERGIC-53, which cycles between the ER and the ERGIC (33). p115 is a scaffold-forming long coiled-coil protein that localizes to the ERGIC (33,74). It recruits a select set of SNARE proteins to budding COPII vesicles (75) and mediates Golgi-associated tethering functions in concert with giantin and GM 130 (76,77).

The ERGIC system of mammalian cells and the ER-Golgi interface region of plant and algal cells have many common features. In both systems the ER export sites bud COPII vesicles that produce small, pleomorphic membrane structures via homotypic vesicle fusion (Figures 3A–C and 6A–D) – as being part of the ERGIC (Figures 3A–C and 6A–C) – as being part of the ERGIC system. However, in light of more recent experimental evidence on the nature of the ERGIC (33), we now interpret the branched tubular structures to be ERGIC-derived tubulovesicular elements that have translocated to the Golgi and are in the process of assembling into a pre-cis cisterna layer (see Figure 2 in (44)). Examples of more mature pre-cis cisterna layers in which the tubulovesicular elements have started to fuse into a C1 cis cisterna can be seen in the electron tomography models depicted in Figure 2A,B of Mogelsvang et al. (80). Based on this information, we propose that the cis cisterna assembly events associated with the C1 cisterna region in plants and algae occur in mammalian cells in two separate cellular locations, the ERGIC and on the surface of the cis-most Golgi cisterna, and that the ERGIC-derived tubulovesicular vesicles are the building blocks of the new cis cisternae.

The extent to which the pre-cis cisterna layers and the C1 cis cisternae of mammalian Golgi are biosynthetically active has yet to be determined in a definitive manner. Cell biology textbooks typically depict α-mannosidase I as a cis cisterna enzyme, but a review of the original literature provides little direct support for this claim for cells that have not been subjected to experimental manipulation. Indeed, several immunolabeling studies have reported that α-mannosidase I (81) and N-acetylglucosamine transferase I (36,82) are located primarily in medial and trans-Golgi cisternae and not in cis-Golgi cisternae of mammalian cells. Based on this information and the data presented in this communication, a re-evaluation of the electron microscopic localization of biosynthetic enzymes in mammalian Golgi by means of cryopreservation methods and well-defined antibodies raised against different native enzyme isoforms is needed.

Materials and Methods

Strains, culturing and plant growth

Cultures of Scherffelia dubia (Pascher), strain M0795 (52) were donated by Dr. Michael Melkonian. This culture is also available as SAG 17.88 (Sammlung von Algenkulturen der Universität Göttingen). Cultures of Chlamydomonas reinhardtii, UTEX 90, were acquired from the UTEX Culture Collection of Algae. S. dubia were grown in modified WARIS medium (WARIS-H), and C. reinhardtii in modified Bristol soil extract medium, at 15°C under 70 μE m⁻² s⁻² light (40W wide spectrum plant & aquarium) on a 14:10-h light:dark cycle (54). Sub-culturing was carried out at a 1:10 dilution of week-old cultures.

Arabidopsis plants and Venus flytrap, Dionea muscipula, (Sturtz and Copeland), were grown at 22°C under long day condition (16h of light/8h of darkness; 100μE.m⁻².s⁻² light). Arabidopsis seedlings were grown
under continuous light (100 μE m⁻² s⁻² light) before dissected before high-pressure freezing. The glands of Venus flytrap were induced to secrete digestive enzymes by stimulation of the trigger hair cells with one large drop (100 μL) of 5% bovine serum albumin (BSA) solution that was placed into each trap.

**High-pressure freezing and freeze substitution**

For *S. dubia* and *C. reinhardtii* cultures, α-mannitol (Sigma) was added to the culture medium as a cryoprotectant to a final concentration of 100 mM one hour prior to freezing. Log-phase growth cultures were centrifuged for 6 min at ~200 g in 15 mL conical tubes in a bench-top centrifuge; 2 μL of this wet pellet was loaded into an interlocking-style brass planchet.

For Venus flytraps, individual traps were removed from the plant with a razor blade. A disposable tissue punch (Technotrade) was used to punch out a circle of gland tissue measuring approximately 1.9 mm in diameter. The bottom leaf cells on this circle were removed by sectioning at a diagonal to the gland cells. This thin disc of tissue was placed into an aluminum hat measuring 2 mm in diameter and 0.3 mm in height. 120 mM α-mannitol (Sigma) or 130 mM sucrose (Malinckrodt) was added to fill the volume of the hat.

All samples were rapidly frozen in a BAL-TEC HPM-010 high-pressure freezer (Technotrade) and immediately transferred to liquid nitrogen.

Freeze substitution was carried out in 2% OsO₄/0.5% uranyl acetate in acetone at ~80°C for 4 days. The samples were then gradually warmed to room temperature over 2 days. Fixed samples were rinsed, removed from the planchets and slowly infiltrated with increasing concentrations of Epon-type resin (Ted Pella) over 4 days (48), placed in Beem capsules (Ted Pella) and polymerized under vacuum at 60°C for 48 h.

Root tips from *Arabidopsis* seedlings were frozen and freeze-substituted as described in Segui-Simarro et al. (83). For protein immunolabeling, frozen root tips were freeze-substituted in acetone with 0.2% uranyl acetate and 0.25% glutaraldehyde at ~90°C for 4 days and slowly warmed to ~60°C for 6 h. After three acetone rinses, samples were infiltrated with Lowicryl HM20 (Electron Microscopy Sciences) at ~60°C as follows: 1 day each in 25, 50 and 75% HM20 in acetone. After three changes of fresh 100% HM20 over 2 days, samples were polymerized at 60°C under UV light for 24 h. All freeze-substitution, Lowicryl embedding and polymerization were performed in a Leica AFS system under controlled time and temperature conditions.

**Immunolabeling of BiP, Manl, GFP, XG and PGA/RG-1**

Eigthy nanometer thick sections of samples embedded in Lowicryl HM20 placed on formvar-coated gold slot grids. The sections were blocked for 30 min with 2% BSA in PBST (phosphate-buffered saline + 0.1% Tween-20). The primary antibodies were diluted 1:20 in 1% BSA in PBST, and the sections were placed on the antibody solutions for 1 h at room temperature. After three rinses in 1% BSA in PBST, sections were incubated with the secondary antibody (anti-rabbit IgG conjugated to 15-nm gold particles or anti-mouse IgG conjugated to 6-nm gold particles, diluted 1:10 in 1% BSA in PBST) for 1 h. Sections were rinsed three times in PBST and distilled water. Finally, the immunolabeled samples were post stained with 3% uranyl acetate in 7% methanol (2 min) and Reynold’s lead citrate (4 min). The GFP antibody was purchased from Santa Cruz Biotechnology (Cat #: SC9998).

**Electron microscopy and dual-axis tilt series imaging**

Three hundred nanometer semi-thick sections were cut on a Leica Ultracut R microscope (Leica). Sections were collected on formvar-coated (EMS) copper electron microscopy slot grids and stained with 2% uranyl acetate in 70% methanol followed by Reynold’s lead citrate. Samples were previewed with a Philips CM10 microscope (Phillips). Dual-axis ±60° tilt series were collected using Serial EM (84) on a 300 kV Tecnai F30 IVEM running at 300 kV (FeI).

**Tomogram reconstruction and modeling**

Tomograms were constructed using an R-weighted back projection algorithm and dual-axis tomograms combined using a warping algorithm with the IMOD software package (85). Dual-axis tomograms from serial thick sections were aligned using the Midaes program within IMOD to produce large reconstructed volumes (78). Tomograms were displayed and analyzed using the imodinfo software package (86). Membranes were modeled as described in Ladinsky et al. (44) and quantitative data was extracted from 3-D models using the imodinfo program in IMOD.

**Identification of α-1,2-mannosidase I knockout mutants and immunoblot analysis of α-1,2-mannosidase**

SALK T-DNA insertion lines that were annotated to contain T-DNAs in both At1g51590 and At3g51590 were obtained from Arabidopsis Biological Resource Center and analyzed by PCR using combinations of gene-specific and T-DNA-specific left border (SALK LB) primers listed in Tables S1 and S2. Integrated DNA Technologies synthesized all primers used in this study.

Total protein was extracted from ten 5-day-old seedlings from each SALK line identified as either heterozygous or homozygous for the T-DNA insertion. The extracted samples were subjected to SDS-PAGE on a 7.5% acrylamide gel, immobilized on a nitrocellulose membrane and this membrane was blocked overnight at 4°C in BLOTTO (2% nonfat dry milk in 20 mM Tris-HCl pH 7.5, 150 mM NaCl) supplemented with 0.02% sodium azide. The portion of the nitrocellulose membrane resolving proteins weighing less than approximately 90 kDa was incubated with an anti-α,1,2-mannosidase antibody diluted 1:100 in BLOTTO for 1 h at room temperature. The blot was washed twice for 5 min each with TBST (1xTBS, 0.1% Tween-20), then incubated 1 h at room temperature with a 1:10,000 dilution of donkey anti-rabbit IgG horseradish peroxidase secondary antibodies (Amersham). The upper portion of the membrane was incubated with a 1:500 dilution of an anti-AtCDA48 polyclonal antibody (56) washed as above, and then incubated with anti-chicken IgY horseradish peroxidase secondary antibodies (Invitrogen). Both membrane portions were washed three times with TBST for 10 min each, and proteins were detected with the enhanced chemiluminescence protein gel blot detection system (GE Healthcare) according to the manufacturer’s instructions.

**Acknowledgments**

We would like to thank Drs David Mastronarde and Tom Giddings, and Mary Morphew for technical advice and guidance, and the members of the Staehelin laboratory and the Boulder Laboratory for 3-D Electron Microscopy of Cells for helpful discussions. We also thank Dr Inhwan Hwang and Dr David Robinson for the Arabidopsis GFP-HDEL overexpression line and the AtSec23 antibody. National Institutes of Health grant GM-61306 to L.A.S and National Science Foundation grant MCB-0958107 to B.-H. K. supported this work. B.S.D was partially supported as part of the Center for Direct Catalytic Conversion of Biomass to Biofuels (C3Bio), an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, Award Number DE-SC0000997.

**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Movie S1**: Serial tomogram of *S. dubia* Golgi and surrounding membrane systems.
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