TRAFFIC JAMS AFFECT PLANT DEVELOPMENT AND SIGNAL TRANSDUCTION

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Analysis of the *Arabidopsis thaliana* endomembrane system has shown that plant cell viability depends on a properly functioning vacuole and intact vesicular trafficking. The endomembrane system is also essential for various aspects of plant development and signal transduction. In this review, we discuss examples of these newly discovered roles for the endomembrane system in plants, and new experimental approaches and technologies that are based on high-throughput screens, which combine chemical genetics and automated confocal microscopy.

PLANT CELL BIOLOGY

TURGOR PRESSURE
Force generated by water pushing outward on the plant cell wall, resulting in plant rigidity. The loss of turgor pressure causes wilting.

PHENOLICS
A diverse group of secondary metabolites. They function in the defence responses against herbivores and pathogens; provide mechanical support; absorb harmful UV light; contribute to taste, odour and flower colour; attract pollinators and fruit dispensers; and can reduce competition by inhibiting the growth of nearby plants.

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Within a plant cell, the vacuole is the most prominent compartment. Occupying up to 90% of the volume of a cell, it is responsible for various processes, some of which are unique to the plant cell[1–3]. The vacuole is the storage site of ions, sugars, polysaccharides, pigments, proteins and even the flavour compounds of fruits and vegetables[4–7]. Vacuoles determine cell size by the osmotic uptake of water and the generation of TURGOR PRESSURE[8,9]. They maintain cellular homeostasis by serving as reservoirs for ions and protons, which in turn regulate the cytosolic pH. As in yeast and animal cells, vacuoles are a site of breakdown and recycling of cellular components, and are therefore important for development, programmed cell death and senescence[10–12]. The vacuole also participates in defence responses, accumulating and sequestering toxic compounds, and also stores defence molecules such as PHENOLICS, ALKALOIDS and protease inhibitors[13,14].

The plant cell vacuole is part of the endomembrane system that also includes the secretory pathway. This system comprises several compartments: the endoplasmic reticulum (ER), Golgi apparatus, *trans*-Golgi network, PRE-VACUOLAR COMPARTMENTS, the vacuole and endosomes (FIG. 1)[15]. In plants, there are at least two kinds of vacuoles. Lytic vacuoles have an acidic pH and are equivalent in function to lysosomes in yeast and mammalian cells, whereas plant-specific protein-storage vacuoles are of neutral pH (REF. 16).

Transport between the compartments of the endomembrane system is accomplished by vesicles. For soluble vacuolar proteins, there are at least two separate delivery routes that are based on the location and peptide sequence of the sorting signal. For example, SWEET POTATO SPORAMIN and BARLEY ALEURAIN carry a sorting signal with an NPIR MOTIF that is found within an amino-terminal propeptide (NTPP) signal[17]. These proteins first enter the endomembrane system at the ER and are then transported through the Golgi apparatus to the *trans*-Golgi network. At the *trans*-Golgi network, a membrane-bound cargo receptor, such as VSR1 (formerly ELP) or BP-80, recognizes the NTPP signal and directs those proteins into clathrin-coated vesicles[18–20]. These vesicles deliver their cargo to the pre-vacuolar compartment, which then fuses with the vacuole. Plant SNARE proteins are involved in this process (BOX 1)[21–23].

Alternatively, some proteins have a vacuolar sorting signal at the carboxyl terminus. Carboxy-terminal propeptides (CTPP) do not have a common sequence motif but are often enriched in hydrophobic amino acids. CTPP cargoes are delivered through a route that is thought to be mechanistically different from the NTPP pathway[24]. Often, different types of sorting signal determine the destination to a specific vacuole. Most NTPP cargoes are delivered into the lytic vacuole, whereas CTPP cargo is sent to pH-neutral storage vacuoles.

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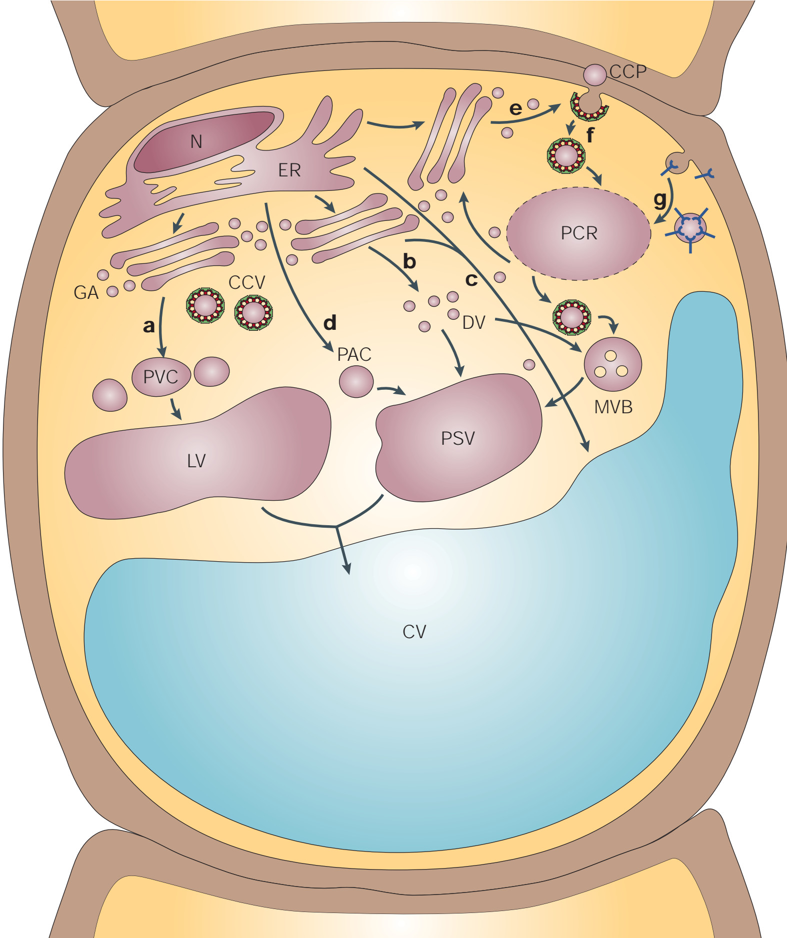

Figure 1 | **The plant endomembrane system.** The plant endomembrane system contains compartments and trafficking components that are conserved among all eukaryotes and some that are unique to plants. A recent report suggests that in BY-2 tobacco cell cultures, multivesicular bodies (MVBs) contain vacuolar sorting receptors, indicating a role as a prevacuolar compartment[105]. **a** | Amino-terminal propeptide (NTPP) pathway. **b** | Carboxy-terminal propeptides (CTPP) pathway. **c** | ER-to-vacuole pathway. **d** | ER-to-PAC-to-vacuole pathway. **e** | Secretion pathway. **f** | CCV endocytosis. **g** | Receptor-mediated endocytosis. CCP, clathrin-coated pit; CCV, clathrin-coated vesicle; CV, central vacuole; DV, dense vesicle; ER, endoplasmic reticulum; GA, Golgi apparatus; LV, lytic vacuole; N, nucleus; PAC, precursor-accumulating compartment; PB, protein body; PCR, partially coated reticulum; PSV, protein-storage vacuole; PVC, pre-vacuolar compartment; SV, secretory vesicle.

Sometimes, the lytic and the protein-storage vacuoles fuse to form one central vacuole[25]. It is possible that these pathways overlap and share common components.

The amino-acid sequences of protein families that mediate protein trafficking are conserved in plants, animals and yeast; however, it is not possible to predict their functions solely on the basis of sequence similarity. The secretory pathway has many unique features in plants, and these include some plant-specific processes, such as formation of the cell plate during cytokinesis, the secretion of cell-wall polysaccharides and protein traffic to plant-cell-specific compartments, such as the protein-storage vacuole. Furthermore, there are several cases where a component of the plant endomembrane system complements its homologue in yeast, but then is found to localize to a different compartment in plant cells[26]. In *Arabidopsis thaliana*, the isolation and characterization of mutations in genes that encode vesicular-trafficking and vacuolar-biogenesis proteins showed that several developmental processes and signal-transduction pathways rely on normal cargo-protein movement within the cell. In this review, we will focus on recent advances in our understanding of the role of the endomembrane system in plant development and signalling.

ALKALOIDS
Generally, nitrogen-containing basic plant compounds that are often used as pharmacological agents, although there are examples of alkaloids that are not basic and/or of plant origin. Alkaloids participate in plant defences and are secreted in response to tissue damage. Compounds include nicotine, caffeine, quinine, cocaine, heroin, codeine and morphine.

The vacuole is necessary for plant viability

The wide-ranging and unique functions of the vacuole in plant cells indicate that this organelle is indispensable; indeed, the recessive *A. thaliana vacuoless 1* (*vcl1*) knock-out mutant is not viable[27]. The *vcl1* allele was isolated in a screen for mutant embryos that contained suspensor cells with altered vacuole morphology. Suspensor cells form a specialized embryonic tissue, which is highly vacuolated and has been proposed to function in the transport of nutrients from the maternal tissues to the developing embryo[28]. The *vcl1* mutant is unable to form vacuoles and mislocalizes the vacuolar contents to the APOPLAST; indeed, localization studies of the vacuole-resident protein aleurain show that it is found in the apoplast instead of the vacuole in this mutant. The absence of vacuoles results in defects in cell elongation and disrupts the orientation of cell division in the embryo, which, in turn, triggers the formation of AUTOPHAGOSOMES. The *VCL1* gene is expressed primarily in dividing and expanding cells. Significantly, the *A. thaliana vcl1* mutant dies at the TORPEDO STAGE of embryonic development. The severity of this complete loss-of-function allele shows that, unlike in yeast, the presence of functional vacuoles and the correct targeting of cellular material to vacuoles are necessary, not only for plant viability, but also for plant cell growth and expansion.

A. thaliana VCL1 is 24% identical to yeast Vps16 (vacuolar protein sorting 16), which is part of the TONOPLAST-associated CLASS-C VPS (C-VPS) PROTEIN COMPLEX that regulates HOMOTYPIC FUSION of vacuoles and docking of vesicles, and is required for the very early stages of vacuole biogenesis[27,29–31]. VCL1 is a peripheral membrane protein and forms a complex with VPS11 and VPS33 (REF. 32). VPS33 is homologous to the *Drosophila melanogaster* protein that causes the *carnation* eye-pigment mutation, and *carnation* is a member of a protein complex that regulates membrane fusion of endosomal compartments[33]. VCL1 also associates with the plant SYNTAXINS SYP21 and SYP22. The complex is localized to the tonoplast and the pre-vacuolar compartment, which indicates that it regulates the fusion of the membranes at the site of the vacuole, and presumably explains why there are no observable vacuoles in the *vcl1* mutant. The lack of vacuoles in *vcl1* embryos probably interferes with nutrient flow through the suspensor, which would starve the developing embryo and trigger the formation of autophagosomes and, eventually, the death of the embryo. Therefore, these experimental data show that vacuoles are essential for plant viability.

Gravitropism

Plants are able to sense and adapt to environmental changes from a fixed location. One example is the

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Box 1 | **The SNARE hypothesis and genomic organization of SNAREs in *Arabidopsis thaliana***

Although the basic machinery for vesicular transport is conserved among all eukaryotic cells, the plant endomembrane system also has unique components. In mammals and yeast, each cell has only one type of vacuole, whereas in plants, different types of vacuole — lytic and protein storage — frequently coexist in one cell[97]. It is possible that this complex vacuolar system requires a more-sophisticated transport machinery in plants than it does in yeast. The correct targeting of proteins to the vacuole or other organelles of the endomembrane system is dependent on the action of SNAREs. Analysis of the genome of *Saccharomyces cerevisiae* has identified 26 SNAREs. In *Arabidopsis thaliana*, there are 60 genes that encode SNAREs, which are sub-grouped into several families[72] — a reflection of the more-complex endomembrane system in plants.

The majority of SNAREs are type II membrane proteins with COILED-COIL DOMAINS that are important for the interaction with other SNAREs. SNAREs are divided into two functional groups: vesicle membrane (v)-SNAREs and target membrane (t)-SNAREs (which are located on the destination membrane). Generally, three t-SNAREs form a *cis*-SNARE complex (a SNARE complex where all the constituents are on the same membrane), which is recognized by a v-SNARE that aligns its coiled-coil region to form a four-helix bundle. The formation of this complex drives the fusion of the target and vesicle membranes, which allows the delivery of cargo protein to the target compartment. Once delivery is complete, the complex is dissociated. Dissociation of the four-helical bundle requires soluble NSF (*N*-ethylmaleimide-sensitive fusion protein) and α-SNAP (soluble NSF attachment protein). After the SNARE complex is dissociated in an energy-requiring process, the v-SNARE is recycled back to its original compartment[98]. An emerging theme in the area of SNARE research is that of substitutional flexibility and genetic redundancy. For example, many (although not all) T-DNA mutants of t-SNAREs, despite the fact that they often represent multi-gene families with high homology, are embryonic lethal[99]. However, as was recently shown with the VTI12 and NPSN11 (novel plant SNARE 11) v-SNAREs, mutants might not have any discernible aberrant phenotype[54,75]. This discrepancy between the two types of SNARE protein might be a reflection of the fact that t-SNARE localization within the cell is often fixed and these proteins are not especially mobile, whereas v-SNAREs can move between subcellular compartments and therefore, as a class, have more functional flexibility[100].

gravitropic response, which ensures that the shoot is positioned to maximize its light-harvesting capabilities (known as the negative response), and that the roots are positioned downward so as to maximize the uptake of water and nutrients (positive response). The molecular mechanisms that underlie the gravitropic response are complex and can be subdivided into signal perception, signal formation, signal transduction and differential growth[34]. The differential growth response is probably mediated by the plant hormone auxin. Mutations that affect auxin synthesis, signalling and perception have gravitropic-response defects[35–38]. Other factors that are known to modulate the gravitropic response in plants are Ca^{2+}, calmodulin, inositol-1,4,5-triphosphate and pH changes; these factors are probably involved in the signal-transduction phase of the gravitropic response[39–43]. In the plant, the site of gravity perception and signal transduction in shoots seems to be the endodermal layer, a single column of cells between the cortex and the stele (FIG. 2)[44]. Shoot endoderm cells contain starch-filled plastids, known as amyloplasts, which function as statoliths that sediment in the direction of gravity.

Although the mechanisms of shoot gravitropic signal transduction are poorly understood, a genetic analysis of this phenomenon is possible. Two *A. thaliana* mutants that are impaired in endodermal development, *scarecrow* (*scr*) and *short-root* (*shr*), have an impaired shoot gravitropic response[45]. Starchless mutants such as *phosphoglucomutase* (*pgm*) also have reduced root and shoot gravitropic responses[46].

In addition, a screen for mutations of *A. thaliana* that are impaired in the shoot gravitropic response yielded seven *shoot-gravitropism-response* (*sgr*) loci[47,48]. All of these mutants have normal PHOTOTROPIC RESPONSES, processes that are also auxin dependent. Two of these mutants, *sgr1* and *sgr7*, are allelic to *scr* and *shr*, respectively. Three other gravitropic mutants have been cloned. *SGR2* encodes a phospholipase A1-like protein (PA-PLA1) that is localized to the tonoplast; *ZIG* (also known as *SGR4*) encodes a vesicle membrane (v)-SNARE, VTI11, and *SGR3* encodes the target membrane (t)-SNARE, SYP22 (REF. 49,50). VTI11 and SGR2 are deficient in the endodermal-specific gravitropic response and have vacuole defects[50]. The *zig* mutant also has several pronounced morphological defects; among these are the distinct 'zig-zag' morphology of the INFLORESCENCE STEM, a smaller size and more-wrinkled leaves compared with wild-type plants. Endodermal-specific expression of both genes complements the gravitropism defect; however, endodermal-specific complementation of the *zig1* allele does not alleviate the morphological defects, such as the 'zig-zag' stem morphology of the mutation[44]. It is noteworthy that VTI11 and SYP22 form a SNARE complex with the SYP5 family of t-SNAREs at the pre-vacuolar compartment[50,51]. The *sgr3*/*syp22-1* mutant has abnormal vacuole morphology in both the endodermal and cortex cell layers[50]. Interestingly, the root gravitropic response in all the above-mentioned mutants seems to be normal.

It is not yet clear how defects in PA-PLA1 or in components of the cellular trafficking machinery inhibit the gravitropic response. Mutations in the *ZIG* and *SGR3/SYP22* genes might cause missorting of cargo proteins, which in turn are responsible for several downstream effects that result in vacuolar abnormalities[52]. As discussed above, SYP22 is part of the *A. thaliana* C-VPS complex that mediates homotypic

PRE-VACUOLAR COMPARTMENT
(PVC). An intermediate compartment between the late endosome and the vacuole, or between the *trans*-Golgi network and the vacuole.

SWEET POTATO SPORAMIN
The major storage protein (up to 80% of the total soluble protein) in the tuberous roots of sweet potatoes.

BARLEY ALEURAIN
A vacuolar thiol protease with high homology to mammalian lysosomal cathepsin H.

NPIR MOTIF
An amino-terminal sequence motif in precursor proteins that is required for correct targeting to the vacuole.

ELP/VSR1
(*Arabidopsis thaliana* epithelial-growth-factor-receptor-like protein). A homologue of pea BP-80 that binds NTPP sequence motifs in a pH-sensitive manner. Recently renamed as VSR1 (vacuolar sorting receptor).

BP-80
An amino-terminal propeptide (NTPP) cargo receptor protein originally purified from clathrin-coated-vesicle fractions that were isolated from pea plants. BP-80 binds NTPP sorting motifs at pH 6.0 and dissociates from them at pH 5.5.

SNARE
(soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptor). SNAREs are a family of membrane-tethered coiled-coil proteins that regulate fusion reactions and target specificity in the vacuolar system. They can be divided into vesicle membrane (v)-SNAREs and target membrane (t)-SNAREs on the basis of their localization.

APOPLAST
The continuous system of intercellular spaces in plant tissues.

AUTOPHAGOSOME
A double-membrane vesicle that engulfs portions of the cytosol, and is ingested by the central vacuole where it is broken down by hydrolytic enzymes.

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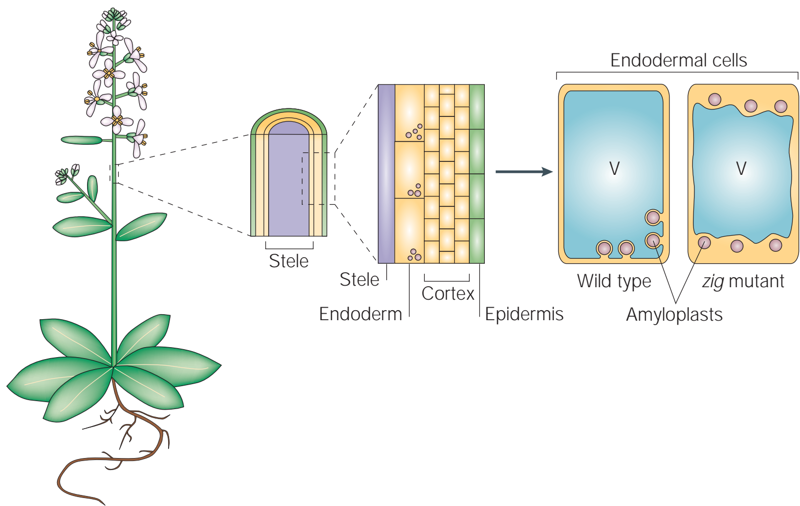

Figure 2 | **The role of vacuoles in shoot gravitropism.** The shoot gravitropic response is complex and is mediated by the endodermal layer of cells. *Arabidopsis thaliana* mutants that affect the development of the endoderm, such as *A. thaliana sgr1* (*shoot gravitropic response 1*)/*scr* (*scarecrow*) and *sgr7*/*shr* (*short-root*), also alter the shoot gravitropic response. Starch-filled amyloplasts function as statoliths that sediment to the bottom of endodermal cells. Mutants that are defective in amyloplast formation or starch biosynthesis also have abnormal gravitropic responses. In the *A. thaliana* gravitropic response mutants *zig* and *sgr2*, the properties of the endomembrane system are altered to the extent that the amyloplasts can no longer sediment to the bottom of the cells. Specifically, in wild-type cells the cytoplasm and the vacuolar membrane seem to surround the amyloplast, whereas in mutant cells the flexibility of the vacuole membrane seems to be diminished and the amyloplasts are pressed against the plasma membrane at the tops, bottoms and sides of cells. Amyloplasts also cannot travel through *trans*-vacuolar strands in the mutants. V, vacuole. Modified with permission from REF. 44 © (2002) Springer Science.

TORPEDO STAGE
One of the stages of *A. thaliana* embryonic development, which also include the preglobular, globular, heart and mature embryo stages. Also known as the mid-maturation stage.

TONOPLAST
The delimiting membrane of the central vacuole.

CLASS-C VPS PROTEIN COMPLEX
A protein complex, first described in yeast, that is required for the docking stage of the homotypic fusion of vacuoles and the heterotypic fusion of vesicles with the tonoplast.

HOMOTYPIC FUSION
The fusion of identical compartments or vesicles.

SYNTAXINS
A subset of SNARE proteins originally isolated from the presynaptic plasma membrane of neuronal and secretory cells.

vesicle fusion[32]. Likewise, PA-PLA1 that is encoded by *SGR2* might regulate vacuole-membrane fluidity and structure by degrading specific phospholipids.

Amyloplasts often travel through *trans*-vacuolar strands when they sediment to the bottom of endodermal cells. A change in the physical properties of the vacuole membrane might impede the movement of amyloplasts in the *sgr2* mutant[52]. Indeed, in both the *zig* and *sgr2* mutants, the amyloplasts are outside the vacuole and are appressed to the plasma membrane, whereas in wild-type cells the vacuole almost completely surrounds the amyloplasts[52] (FIG. 2). Whether there is a relationship between the trafficking components and PA-PLA1 is not clear either. The two classes of genes might function independently, or PA-PLA1, or other proteins that regulate PA-PLA1 function, might be transported to the vacuole by a *zig*- and *sgr3*-dependent pathway[52]. Nevertheless, the conclusion to be drawn from this mutant screen is that two proteins that are involved in vacuolar biogenesis are also involved in gravitropism.

Autophagy

Four genes of *A. thaliana*, *VTI11*, *VTI12*, *VTI13* and *VTI14*, are annotated as homologues of Vti1, a yeast v-SNARE that is required for several transport pathways to the yeast vacuole. VTI11 participates in the plant gravitropic response, as discussed above, and only *VTI11* and *VTI12* are expressed at detectable levels in *A. thaliana*. Even though these two proteins are 60% identical at the protein level, when expressed in the yeast *vti1* mutant, they complement different pathways. VTI11 complements Golgi-to-prevacuolar transport, and VTI12 substitutes in the vacuolar import of alkaline phosphatase and in the cytosol-to-vacuole transport pathway, which imports aminopeptidase I into the vacuole in yeast[23].

It had been shown that VTI12 is part of a SNARE complex with VPS45 (a Sec1-family member) and SYP4- and SYP6-family members (t-SNARE proteins). This complex seems to be localized to the *trans*-Golgi network[53] (FIG. 3). An *A. thaliana* T-DNA-insertion mutant, *vti12*, has no visible phenotype under nutrient-rich growth conditions[54]. However, when grown under nutrient-poor conditions and subjected to the DETACHED-LEAF ASSAY, *VTI12* shows an accelerated senescence phenotype, similar to those already shown for *A. thaliana* plants that are mutated in the homologues for yeast autophagy (FIG. 3)[54–56]. Therefore, in *A. thaliana*, it is hypothesized that VTI12 has a role in autophagosome formation and/or autophagosome docking and fusion.

VTI11 helps to establish tissue specificity

A double mutant of *zig* and *vti12* lends further insight into the function of VTI11 and the nature of genetic redundancy in *A. thaliana*[54]. The homozygous double mutant is embryonic lethal. Interestingly, *VTI12*/*vti12*$^{+/-}$ *zig*/*zig*$^{-/-}$ plants have an 'enhanced *zig*' phenotype. Mutants of *zig* have numerous morphological defects; they are smaller than wild-type plants, have wrinkled leaves, and the inflorescence stem has a distinctive 'zig-zag' morphology[49]. The 'enhanced *zig*' plants are more wrinkled than *zig* plants, have an aberrant PHYLLOTAXY, sometimes develop FASCIATED inflorescence stems, and are also delayed in their development compared with the single-mutant *zig* plants. These results show that at least some VTI-family protein is required for plant viability, and also indicates that these SNAREs can substitute for one another in their respective SNARE complexes (FIG. 3)[54].

There are further defects in plants with the enhanced *zig* phenotype. They show a marked loss of discernible tissue types. For example, in longitudinal cross-sections, it is difficult to distinguish between the cortex and endoderm cell layers. In these cross-sections, the vasculature, especially the phloem tissue, shows an almost complete loss of spatial pattern and forms a nearly continuous ring of phloem cells. In addition, *zig* plants have a reduced ability to conduct whole-plant transport of auxins. This defect does not seem to be linked to defects in vesicle cycling of auxin-efflux carrier proteins — known as PIN proteins (see below). Instead, it seems to be a direct effect of the vascular development defects[54]. Taken together, these results indicate that one of the functions of the VTI11-dependent transport pathway might be to contribute to the establishment and maintenance of tissue identity. A complete loss of VTI11 protein results in the *zig* phenotype. The VTI12 SNARE can partially substitute for VTI11, but a further reduction in the amount of VTI12 protein in the 'enhanced *zig*' double mutant causes a markedly more severe phenotype with extensive loss of tissue identity[54].

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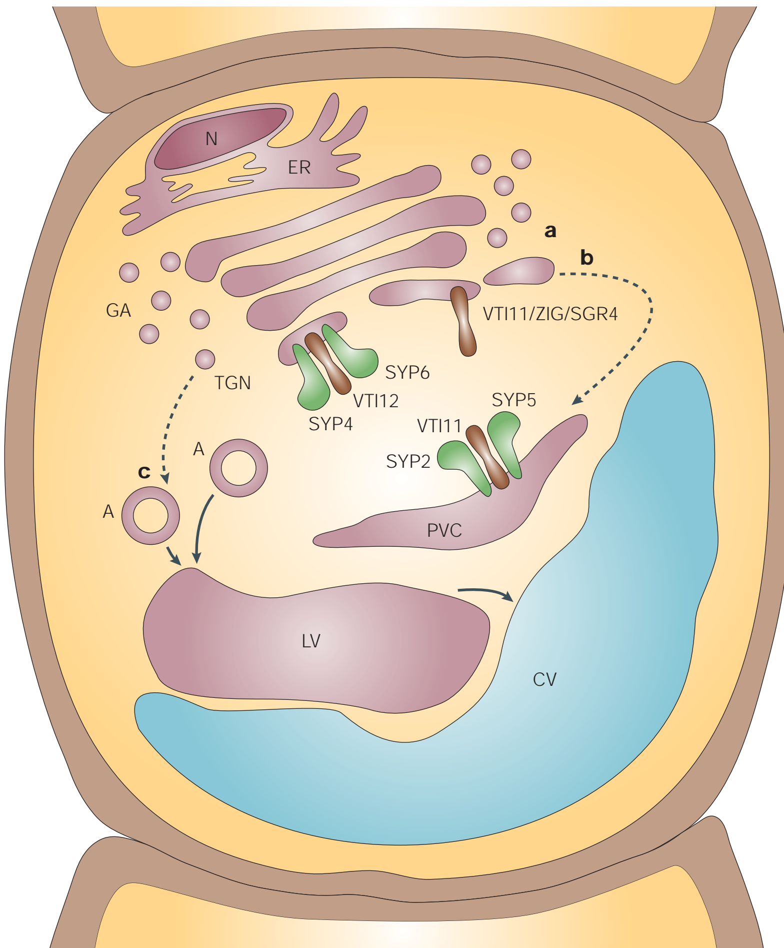

Figure 3 | **The VTI family of v-SNAREs.** The *A. thaliana* VTI family of v-SNAREs contains two members, VTI11 and VTI12, which are expressed in detectable amounts. VTI11 forms a SNARE complex at the pre-vacuolar compartment (PVC) with members of the SYP2 and SYP5 families of t-SNAREs, and VTI12 forms a complex on the *trans*-Golgi network (TGN) with members of the SYP4 and SYP6 families of t-SNAREs. Both VTI11 and VTI12 can substitute for each other in their respective SNARE complexes, at both the molecular and functional levels. VTI11 has been shown to have a role in the gravitropic response and also contributes to the establishment and maintenance of tissue identity. VTI12 participates in autophagosome formation and/or autophagosome docking and fusion with the central vacuole. The compositions of the individual SNARE complexes that are associated with these different pathways are not known. **a** | Gravitropism pathway. **b** | Cell-type-specific pathway. **c** | Cytoplasm-to-vacuole transport (CVT)/autophagy pathways. A, autophagosome; CV, central vacuole; ER, endoplasmic reticulum; GA, Golgi apparatus; LV, lytic vacuole; N, nucleus.

COILED-COIL DOMAIN
A protein structural domain that mediates subunit oligomerization. Coiled coils contain between two and five α-helices that twist around each other to form a supercoil.

PHOTOTROPIC RESPONSE
Plant growth in the direction of light.

INFLORESCENCE STEM
The post-vegetative phase of stem growth that produces floral organs.

Polar auxin transport and vesicle cycling

The transport of auxin is instrumental in setting up asymmetric growth responses and tropisms. This is accomplished by the asymmetric localization of auxin-influx and -efflux carrier proteins[57,58]. Auxins are synthesized in the uppermost portions of the plant and are transported both through the vascular system and through single files of cells in a process known as polar auxin transport. Polar auxin transport is mediated by influx and efflux carrier proteins, which are localized to the plasma membrane in an asymmetric manner. The localization of auxin-efflux carriers has been shown to be reliant on an actin-dependent vesicle transport pathway, but not one that is VTI dependent[54]. These findings were based on the observation that the PIN1 auxin-efflux carrier protein does not show polar localization in *A. thaliana gnom* embryos[59]. Mutations in a gene known as *gnom* are embryonic lethal and cause a loss of cell alignment along the embryonic axis. Treatment of *Brassica juncea* embryos with high amounts of auxin or inhibitors of polar auxin transport results in a phenocopy — the *gnom* phenotype — which indicates a link between GNOM function and auxin transport[60]. The *GNOM* gene encodes a GDP/GTP-exchange factor (GEF) for small G proteins of the ARF class (ARF-GEF)[59,61]. ARF proteins are regulators of cargo selection and vesicle budding. GNOM is also sensitive to brefeldin A (BFA), an inhibitor of vesicle trafficking[62] (BOX 2).

Treatment of wild-type seedlings with BFA causes both PIN1 and PIN3 to re-localize from the plasma membrane to an intracellular compartment. This re-localization also takes place in the presence of protein synthesis inhibitors, so the re-localized efflux carrier protein originates from the plasma membrane[63,64] (FIG. 4). Actin-depolymerization agents, such as cytochalasin D and latrunculin B, prevent the internalization of PIN proteins as well as their recycling in the presence of BFA, which indicates that the vesicle transport of PIN proteins is dependent on the actin cytoskeleton. Finally, although polar auxin-transport inhibitors do not, by themselves, have significant effects on PIN protein localization, when either applied prior to or in conjunction with BFA, PIN1 remains at the plasma membrane or in an intracellular compartment when BFA is washed out and the inhibitors remain[63].

The same effects are observed when the trafficking of non-auxin-efflux carrier proteins, such as KNOLLE (a SNARE protein) and the plasma membrane H^+-ATPase, is examined. These results raise the possibility that auxin-efflux inhibitors (AEIs) exert their effects by altering the general mechanism of vesicle cycling, as opposed to a direct interaction and inhibition of PIN proteins[63]. The interpretation of this last finding is controversial. At concentrations that impede auxin transport, the localization of PIN1 at the plasma membrane is not significantly reduced. Nor do AEIs affect actin filaments, microtubule arrangements or ER structure, as BFA does[65]. Furthermore, BFA and the AEI NPA have different dose–response curves. When seedlings are treated with AEIs, PIN1 does not re-localize to the endosomes after subsequent treatment with BFA; when BFA is washed out, AEIs prevent the return of PIN1 to the plasma membrane[63] (FIG. 4). It seems that AEIs have some effect on endosomal cycling to the plasma membrane, but probably they have a different site of action from BFA[58]. So, polar auxin transport is mediated by the endomembrane trafficking system.

Cytokinesis

In plants and animals, the cellular mechanisms for segregation of the chromosomes during mitosis and meiosis are well conserved, but there is significant divergence in the processes of CYTOKINESIS. In animal cells, a contractile actomyosin-based ring constricts the existing plasma membrane inward, forming a cleavage furrow, and

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Box 2 | Vesicular cycling and brefeldin A action

Brefeldin A (BFA) has been a valuable tool for the study of vesicular trafficking in animal and plant cells. BFA is a macrocyclic lactone of fungal origin[101]. The molecular target of BFA in both plant and animal cells seems to be a Sec7-type GTP-exchange factor that catalyses the activation of the GTPase ARF [102]. Activated ARFs recruit COPI COAT PROTEINS and CLATHRIN to form transport vesicles. Treatment of both mammalian and plant cells with BFA causes the redistribution of Golgi enzymes to the endoplasmic reticulum (ER). However, there are differences in the BFA response in mammalian and plant cells. In mammalian cells, the loss of COPI coats causes the formation of membrane tubules that eventually fuse with ER. By contrast, in plant cells, BFA treatment does not cause tubulation of the Golgi apparatus. The Golgi stacks initially retain their morphology except for the *cis*-cisternae. The remaining cisternae fuse with the ER to form hybrid stacks, or fuse with other Golgi to form oversized Golgi complexes that are continuous with the ER. These structures are unique to plants. Other studies have described a plant-specific 'BFA compartment', which is caused by the disintegration of the Golgi into a mass of tubules and vesicles. Experiments with markers from the late-Golgi and *trans*-Golgi network do not label the ER in BFA-treated cells, which indicates that BFA causes the Golgi to 'split' in a horizontal manner, whereby the *cis*-cisternae are absorbed into the ER, and the *trans*-cisternae and *trans*-Golgi network form the unique BFA compartment[103]. The recent discovery that BFA prevents auxin-efflux carrier proteins — the PIN proteins — from cycling between the plasma membrane and endosomes indicates that endocytic vesicles also contribute to the BFA compartment[63].

Not all ARF-GEFs are sensitive to BFA inhibition. Sensitivity is conferred by a specific conserved methionine residue in the catalytic domain[104]. The ARF-GEF GNOM, which is necessary for proper PIN1 localization in *A. thaliana*, is sensitive to BFA[63]. Geldner *et al.* engineered a BFA-resistant variant of GNOM and transformed it into a gnom-segregating population[62]. The engineered GNOM construct rescued homozygous mutant plants, and PIN recycling is rendered BFA resistant in rescued seedlings. GNOM is localized to the endosomes, and the BFA-resistant variant also retains the ability to mediate auxin transport and auxin-related physiological responses[62]. Therefore, the use of BFA indicates that GNOM regulates the structure and function of endosomes and vesicle traffic for polar auxin transport and, perhaps most significantly, shows that it is possible to use BFA sensitivity as a tool to analyse vesicle transport pathways.

T-DNA
DNA transferred from *Agrobacterium tumefaciens* and stably integrated into plant genomes. The insertion is random and might therefore disrupt genes, causing a mutation at the insertion point.

DETACHED-LEAF ASSAY
Standard assay used to evaluate the effects of plant stress induced by pathogens or mutations.

PHYLLOTAXY
The spatial arrangement of leaves around the plant stem.

FASCIATED
A defect in stem development where normally cylindrical stems become flattened.

ARF-GEF
(guanine nucleotide-exchange factor (GEF) for small G proteins of the ARF class). ARFs belong to the Ras superfamily of small GTP-binding proteins. GEFs mediate the conversion of GTP to GDP.

targeted addition of new membranes is required for cleavage-furrow introgression and cell separation. By contrast, cytokinesis in higher plants starts in the centre of the dividing cell with the formation of the cell plate, which grows outward and fuses with the parental plasma membrane. Trafficking of Golgi-derived vesicles to the site of cell-plate assembly is mediated by a cytoskeletal structure, known as the phragmoplast. The initiation and maturation of the cell plate probably proceeds by homotypic membrane fusion[66].

During embryogenesis in *A. thaliana*, there is a pattern of cell division and the cell shape changes. A screen for mutations that affect the body organization of *A. thaliana* seedlings yielded the seedling-lethal *knolle* (*kn*) mutant[67]. *kn* mutants have malformations in the epidermal cell layer that are due to abnormal cell divisions and enlargements. The embryos are composed of multinucleate cells with incomplete cross walls. The *kn* mutation seems to disrupt the normal pattern of embryogenesis by altering the plane of cell division, the rate of cell division and cell shape.

Positional cloning of the *KN* gene showed that it encodes a syntaxin with homology to the *A. thaliana* t-SNARE SYP111 (REF. 67). KN is membrane associated, localized to the plane of division, and is a cytokinesis-specific syntaxin[68]. *KN* mRNA is most abundant in tissues with rapidly dividing cells, such as flowers and developing SILIQUES[67,69]. The *KN* gene is very tightly cell-cycle regulated; it must be highly transcribed during M phase to produce sufficient protein, but its mRNA must be degraded rapidly to prevent the accumulation of protein following cytokinesis[69]. When expressed from non-native promoters that are active in both proliferating and non-proliferating cells, KN is mis-targeted to the plasma membrane and seems not to cause any aberrant phenotype. Protein-localization experiments show that it is present primarily during the mitosis (M) phase and telophase of the cell cycle, where it accumulates in a punctuate pattern during the M phase and localizes to the plane of division in telophase[69].

The *A. thaliana* keule (*keu*) mutant has a similar, although slightly weaker phenotype compared with *kn*, whereas *kn keu* double-mutant embryos show a significantly more severe cytokinesis defect than either single mutant[70]. The double mutant completely lacks internal cell walls, which results in a single cell containing up to 30 nuclei[71].

KEULE is a member of the Sec1 protein family. These proteins regulate vesicle docking and fusion by interacting with syntaxins[70]. Based on *in vitro* protein-binding experiments and genetic-interaction studies, it is probable that KN and KEU interact *in vivo*[70]. KEU is required for cytokinesis throughout the life cycle of the plant and is also found primarily in adult meristematic tissue. KEU is also required for root-hair growth. The *A. thaliana* genome contains six genes that are annotated as members of the KEU/Sec1 family. However, there are also 24 genes that are annotated as syntaxins[72]. Therefore, it is probable that KEU interacts with syntaxins other than KN, but it is through KN that it transduces cell-cycle signals to the cytokinesis vesicle-fusion machinery[70,71].

SNARE complexes are composed of four helical bundles (BOX 1). Generally, they contain one v-SNARE helix and three t-SNARE helices, but can also be composed of one v-SNARE, one t-SNARE and a two-helical SNAP25 protein. SNARE complexes also contain a Sec1 regulatory protein. Members of the SNARE superfamily of proteins are often characterized on the basis of either a conserved Q or R residue in their SNARE helices. All the SNARE proteins discussed in this review, whether v- or t-SNAREs, are Q-containing SNAREs[73].

The cytokinesis-specific complex that contains KN (t-SNARE) and KEU (Sec1-type protein) also contains a SNAP25 homologue, SNAP33 (SNP33)[74], which is found primarily at the plasma membrane and cell plate of dividing cells, where it colocalizes with KN. The *snp33* mutant has incomplete cell walls and the *snp33 keu* double mutant is synthetically embryonic lethal, which is also the phenotype of a *kn keu* double mutant[74].

In the cytokinesis-specific KN complex, the fourth helix is possibly NPSN11 (novel plant SNARE 11; REF. 75). Homologues of the NPSN family are absent from yeast and animal cells; however, it has recently come to light that there are possible homologues in DIATOMS and OOMYCETES (A. Sanderfoot, personal communication). NPSN11 is highly expressed in tissues that contain actively dividing cells, and is localized predominantly to

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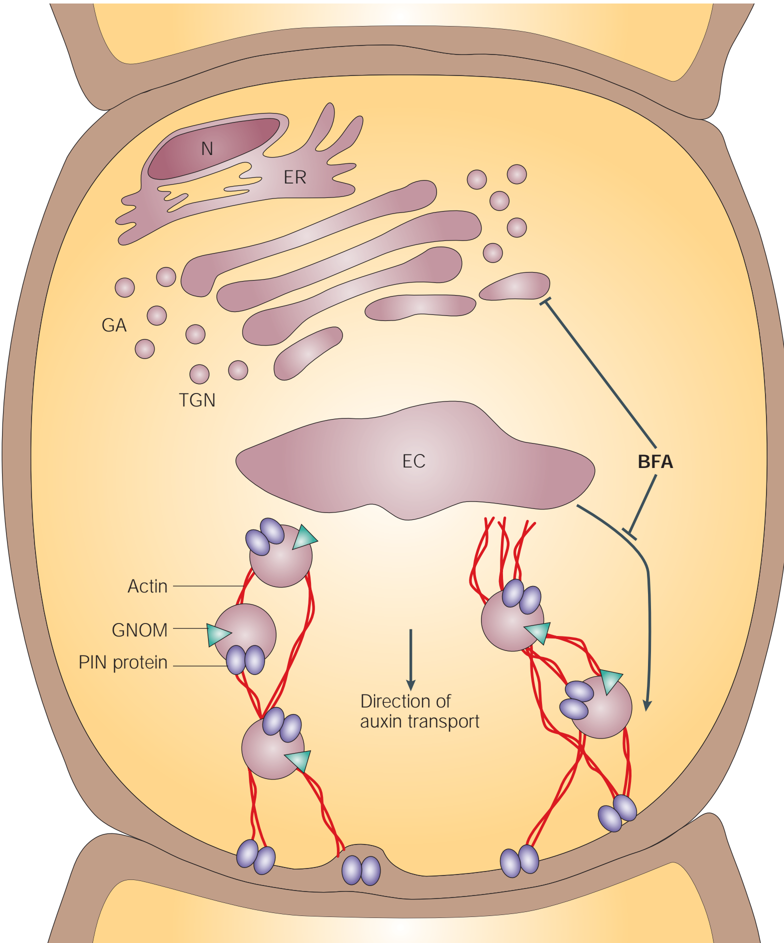

Figure 4 | **Polar auxin transport and vesicle cycling.** A model that illustrates the cycling of the PIN auxin-efflux carrier proteins shows the transport of auxin to the basal plasma membrane, the site of PIN1 localization. PIN3 (not shown), which is involved in the shoot gravitropic response, is localized to the lateral portion of the plasma membrane. PIN1 vesicles cycle between endosomal compartments and the plasma membrane in an actin-dependent manner. Brefeldin A binds GNOM, a guanine nucleotide-exchange factor (GEF) for small G proteins of the ARF class (ARF-GEF), which prevents vesicle transport between the endosomal compartment and the plasma membrane, resulting in the internal accumulation of PIN1 protein. BFA also targets the Golgi apparatus. BFA, brefeldin A; EC, endosomal compartment; ER, endoplasmic reticulum; GA, Golgi apparatus, N, nucleus; TGN, *trans*-Golgi network. Adapted from REF. 106 © (2001) Macmillan Magazines Ltd.

CYTOKINESIS
The distribution of cytoplasm to daughter cells following nuclear division.

COPI COAT PROTEIN
Coat protein of vesicles that are involved in ER and Golgi trafficking.

CLATHRIN
A protein that coats vesicles that originate on the *trans*-Golgi network (TGN) or plasma membrane.

SILIQUES
The fruit of *A. thaliana* and other cruciferous plants.

the cell plate in dividing cells[75]. The results of co-immunoprecipitation experiments show that NPSN11 interacts with KN, therefore NPSN11 seems to contribute the fourth helix to the cytokinesis-specific SNARE complex. The homozygous *npsn11* mutant has no obvious phenotype, which indicates that other members of the NPSN protein family might be able to substitute for the loss of NPSN11 in the mutant.

In yeast and mammals, Cdc48 and p97, respectively — both AAA PROTEINS — participate in a wide array of pathways, including the regulation of SNARE-complex assembly and integrity, although the mechanisms by which they interact with SNARE proteins are not well understood[76]. Mammalian and yeast p97 and Cdc48 proteins have been implicated in cell division and are involved in the homotypic membrane fusion of ER, nuclear and mitotic Golgi fragments. In *A. thaliana*, most of the cytosolic CDC48 is isolated as a hexamer and is associated with other factors that form a higher-order hetero-oligomeric complex[77]. CDC48 colocalizes to the cell-division plane with KN and SYP31. SYP31 is found in defined, punctate membrane structures during interphase and is mobilized during cytokinesis to the division plane. *In vitro* binding studies show that CDC48 specifically interacts with SYP31 (a plant orthologue of syntaxin 5) but not KN, in an ATP-dependent manner. KN assembles into a large 20S complex with the adaptor protein Sec18/NSF and the adaptor protein α-SNAP. So, it seems that at least two multiple-trafficking pathways function at the plane of division during cytokinesis (FIG. 5). It is clear that the KNOLLE pathway is required for cell-plate formation. However, it is not clear whether CDC48–SYP31 functions in parallel with the KNOLLE/NSF pathway during cell-plate assembly, or if it mediates the assembly of some other membrane system at the division plane[77].

Because many of the *A. thaliana* cytokinesis-defective mutants are embryonic or seedling lethal, a screen was carried out for weaker mutants to identify further components of the plant cytokinesis machinery. A conditional mutant that affects cytokinesis in the guard mother cells of STOMATA, *stomatal cytokinesis-defective 1-1* (*scd1-1*), was found to encode a protein containing an amino-terminal DENN domain and eight carboxy-terminal WD-40 REPEATS. DENN domains are often found in proteins that interact with members of the Rab family of small GTP-binding proteins, which in turn regulate vesicle trafficking. Examination of *scd1-2* mutant cells shows a significant accumulation of non-fused vesicles compared with wild-type cells. It is not yet known whether SCD1 interacts with either of the cytokinesis-specific SNARE complexes described above, but its isolation nonetheless underscores the importance of vesicle trafficking in plant cell cytokinesis[78].

Abscisic acid and stress responses

Abscisic acid (ABA) is a plant hormone that mediates stress responses to conditions such as drought, excessive salt and cold. For osmotic stress tolerance there is evidence that these effects are exerted through non-transcriptional pathways that regulate the opening and closing of the stomata[79]. ABA signals are transduced through inositol-1,4,5-triphosphate, reactive oxygen species and cyclic ADP-ribose[80]. These intermediates lead to a rise in the Ca^{2+} concentration in the cytosol, which in turn affects stomatal opening and closing. ABA-induced chloride currents in *Xenopus laevis* oocytes require the mRNA that encodes Nt-Syr1, a homologue of yeast syntaxins[81]. Pharmacological studies have shown that *Clostridium botulinum* neurotoxin type C (Bot N/C), which inhibits syntaxin-dependent vesicle fusion by binding and cleaving syntaxin proteins, can also inhibit ABA control of ion channels in tobacco guard cells[81]. Expression of a dominant-negative fragment of Nt-Syr1 indicated that this syntaxin mediates traffic between the Golgi complex and the plasma membrane[82].

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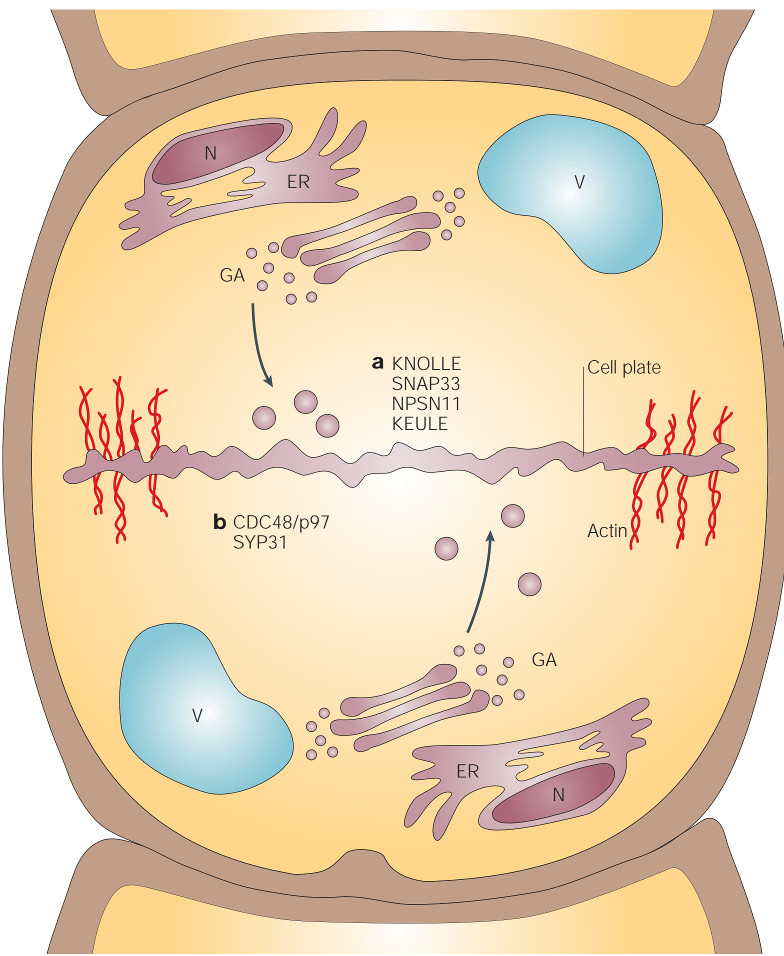

Figure 5 | **Cytokinesis pathways.** There are at least two vesicle transport pathways that are associated with plant cell cytokinesis. One is defined by a SNARE complex containing the KNOLLE and SNAP33 t-SNAREs, the NPSN11 v-SNARE and the Sec1-family protein KEULE (**a**). Another pathway is defined by a complex that contains SYP31 and the *Arabidopsis thaliana* CDC48/p97 (**b**). ER, endoplasmic reticulum; GA, Golgi apparatus; N, nucleus; V, vacuole.

DIATOM
Unicellular alga, the cell wall of which is composed of silica.

OOMYCETES
A phylum of filamentous protists that includes downy mildews and water moulds.

AAA PROTEINS
ATPases that are associated with various cellular activities.

STOMATA
Pores in the plant cell epidermis that are composed of two guard cells. Osmotic pressure regulates the size of the guard cells, which in turn control the size of the stomatal opening. The size of the stomatal opening regulates the rate of gas exchange.

The mutant *osm1* (*osmotic-stress-sensitive mutant 1*) was identified in a screen for salt-tolerant mutants in an *A. thaliana* collection of T-DNA mutants[83]. *osm1* is sensitive to several salts and soil desiccation and seems to control stomatal responses to ABA. The *osm1* gene was identified as a mutant allele of the t-SNARE *SYP61*. Although it is not a null allele, the observed phenotype is complemented with a wild-type copy of the *SYP61* gene. It is not clear precisely how SNARE proteins might control guard-cell ion-channel activity, or if indeed this function is related to their role in vesicle trafficking. The action of ABA significantly increases endocytosis and causes a reduction in the plasma membrane surface area during stomatal closure[82]. Furthermore, from mammalian systems there is evidence that links the activity of sodium channels and H+-ATPase to vesicular trafficking. Human syntaxin 1A (SYN1A) seems to inhibit epithelial sodium-channel-mediated sodium entry into cells, possibly as part of a larger mechanism that decreases the number of sodium channels at the plasma membrane[84].

Alternatively, syntaxins have been shown to function in a regulatory and non-trafficking role in mammalian systems. In humans, syntaxin 1A inhibits CFTR (cystic fibrosis transmembrane conductance regulator) chloride channels by protein–protein interactions: syntaxin 1A binds to the amino-terminal cytoplasmic tail of the CFTR-channel protein and is essential for inhibiting the chloride current[85]. CFTR binds the domain of syntaxin 1A that is necessary for a membrane fusion reaction, so it is possible that the binding activity of CFTR regulates the functional properties of syntaxin 1A as well. It might be possible that in *A. thaliana*, OSM1/SYP61 functions in a similar fashion in the regulation of turgor pressure in guard cells, therefore expanding the range of functions that are carried out by vesicular trafficking proteins to regulatory roles.

SNAREs and plant pathogen resistance

A. thaliana is naturally resistant to many pathogens of other plants, a phenomenon known as non-host resistance. For example, barley powdery mildew affects barley but not *A. thaliana*. This non-host resistance was used to isolate *pen* (*penetration*) mutants that permit the penetration of *A. thaliana* cells by barley powdery mildew but not development of the full-blown disease[86]. The gene that is responsible for the *pen* phenotype encodes syntaxin SYP121. In wild-type plants, SYP121 is localized to the plasma membrane. A functional barley homologue, ROR2, was isolated on the basis of its role in penetration resistance to a barley powdery-mildew pathogen. ROR2 is also localized to the plasma membrane and forms complexes with a SNAP25 homologue of barley, SNAP34. At the mechanistic level, penetration resistance is not well understood and these observations point to the importance of endomembrane trafficking in events that mediate penetration resistance, possibly by delivering toxins or some other biologically active compounds to the cell wall[86]. Future analysis of vesicular contents should be informative in understanding the mechanism of disease resistance in plants. This example of functional similarity between a syntaxin from a crop plant (barley) and the syntaxin from a model plant (*A. thaliana*) is relatively rare at this point. However, it highlights the utility of *A. thaliana* as a model plant for economically valuable improvements of crops.

Perspectives

A functioning vacuole and an intact vesicular trafficking system are necessary for plant cell viability and function. Perturbation of the trafficking machinery often impedes vital cell processes such as cytokinesis, the response to plant hormones and the development of tissue specificity. However, the studies described herein also highlight several new challenges that face the study of vacuolar biogenesis and the vesicular trafficking machinery. Many of the genes that encode proteins that mediate endomembrane trafficking are either single-copy genes or members of gene families, which complicates the assignment of functions to individual proteins. Unfortunately, the knockout mutations of many of these genes are often either embryonic lethal or have no obvious abnormalities.

The recent development of several new tools and methods for examining the morphology and function of the

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WD-40 REPEAT
Protein motif that is composed of a 40-amino-acid repeat that forms a β-propeller sheet. Proteins that contain WD-40 repeats participate in a wide array of cellular functions, including G-protein-mediated signal transduction, transcriptional regulation, RNA processing, and regulation of vesicle formation and trafficking.

vacuolar and endomembrane system will alleviate some of these limitations. Among these are the isolation of four groups of mutants with tonoplast-localized green fluorescent protein (GFP) fusion proteins, which will be useful for the analysis of vacuolar biogenesis and the development of high-throughput confocal microscopy with specialized culture plates for germinating and growing seedlings[87]. One of the interesting observations of these studies is that the endomembrane systems of the shoots and roots are not coupled, a factor that will have to be taken into account when designing future studies[87].

Large, diverse, chemical libraries of small organic molecules are now commercially available, which allow a new type of genetic screen to be undertaken. When chemicals from these libraries are individually applied to cell cultures or to intact organisms, it is feasible to identify a particular chemical that perturbs a specific protein or family of proteins. Using this strategy, it should be possible to draw conclusions about the function or functions of an entire family of proteins, or to engineer a single member of a gene family to be resistant to a chemical inhibitor, thereby simultaneously isolating and highlighting its function separately from the other family members. This approach was used to study GNOM, a member of the ARF-GEF family in *A. thaliana*[62] (BOX 2), and several proteins from other systems, such as Cla4 and Cdc28 in *S. cerevisiae*, and v-Src in mouse fibroblasts[88–90]. It might also be possible to study the function of a gene for which a null allele (caused by a T-DNA insertion) is lethal. The dosage of an inhibitory chemical can be adjusted to obtain a sub-lethal inactivation of the protein. It is also possible to apply such a chemical at any point during development. The biggest challenge facing the wide-scale use of chemical genetics is in the identification of target proteins or components of particular pathways. *A. thaliana* offers the opportunity to identify target pathways by genetics. For example, a chemical-genetic approach was recently used to identify a new upstream negative regulator of an auxin response[91].

The use of chemical-genetic screens might allow us to pinpoint components of vesicular trafficking pathways that heretofore evaded identification through traditional genetic screens[92,93]. The large-scale use of chemical-genetic screens will require the concomitant development of high-throughput methods to identify chemicals that modify trafficking components. Ultimately, the knowledge that is accumulated from traditional and chemical-genetic screens, along with the development of further high-throughput methods and computer modelling techniques can be used to better understand the interactions between trafficking pathways[94]. Recently, such 'systems biology' methods were used to simulate and quantify Ran-mediated nucleocytoplasmic transport in *X. laevis* egg extracts[95]. The constructed model estimated steady-state flux of Ran across the nuclear envelope and predicted a Ran gradient between the two compartments — predictions that were later verified experimentally[96]. A similar approach could be used to study cargo loading and fluxes in endomembrane trafficking pathways.

It is clear that the endomembrane trafficking system does not just deliver cargo. It is intimately involved in signal transduction and development. The new approaches described in this section will increase our opportunities to discover new connections between trafficking, plant development and signal transduction, and marks the beginning of understanding these networks of pathways.

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