REVIEW ARTICLE

The role of Rab GTPases in cell wall metabolism

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Abstract

The synthesis and modification of the cell wall must involve the production of new cell wall polymers and enzymes. Their targeted secretion to the apoplast is one of many potential control points. Since Rab GTPases have been strongly implicated in the regulation of vesicle trafficking, a review of their involvement in cell wall metabolism should throw light on this possibility. Cell wall polymer biosynthesis occurs mainly in the Golgi apparatus, except for cellulose and callose, which are made at the plasma membrane by an enzyme complex that cycles through the endomembrane system and which may be regulated by this cycling. Several systems, including the growth of root hairs and pollen tubes, cell wall softening in fruit, and the development of root nodules, are now being dissected. In these systems, secretion of wall polymers and modifying enzymes has been documented, and Rab GTPases are highly expressed. Reverse genetic experiments have been used to interfere with these GTPases and this is revealing their importance in regulation of trafficking to the wall. The role of the RabA (or Rab11) GTPases is particularly exciting in this respect.

Key words: Cell wall, fruit softening, pollen tube, Rab GTPase, root hair, root nodule, trafficking, vesicle.

Introduction

Control of the process of trafficking has been implicated in the control of a wide range of cellular and physiological processes, including gravitropism, polar auxin transport, autophagy, cytokinesis, abscisic acid (ABA) and stress responses, and pathogen resistance (Surpin and Raikhel, 2004), and cell wall metabolism is clearly another important example. The vitally important process of how the cell wall is synthesized and modified has been difficult to study at the molecular level because it occurs partly in the apoplast. Attempts to study cell wall modification by inhibiting single enzymes have met with limited success (Brummell, 2006) and it is clear that this is partly because the process needs to be highly coordinated. The elements involved are produced in different parts of the cell: cellulose at the plasma membrane (PM), other polysaccharides in the Golgi apparatus, and the enzymes responsible for both synthesis and modification on the endoplasmic reticulum (ER). Coordination of the delivery of these elements to the apoplast at the correct time must, therefore, involve trafficking through the endomembrane system.

Studies involving reverse genetic approaches, mostly, but not exclusively, by blocking the action of Rab GTPases, have now reached an exciting stage where the results from several systems are revealing their central importance in cell wall metabolism, both in highly polarized cells such as root hairs and pollen tubes and in processes such as fruit ripening. This review sets out to examine the processes by which materials destined for the cell wall reach their destination and the extent to which reverse genetic approaches, supplemented by other evidence, have established the central importance of Rab GTPases in regulating these processes.

Rab GTPases help to regulate different vesicle trafficking pathways

Most macromolecules that are secreted from the cell are transported by vesicles through the endomembrane system. Polysaccharides are synthesized at the cell membrane or in the Golgi apparatus (see later section) and secreted proteins are usually synthesized on the ER and transported via the Golgi apparatus and the trans-Golgi network (TGN) on their way to the PM (Hanton and Brandizzi, 2006; Fig. 1).
Within this trafficking system, transport is by vesicles that bud from one organelle and fuse with another, and these may move in an anterograde or retrograde direction. Fusion is promoted by complementary SNAREs (soluble N-ethylmaleimide-sensitive factor attachment proteins), the v-SNAREs on the vesicle membrane and the t-SNAREs on the target membrane, which are also often referred to as syntaxins or SYP proteins. The role of SNAREs in plants has been reviewed (Sutter et al., 2006).

However, it is thought that Rab GTPases are one regulatory factor in the fusion of the correct vesicle with the correct target membrane, and in mammalian systems there are 41 subclasses of these, each of which regulates docking of different vesicles with their target membranes (Zerial and McBride, 2001). In Arabidopsis thaliana, the 57 Rab GTPases have close homology to only eight of the types found in mammals (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003), and the same eight groups are also found in other plants (Zhang et al., 2007; Abbal et al., 2008; Table 2) though the total number of Rabs in grapevine was about half that in Arabidopsis. However, these eight have many more members than the corresponding mammalian groups and they have been renamed RabA, RabB, RabC, RabD, RabE, RabF, RabG, and RabH, and further divided into subclades (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003; Table 1). Most notably, the two mammalian Rab11 GTPases correspond to the Arabidopsis RabA clade, which has 26 members, divided into six subgroups (RabA1 – RabA6). This has led to the hypothesis that these subgroups may have evolved different functions (Rutherford and Moore, 2002), which means that we cannot be sure of the functions of these types in plants, though the evidence so far suggests that some groups have largely similar functions to their mammalian counterparts (Vernoud et al., 2003; Molendijk et al., 2004).

Different Rab GTPases regulate trafficking between different membrane compartments, and assumptions based upon homology to mammalian Rabs are now being supplemented by evidence from plant systems so that, with the exception of RabC (Rab18) for which evidence is still scarce, it is possible to predict which ones may be involved in regulating transport to and from the cell wall (Table 1, Fig. 1). RabF (corresponding to Rab5) and RabG (Rab7) proteins are expected to be associated with endocytosis, and evidence suggests that RabG is indeed involved in internalization of dye into the vacuole (Mazel et al., 2004), though this is a process that would involve trafficking steps through several compartments. Similarly, different RabFs may be involved in either endocytosis or vacuolar trafficking (Ueda et al., 2001; Grebe et al., 2003; Sohn et al., 2003; Bolte et al., 2004; Kotzer et al., 2004; Lee et al., 2004). RabE is related to the Golgi to PM-associated Rabs of mammals such as Rab8 and seems to have the same role in plants (Zheng et al., 2005). RabD

Fig. 1. Simplified schematic representation of the trafficking pathways to and from the cell wall. Pathways are shown as arrows with probable cargoes indicated. The Rab GTPases probably involved at each step are indicated in parentheses. Pathways to and from the vacuole have been omitted for simplicity. TGN/EE refers to the trans-Golgi network and early endosomal compartments which now seem to partially overlap in plant cells and possibly function in both endocytosis and exocytosis.

RabB Rab2 cis-Golgi. ER→Golgi trafficking PM→endosome and/or PVC→vacuole. Osmotic stress

RabC Rab18 Lack of evidence

RabD Rab1 ER→Golgi trafficking

RabE Rab8/10/12 Constitutive exocytosis, especially in polarized cells. TGN→PM PM→vacuole? Osmotic stress

RabF Rab5/22 Early exocytic pathway. Early endosome fusion

RabG Rab7 Late endocytic pathway Intraendosomal and endosome→lysosome transport

RabH ab6 Retrograde intra-Golgi and Golgi→ER transport Stress responses. PVC/late endosome→vacuole trafficking

*See text for discussion and references.

PVC, pre-vacuolar compartment.

(Rab1) and RabB (Rab2) are expected to be involved in ER to Golgi transport, and plant studies so far confirm this (Batoko *et al.*, 2000; Cheung *et al.*, 2002). RABH (Rab6) will complement its yeast homologue, *ypt6*, which is implicated in retrograde Golgi to ER transport (Bednarek *et al.*, 1994). The role of the RabA (Rab11) GTPases has been the most difficult to assign. The mammalian Rab11 GTPases are traditionally associated with trafficking (Zerial and McBride, 2001) though there are also reports indicating that they can be involved in exocytosis from the TGN (Chen *et al.*, 1998). Evidence from plants is suggesting that at least some groups are associated with transport to the cell membrane (Ueda *et al.*, 1996; Lu *et al.*, 2001; Inaba *et al.*, 2002; de Graaf *et al.*, 2005; Chow *et al.*, 2008; Rehman *et al.*, 2008). As discussed in detail later, one of the exciting developments is that this class may be vital for trafficking to the cell wall.

**Cell wall synthesis and modification**

Before considering how trafficking to the cell wall may be regulated, it is necessary to consider the components of the cell wall itself and how they are made. These will be discussed only briefly, and readers interested in a detailed treatment of the structure and experimental evidence should consult recent reviews (O’Neill and York, 2003; Brummell, 2006). There are three main groups of polysaccharides in the plant cell wall. Cellulose (β-1,4-D-glucan) chains are grouped into crystalline microfibrils embedded in a matrix of other polysaccharides. The second class of polysaccharides, the matrix glycans or hemicelluloses, comprises four main types: xyloglucans, gluconoarabinoxylans, glucomannans, and mixed linkage glucans (MLGs). The third class of wall polysaccharides are the pectins, which again fall into four main types: homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. The synthesis of these polysaccharides by membrane-associated enzymes has again been extensively reviewed (Doblin *et al.*, 2003; Lerouxel *et al.*, 2006; Scheller *et al.*, 2007). Cellulose is synthesized by hexameric rosette complexes in the PM. The catalytic subunits are encoded by the *CESA* genes, but other associated proteins also seem to be needed. The pioneering study of Northcote and Pickett-Heaps (1966) employed [3H]glucose to show that pectic polysaccharides move through the Golgi from *cis* cisternae to *trans* cisternae, then through the TGN and Golgi-derived vesicles to the primary cell wall. Since then, antibodies have been used to show localization of xyloglucans and pectin in the Golgi cisternae and the TGN, and isolated Golgi preparations have been shown to incorporate labelled sugars into MLGs, xyloglucan, gluconoarabinoxylan, glucanmannan, and galactomannan (for references, see Doblin *et al.*, 2003). There is, however, some doubt about the site of synthesis of MLGs because two recent immunolabelling studies have indicated that these are synthesized at the PM (Philippe *et al.*, 2006; Wilson *et al.*, 2006). Glycan synthases, which make the backbones, and glycosyl transferases, which add side chains, have also been found in the Golgi bodies (Doblin *et al.*, 2003; Scheller *et al.*, 2007). Glycan synthases are type III membrane proteins with multiple membrane-spanning regions and the glycosyl transferases are type II membrane proteins. Therefore, it is clear that all of these enzymes must be correctly targeted and trafficked to the correct membrane (Fig. 1). The Golgi-synthesized polysaccharides too must be correctly transported to the apoplast, so that vesicle trafficking must play an important role in cell wall synthesis.
Table 2. Fruit-related expression profiles of Rab genes in the Df1 tomato gene index

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Type of Rab</th>
<th>Expression in different stages of fruit development</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Ovary Developing/mature green fruit</td>
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Classification of Rab GTPases is according to Rutherford and Moore (2002).
<sup>b</sup> Rab1C of Loraine et al. (1996) (see text).
<sup>c</sup> Rab1A of Loraine et al. (1996) (see text).
<sup>d</sup> Rab1B of Loraine et al. (1996) (see text).
<sup>e</sup> Rab8 of Zegzouti et al. (1999) (see text).
<sup>f</sup> Rab11a of Lu et al. (2001) (see text).
Recent data throw some light on the potential importance of vesicle trafficking in regulating cellulose synthesis. Certain enzymes are recycled within the cell, and this may be a means of regulating synthesis. By employing a freeze-fracture technique on cultured *Zinnia elegans* cells, Haigler and Brown (1986) have shown that cellulose synthase (CESA) rosettes are present in the Golgi and Golgi-derived vesicles throughout cell wall deposition, suggesting that the system is dynamic and new rosettes need to be constantly inserted into the wall. Dunkley et al. (2006) have also localized CESA to the Golgi by a membrane fractionation technique. CESA proteins in young developing xylem cells are localized intracellularly, whereas in older cells that are synthesizing cellulose more actively they co-localize with bands of microtubules at the cell surface that mark sites of cell wall deposition. The intracellular pattern is consistent with some or all being in the ER (Gardiner et al., 2003). In the absence of one of the three subunits, the proteins appear to stay in the ER, indicating that a complex must be assembled before it can be transported to the site of synthesis. Disruption of the microtubules causes the proteins to redistribute intracellularly.

Yellow fluorescent protein (YFP) fusions have been used to visualize CESA proteins in the Golgi and in structures that may be secretory vesicles. In the PM, the fusion proteins assemble into structures, assumed to be rosettes, that move along microtubules. Inhibition of cellulose synthesis by isoxaben causes the YFP fluorescence at the PM to disappear (Paredes et al., 2006). It is possible that these are recycled to the Golgi, either continuously or to be stored. Green fluorescent protein (GFP)–CESA is found primarily in the PM in slowly growing hypocotyl cells but has a more intracellular distribution in larger cells further down the hypocotyls (Johansen et al., 2006). KORRIGAN, a β-1,4-glucanase associated with the cellulose synthetic rosettes and necessary for their action, also appears to cycle through different compartments depending upon the age of the cell and its growth rate (Robert et al., 2005). Therefore, cellulose synthesis may be regulated not only by the synthesis of CESA proteins but also by their redistribution away from the PM, and perhaps even by their subsequent recycling back to the PM.

Pectin polysaccharides themselves also seem to be recycled in dividing cells. Antibodies have been raised against cross-linked rhamnogalacturonan–borate complexes that are believed to form only after delivery to the cell wall. These have revealed that these pectic polysaccharide complexes may be recycled back into the cell and may then be inserted into the growing cell plate (Baluška et al., 2002, 2005; Dhonukshe et al., 2006).

The wall also contains structural proteins, which may act to stiffen the wall by peroxidase-mediated cross-linking (Passardi et al., 2004). Recent evidence confirms their importance because modifying levels of hydroxyproline-rich glycoproteins in the wall can change stem thickness (Roberts and Shirsat, 2006). Extensins are induced by elicitors and by pathogen attack (Bowles, 1990), and, recently, Wei and Shirsat (2006) have shown that transgenic plants with increased levels of these proteins have reduced pathogen invasiveness. As expected for secreted proteins, these all have N-terminal signal sequences and glycosylation has been shown to occur in the Golgi (Johnson et al., 2003).

Therefore, it is clear that both polysaccharides and proteins must be transported to, and sometimes from, the cell surface in a regulated manner. It is also clear that this transport must be via the Golgi apparatus through a classical vesicle trafficking route (Fig. 1). However, there must be more than one route and more than one control. Homann and Tester (1997) showed that exocytosis in barley aleurone protoplasts could be by a Ca\(^{2+}\)-independent pathway or by a Ca\(^{2+}\)-dependent one requiring GTP-binding protein. This indicates that control may be by classical signalling molecules and by GTPases. Further evidence for differentially regulated pathways comes from the finding that one of two SNARES involved in trafficking to the PM is implicated in ABA-responsive secretion (Leyman et al., 1999, 2000). New tools for studying the involvement of different SNAREs in different pathways (Tyrrell et al., 2007) offer the probability of great strides in this exciting area. Different trafficking routes may also carry different constituents of the cell wall. In pea seedlings, a low concentration of brefeldin A was shown to inhibit incorporation of matrix polysaccharides and cellulose, but only slightly inhibited incorporation of labelled amino acids into PM and wall proteins (Lanubile et al., 1997). Blocking vesicle docking by blocking the action of an ABA-inducible syntaxin in tobacco leaf protoplasts did not inhibit polysaccharide incorporation into the wall and actually slightly increased cellulose incorporation (Leucci et al., 2007). However, the transport of secreted proteins was reduced by half. Therefore, there seem to be parallel routes to the apoplast that may be controlled independently and by different mechanisms, and these may carry different cargoes.

Following cell wall synthesis, there are numerous circumstances in which the wall should not remain rigid. One of these is cell expansion, during which it is necessary for some of the bonds between primary wall polymers to be selectively loosened and reformed in a controlled manner to allow it to stretch (Cosgrove, 2003). Cell wall modification is also integral to fruit ripening, and there are many developmental processes where cells need to separate, and these include growth of pollen tubes through the style, abscission of leaves flowers and fruit, dehiscence of pods and anthers, emergence of lateral roots through the cortex and radicles through the endosperm, formation of leaf mesophyll, aerenchyma, hydathodes, laticifers, stomata, and tracheary elements,
and the shedding of root caps (Roberts et al., 2002). All of these processes involve hydrolytic enzymes and other key proteins such as expansins.

With the advent of genomics, many families of glycoside hydrolases have been identified and these are involved not only in cell wall polysaccharide synthesis and degradation during cell separation, but also in cellulose synthesis, cell division, storage reserve mobilization, defence, symbiosis, secondary metabolism, and signalling (Minic, 2008). It would normally be expected that proteins bound for the cell wall would be transported via vesicles, but this has been very little studied. Recent analyses of apoplastic or cell wall protein fractions have identified many glycoside hydrolases, and the majority of these were predicted to have signal peptides (Chivasa et al., 2002; Watson et al., 2004; Boudart et al., 2005; Kwon et al., 2005; Bayer et al., 2006; Zhu et al., 2006), which would indicate that they entered the endomembrane system during synthesis. Therefore, it would be expected that they would be trafficked via vesicles and therefore might potentially be regulated in part by Rab GTPases.

Alteration of cell wall metabolism by wounding is an interesting specialized case where recent work has shed some light. The response to wounding is mediated by a number of local and systemic signals including nitric oxide, systemin, jasmonates, ABA, ethylene, and cell wall-derived oligosaccharides (León et al., 2001; Paris et al., 2006). Whereas some signalling oligosaccharides may be produced mechanistically, one particular wound-inducible polygalacturonase (PG) from tomato leaves may be responsible for the production of oligogalacturonide signalling molecules (Bergey et al., 1999). Callose synthesis is a major response to wounding and, following wounding, β-1,3-glucan synthase appears at sites of callose synthesis on the PM. The silencing of callose synthase genes prevents wound-induced callose synthesis (Jacobs et al., 2003; Nakashima et al., 2003). A wide range of other glycan synthases and transferases, as well as expansins and structural wall proteins, are also induced by wounding (Dimmer et al., 2004; Guzzardi et al., 2004; Park et al., 2006a; Reilly et al., 2006), though we cannot be sure that all of these are directly involved in wound repair. In fruit, the situation is rather different because in normal fruit ripening, cell wall hydrolases are produced in response to ethylene (see next section). However, wounding of tomato fruit has been shown to halt production of some of these (Chung et al., 2006).

Rab GTPases presumably must be involved in trafficking of wound-induced cell wall metabolic enzymes, but this has been little studied. In addition to inducing the production of Rab GTPases in ripening fruit (see next section), ethylene induces Rab11 and Rab8 in pea hypocotyls and Arabidopsis leaves (Moshkov et al., 2003a, b). However, a direct connection between Rab GTPases and wound repair must await further study.

Selected systems

There are several systems where the key role of vesicle trafficking in synthesis and modification of cell walls has been well studied. Some of the evidence is indirect; typically this involves a correlation between the synthesis of key trafficking elements such as Rab GTPases with the process of wall growth and modification. More excitingly, in the last few years, evidence has emerged from reverse genetic approaches to suggest that these processes may be controlled at the level of trafficking. Three of these are considered here.

Fruit

One of several obvious changes that occur during fruit ripening is softening of the fruit. This is caused by changes in the cell wall brought about by the synthesis and secretion of enzymes and also due to synthesis of new cell wall materials (for a review, see Brummell, 2006). However, this process is extremely complex, as shown by attempts to reduce fruit softening by the use of antisense transgenes to inhibit the action of single enzymes. In tomato fruit, the use of gene silencing to inhibit the synthesis of PG reduced PG levels to as little as 1% and inhibited pectin depolymerization but had only very small effects upon fruit firmness (Sheehy et al., 1988; Smith et al., 1988, 1990; Kramer et al., 1992; Langley et al., 1994). Similarly, inhibition or overexpression of pectinesterase (PE; Tieman et al., 1992; Hall et al., 1993), β-glucanase (Lashbrook et al., 1998; Brummell et al., 1999a), and xylanoglucan endotransglycosylase (de Silva et al., 1994) had little or no effect upon fruit softening, though suppression of galactanase gene expression reduced softening by 40% (Smith et al., 2002) and inhibition of the ripening-specific expansin reduced softening by up to 20% and increased the degree of pectin polymerization (Brummell et al., 1999b). Therefore, most of these attempts have met with limited success, and a recent study has even shown that inhibition of PE in tomato causes cell walls to soften more quickly (Phan et al., 2007). Recent work on pollen tubes has also shown that regulation of cell wall stiffness by PE and PE inhibitors is complex (Röckel et al., 2008; see later section).

Therefore, it is clear that this process of wall disassembly is highly coordinated and requires many enzymes acting in concert. The prospects for effecting biotechnological improvements in post-harvest quality of fruit are better using approaches that inhibit more than one enzyme simultaneously. When plants silenced for PG and those silenced for expansin were crossed, the effects were synergistic and the fruits were firmer than in either single line (Powell et al., 2003) and gave more viscous paste (Kalamaki et al., 2003). Inhibition of ethene biosynthesis, which would in turn inhibit the synthesis of a range of ethene-inducible enzymes, did cause dramatically firmer fruit (Hamilton et al., 1990) though, unfortunately,
inhibition of ethene production can also affect colour and flavour development. The inhibition of vesicle trafficking to the apoplast offers the potential of inhibiting many cell wall hydrolases at once and also the deposition of new polymers. This could lead to new biotechnological applications in post-harvest technology.

Elements of the secretory machinery, notably Rab GTPases, have been shown to be expressed preferentially in fruit (Loraine et al., 1996; Zainal et al., 1996; Zegzouti et al., 1999; Lu et al., 2001; Park et al., 2006b; Abbal et al., 2008), often in a ripening-related and ethene-regulated manner. Three Rab1 proteins from tomato are expressed strongly in fruit (Loraine et al., 1996). One of these (LeRab1C) is expressed most strongly in expanding green fruit, and the other two (LeRab1A and LeRab1B) more strongly in ripe fruit. However, the protein products of these two genes may not be totally redundant in their functions because prenylation at the C terminus is important for the function of Rab GT Pases, and these two differed in their ability to interact with geranylgeranyl transferases from yeast, mammalian cells, and plant cells. Thus, although they are all the same class of protein and would, therefore, be expected to regulate ER–Golgi transport and they are all expressed in fruit, there may be subtle differences in their roles. Rab8 and Rab11 genes have also been found to be preferentially expressed in tomato fruit (Zegzouti et al., 1999; Lu et al., 2001) and, subsequently, the same classes have been found in apple fruit (Park et al., 2006b). Examination of the tomato TIGR database (Table 2) shows that many of the Rab expressed sequence tags (ESTs) in the database have been recovered from carpel or fruit pericarp tissues at various stages of development.

It has been suggested that Rab GTPases enable or regulate secretion of the enzymes that modify the cell wall to bring about fruit softening (Loraine et al., 1996; Zainal et al., 1996). This has been investigated for the Rab11 class, first identified in ethene-treated mango fruit mesocarp (Zainal et al., 1996). A tomato orthologue (LeRab11a) was silenced by transformation with antisense constructs. The transgenic tomato plants had fruit that remained firm for a very long period, and it was shown that the levels of extractable PG and PE were reduced in these fruit (Lu et al., 2001).

The Rab11 antisense tomato plants also showed physiological and developmental differences, including altered floral organ identity, highly branched inflorescences, rumpled leaves, ectopic shoots from leaves, determinate growth, reduced apical dominance, and altered ethene production. These effects are reminiscent of developmental abnormalities and altered hormone levels seen in studies where Rab11 GTPases have been overexpressed (Kamada et al., 1992; Sano et al., 1994; Aspuria et al., 1995) and are not likely to be due to altered trafficking to the wall but instead it has been argued (Lu et al., 2001) that these may be the result of misdirection of homeobox proteins, hormone carriers, and/or hormone receptors. However, there was not a general failure of trafficking in LeRab11a antisense tomatoes because, although a constitutive cauliflower mosaic virus (CaMV) 35S promoter was used to drive the antisense construct, the plants grew at the normal rate, and leaves and fruit reached the usual size. Again, this indicates that there is some subtlety to the regulation of secretion to the cell wall. In a more recent study using tobacco leaf protoplasts, LeRab11a–GFP fusions were shown to be associated with the TGN, and dominant-negative mutant versions of the Rab gene also inhibited exocytosis of secreted GFP (Rehman et al., 2008). Therefore, tomato Rab11a does seem to be involved in trafficking from the Golgi to the PM, though the distinctiveness of the endosome and the TGN has recently been questioned (Dettmer et al., 2006; Lam et al., 2007) and the exact route by which material is trafficked through post-Golgi endomembrane compartments is unclear. Both the study by Rehman et al. (2008) and a recent study by Chow et al. (2008) on Arabidopsis root tips indicate that there may be several partially overlapping routes that are regulated, in part, by several classes of RabA GTPases. Chow et al. (2008) showed that fluorescently labelled AtRabA2 and AtRabA3 both label the cell plate, and dominant-negative mutants of these Rabs show disruption of cell division patterns indicating that these are involved in trafficking to the newly forming cell plate in mitosis. One dominant negative also targets the cell periphery in interphase cells, so there might be an involvement in Golgi to PM trafficking too. The fluorescently labelled AtRabA2 and AtRabA3 completely co-localized with an endocytic marker FM4-64 and partially co-localized with a TGN marker VHA-a1. Conversely, Rehman et al. (2008) showed that the GFP fusions of LeRab11a (an AtRabA1 homologue) co-localized completely with the TGN marker Venus-Syp61 and only partially with FM4-64. Whether either of these overlapping post-Golgi compartments corresponds to classic TGN or classic early endosomes is unclear, though it is interesting that material trafficked to the cell plate appears to come partly from endocytosis of material previously incorporated into the cell wall (Baluska et al., 2002, 2005; Dhonukshe et al., 2006). Clearly, elucidation of the relationships between these compartments requires further work, especially as the two main studies reported here are from different systems.

Rehman et al. (2008) went on to show that inhibiting the syntaxin SYP121 pathway as well as Rab11a caused a greater inhibition of trafficking than just the LeRab11a dominant-negative mutant alone, whereas inhibition of SYP122 did not. This indicates that SYP122 and Rab11a are probably involved in the same pathway, but SYP121, which has been implicated in ABA-responsive secretion (Leyman et al., 1999, 2000), may be involved in
a different pathway. This may shed some light on one way in which subtlety of regulation of trafficking to the cell wall may be generated.

**Root nodules**

The formation of root nodules is a process that involves both growth and breakdown of the cell wall at several stages. Formation of the nodule involves cell division and cell expansion, and take up of the symbiotic bacteria involves the formation of a novel tip-growing structure, the infection thread. Loosening of cell walls will be necessary as bacteria enter the root hair, as the infection thread grows through cortical cells and as the bacteria are released from the infection thread.

A PG and a PE have been found in *Sinorhizobium meliloti–Medicago* symbiotic tissues. In both cases these are encoded by genes that are distinct from, but very closely related to, those expressed in pollen (Muñoz et al., 1998; Rodriguez-Llorente et al., 2004). The PG is also localized at the tips of uninoculated root hairs (Rodriguez-Llorente et al., 2003b), and the authors proposed that these genes have evolved from those that support tip growth of pollen to support tip growth of the infection thread. Promoter–β-glucosidase (GUS) fusion studies from the same laboratory (Muñoz et al., 1998; Rodriguez-Llorente et al., 2003a) have shown that the PG gene is expressed in nodule primordia, young nodules, and the invasion zone of mature nodules. Because the gene is also expressed in lateral root primordia, it is clearly not confined to a role in infection thread growth and may be secreted to loosen cell walls of normal growing cells within the nodule.

More direct investigations of control of secretion in root nodules have centred on the role of Rab GTPases. Cheon et al. (1993) identified a Rab7 GTPase and a Rab1 GTPase in the nodules of soybean, and later Borg et al. (1997) used degenerate probes to isolate a total of 29 Rab cDNAs from 3-week-old nodules of *Lotus japonicus*. These included seven copies of the ER–Golgi implicated Rab1, and five Rab8 and 10 Rab11 cDNAs, each of which seems to be implicated in secretion from the Golgi/TGN to the apoplast (Rutherford and Moore, 2002; Vernoud et al., 2003). Whilst many of these were not established as being specific to root nodules, Schiene et al. (2004) showed, using western blots, that *Medicago sativa* Rab11f is specific to nodules. One Rab11 has recently been implicated in nodulation by specific rhizobia (Meschini et al., 2008).

In a reverse genetic approach to test the importance of Rab1 and Rab7 in nodulation, Cheon et al. (1993) transformed antisense constructs under the control of a nodule-specific leghaemoglobin promoter into hairy roots. Both antisense Rab genes caused smaller nodules with reduced nitrogenase activity. The Rab7-suppressed nodules showed a perinuclear accumulation of late endosomes and multivesicular bodies. The Rab1 antisense nodules had fewer bacteroids per cell, release of bacte-roids into the central vacuole, and reduced expansion of infected cells. Since this was a very early study, and the first that I am aware of to show an effect on cell expansion due to inhibition of an element of the secretory machinery, it would be interesting to see further investigations of this phenomenon in root nodules.

**Apical growth systems**

Two of the best studied experimental systems involving secretion to the cell wall are the tip growing cells: pollen tubes and root hairs. These structures grow at a prodigious rate and traffic large quantities of new material to the growing tip (for recent reviews, see Šamaj et al., 2006; Campanoni and Blatt, 2007). Caution must be exercised in generalizing from these systems because they are very specialized and growth of the cell is highly polarized. Also, most other systems studied have involved trafficking of enzymes that modify cell walls, rather than new wall material.

However, there seem to be some similarities between these systems and fruit ripening. Studies involving gene silencing to inhibit elements of the trafficking machinery have shown that similar Rab GTPases are implicated (see below). In pollen tubes, there is also evidence for trafficking of cell wall-modifying proteins, including PE (Bosch et al., 2005) and β-expansin (Cosgrove, 2000). Jiang et al. (2005) found that the VANGUARD1 gene encodes a PE expressed in pollen tubes, and the vgd1 mutant has reduced fertility because of impaired growth through the style tissue. They propose that this PE is secreted out of the pollen tube tip and modifies the walls of the stylar cells to facilitate the growth of the tube through the style. This is supported by evidence that externally applied pectinase and PE could reduce wall stiffness and promote tip growth in *Solanum chacoense* pollen tubes (Parre and Geitmann, 2005). When an antisense transgene was used to silence expression of a pollen-specific PE in tobacco, genetic segregation ratios were perturbed and growth of the pollen tube through the style was inhibited whereas growth *in vitro* was unaffected (Bosch and Hepler, 2006). In this case, the authors argued that the PE is more important in signalling than in modification of the style cell walls but, in either case, the enzyme would need to be secreted through the cell membrane. PE is not the only protein secreted in this way. Maize pollen (Cosgrove et al., 1997) and the pollen of other grasses (Cosgrove, 2000) secrete β-expansin. Because the maize expansin is secreted in such large quantities, it is very soluble, and was shown to loosen the cell walls of grasses, it seems probable that the expansin functions to loosen the cell walls of the stylar tissue to allow the pollen tube to penetrate more readily. Interestingly, the pollen tubes of dicotyledonous plants do not secrete this type of expansin and dicot cell walls are not susceptible to it (Cosgrove et al., 1997).
Trafficking during the growth of the cell wall has also been extensively studied in both pollen and root hairs. Inhibition of vesicle formation by brefeldin treatment of pollen caused reduced pollen germination, retarded pollen tube growth, abnormal pollen shape, and altered cell wall composition and structure (Wang et al., 2005). In particular, the protein and polysaccharide content of the wall, especially the pectin content, was reduced at the tube apex. In this study, brefeldin treatment inhibited exocytosis but, more unexpectedly, stimulated endocytosis. This led Wang et al. (2005) to note that cross-linked pectin has been shown to be recycled in root meristematic cells (Baluška et al., 2002, 2005). Perhaps, therefore, such recycling is important in maintenance of the plasticity of the apical wall, and increased endocytosis could account for the reduced pectin content of the wall. This idea is supported by the observation that in latrunculin-treated root hairs, the inhibition of actin-driven movement of endosomes was coincident with cessation of tip growth (Voight et al., 2005). These authors proposed that the prevention of pectin recycling might also be the cause of growth cessation in this system. Exciting recent data show that several different PE isoforms and several PE inhibitors are produced in Arabidopsis pollen tubes. Some of these accumulate differentially at the tip and the flanks of the tube and may regulate the extensibility of the wall by changing the esterification of the pectins. This differential accumulation appears to involve not only exocytosis of new proteins but also endocytosis of PE inhibitor from the flanks of the tube (Röckel et al., 2008). Therefore, regulation of vesicle trafficking is crucial to maintaining appropriate cell wall extensibility.

The roles of elements of the trafficking machinery and particularly Rab GTPases have also been investigated in these tip growing systems. AtRabF1 and AtRabF2a were shown to associate with endosomes in root hairs and other tissues (Voight et al., 2005), and the ER–Golgi-associated AtRabB1c (Rab2B) was shown to have a high level of expression in pollen (Moore et al., 1997). A tobacco gene of the same class, NtRab2, is highly expressed in pollen and also expressed in root hairs and other tissues that secrete enzymes or other material to the cell wall: young seedlings, pistils, and germinating seeds (Cheung et al., 2002). A dominant-negative mutant of this gene blocked ER–Golgi trafficking and prevented delivery of membrane proteins and invertase to the cell surface. This same mutant inhibited pollen tube growth, strongly implicating this Rab GTPase in control of trafficking of new cell wall material to the growing tip.

Once again, the Rab11/RabA class of GTPases has been best characterized. MtRab11G and AtRabA4b have been shown to be expressed in root hairs of Medicago and Arabidopsis (Covitz et al., 1998; Preuss et al., 2004). AtRabA4b was shown to localize to the tips of root hairs only when actively growing, but did not co-localize with either Golgi or the TGN. AtRabA5c (previously called Ara-4) is expressed in pollen tubes and was shown to co-localize with the Golgi cisternae, the TGN, and Golgi-derived vesicles (Ueda et al., 1996). NtRab11b localizes to the cone-shaped region, almost totally occupied by vesicles, at the tip of tobacco pollen tubes. This GTPase seems to be a key element in correctly delivering material to the growing cell wall because a dominant-negative mutant, when overexpressed in pollen, caused a reduced growth rate of the pollen tube and reduced fertility (de Graaf et al., 2005). It also plays some role in orienting growth because the same mutant induced a wandering deformation in the pollen tubes. These phenotypic effects were shown to be mediated by the inhibition of exocytic and endocytic vesicle targeting to the cone region and reduced delivery of cell wall proteins to the apoplast. T-DNA knockouts of several Arabidopsis Rab11/RabA GTPases gave no comparable phenotype (de Graaf et al., 2005), and it is possible that this reflects functional redundancy because at least 10 RabA genes are expressed in pollen at roughly similar levels (Pina et al., 2005). Rab8 is also classically implicated in Golgi–PM transport, but its involvement in these systems remains to be determined.

How are Rab GTPases involved in growth orientation? This may be directly, through interaction with the exocyst, but Ca2+ signalling, which is known to play a role in orientation of growth, may also be involved. In a yeast two-hybrid system, AtRabA4b was shown to interact specifically with the phosphatidylinositol-4-OH kinase, PI-4Kβ1, but not other classes of PI-4Ks, whereas the dominant-negative mutant form of RabA4b would not interact (Preuss et al., 2006). AtRabA4b and PI-4Kβ1 were also shown to co-localize in root hairs. In the same system, PI-4Kβ1 interacted specifically with the calcineurin-like Ca2+ sensor, AtCBL1, but not AtCBL3 or AtCBL5. PI-4Kβ1/PI-4Kβ2 T-DNA double insertion mutants had abnormal root hairs, and treatment with the ionophore A23187 to disrupt the Ca2+ gradient in the root hair caused dispersion of RabA4b away from the tip (Preuss et al., 2006). A further study (Thole et al., 2008) has shown that ROOT HAIR DEFECTIVE4 encodes a phosphatidylinositol-4-phosphate phosphatase that co-localizes with RabA4b and may also help to regulate phosphatidylinositol-4-phosphate levels in the root hair tip. Therefore, it is clear that Ca2+ signals are directly linked to Rab action.

Although these tip growth systems are difficult to dissect because inhibition of related factors such as endocytosis and signalling by Rop GTPases also affects growth (see Cole and Fowler, 2006; Samaj et al., 2006; Campanoni and Blatt, 2007), there does seem to be evidence for the importance of control of secretion to the
cell wall in these tip growing systems and a possible regulatory role for Rab GTPases.

Concluding remarks

It is now clear that vesicle trafficking is necessary for cell wall synthesis in several ways. The enzymes that synthesize the polysaccharides need to be trafficked to the cell wall, and it seems that the retrograde trafficking of cellulose synthase to storage compartments may be an important regulatory step. In addition, the polysaccharides themselves need to be trafficked to the wall and also recycled so that they can be re-used when rapid cell wall synthesis is required. Blocking of trafficking to the apices of pollen tubes and root tips causes inhibition of tip growth. Cell wall hydrolases and other wall-modifying enzymes also need to be trafficked to the cell wall, and inhibition of vesicle trafficking in fruit causes a reduction in enzyme levels in the walls and a reduction of softening.

Rab GTPases have long been considered key players in the regulation of vesicle trafficking in animal systems and their roles are now being evaluated in plants. Based on animal models, the Rab11/RabA class of GTPase was thought to be involved in recycling to endosomes (Zerial and McBride, 2001). However, the Rab11 clade in Arabidopsis has 26 members compared with the two in mammals, and this has led to speculation that they have diversified structurally and functionally (Rutherford and Moore, 2002). Based upon the effect of inhibiting these, several of them now seem to be implicated in trafficking to the cell wall. First of all, a tomato RabA1 homologue is required for trafficking to the walls and a reduction of softening caused by gene silencing with a constitutive promoter have interfered with production or delivery of PE (Jiang et al., 2001) and have produced specific phenotypes in specific organs, the overall size and rate of growth of the plants have been normal. Many of these genes are differentially regulated and belong to gene families so this might potentially explain the phenomenon. However, several Rab GTPases have been shown to be expressed in a range of actively growing or highly secretory tissues (Lu et al., 2001; Cheung et al., 2002) and inhibition of LeRab11A did not inhibit overall growth (Lu et al., 2001). It is also possible that different proteins and polysaccharides destined for the cell wall are delivered by different vesicles, and support for this has recently emerged (Leucci et al., 2007; Rehman et al., 2008).

Trafficking is important for the location of hormone receptors and other elements of the signalling machinery and for localization of ion channels. Interference with these processes would be expected to have effects upon cell wall metabolism, and this is probably important in tip growing systems (see Cole and Fowler, 2006; Šamaj et al., 2006; Campanoni and Blatt, 2007) and has also been observed in plants with a silenced tomato fruit Rab GTPase (Lu et al., 2001). Therefore, future studies must aim to distinguish between direct control of trafficking to the cell wall and more global effects due to altered signal transduction. However, despite this reservation, it seems clear that trafficking pathways, and Rab GTPases in particular, must play a role in controlling cell wall synthesis and modification.

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