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Tomato Rab11a Characterization Evidenced a Difference Between SYP121-Dependent and SYP122-Dependent exocytosis

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The regulatory functions of Rab proteins in membrane trafficking lie in their ability to perform as molecular switches that oscillate between a GTP- and a GDP-bound conformation. The role of tomato *Le*Rab11a in secretion was analyzed in tobacco protoplasts. Green fluorescent protein (GFP)/red fluorescent protein (RFP)-tagged *Le*Rab11a was localized at the *trans*-Golgi network (TGN) in vivo. Two serines in the GTP-binding site of the protein were mutagenized, giving rise to the three mutants Rab11S22N, Rab11S27N and Rab11S22/27N. The double mutation reduced secretion of a marker protein, secRGUS (secreted rat β-glucuronidase), by half, whereas each of the single mutations alone had a much smaller effect, showing that both serines have to be mutated to obtain a dominant negative effect on *Le*Rab11a function. The dominant negative mutant was used to determine whether Rab11 is involved in the pathway(s) regulated by the plasma membrane syntaxins SYP121 and SYP122. Co-expression of either of these GFP-tagged syntaxins with the dominant negative Rab11S22/27N mutant led to the appearance of endosomes, but co-expression of GFP-tagged SYP122 also labeled the endoplasmic reticulum and dotted structures. However, co-expression of Rab11S22/27N with SYP121 dominant negative mutants decreased secretion of secRGUS further compared with the expression of Rab11S22/27N alone, whereas co-expression of Rab11S22/27N with SYP122 had no synergistic effect. With the same essay, the difference between SYP121- and SYP122-dependent secretion was then evidenced. The results suggest that Rab11 regulates anterograde transport from the TGN to the plasma membrane and strongly implicate SYP122, rather than SYP121. The differential effect of *Le*Rab11a supports the possibility that SYP121 and SYP122 drive independent secretory events.

Keywords: Dominant negative mutants — Exocytosis — Plasma membrane — Rab11 — Syntaxin.

Abbreviations: ANOVA, analysis of variance; BFA, brefeldin A; CA, constitutively active; DN, dominant negative; EE, early endosome; ER, endoplasmic reticulum; GFP, green fluorescent protein; PEG, polyethylene glycol; PM, plasma membrane; PVC, pre-vacuolar compartment; RFP, red fluorescent protein; SecRGUS, secreted rat β-glucuronidase; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; TBS, Tris-buffered saline; TGN, *trans*-Golgi network; TMD, transmembrane domain; YFP, yellow fluorescent protein.

Introduction

Proteins to be secreted are transported by the secretory pathway. They are synthesized at the endoplasmic reticulum (ER), pass through the Golgi apparatus and are conveyed to the outside of the cell. Molecules are transported from one compartment to the other along this route by vesicles. The secretory pathway or endomembrane system plays an important role in the biogenesis of the cell wall, plasma membrane (PM) and vacuoles. It also contributes to the control of development and to the responses to biotic and abiotic stresses (Surpin and Raikhel 2004). Rab proteins are important signal transducers and essential elements of the membrane trafficking machinery. They have been found in all eukaryotes, constitute the largest family of small monomeric GTPases in the Ras superfamily (Pereira-Leal and Seabra 2001) and are ubiquitously expressed. Like other Ras-related GTPases, Rab proteins are prenylated and exist in both a soluble pool and bound to the cytosolic face of membranes. They cycle between a mainly cytosolic, inactive, GDP-bound, and a membrane-associated, active, GTP-bound form. This conformational change to the active form regulates trafficking events in response to regulatory factors (Surpin and Raikhel 2004, de Graaf et al. 2005, Scapin et al. 2006). Specific Rab GTPases associate with a particular endomembrane compartment, and are involved in specific vesicle transport steps (Armstrong 2000, Zerial and McBride 2001, Pfeffer and Aivazian 2004, Seabra and Wasmeier 2004).

Many Rab homologs have been identified from different plant species, including 57 Rabs in the *Arabidopsis thaliana* genome. Based on sequence homology,

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the *Arabidopsis* Rabs are grouped in eight functional families that may be further divided into 18 structurally different subclasses (Rutherford and Moore 2002, Vernoud et al. 2003). Many T-DNA insertional mutants are available in these *Arabidopsis* genes, but their functional analysis has yet to be reported.

The GDP–GTP exchange regulatory mechanism allows the equilibrium between the active and inactive forms to be manipulated. Mutations in Ras-related GTPases at specific positions can lock the proteins in the GTP-bound or GDP-bound form, generating constitutively active (CA) and dominant negative (DN) mutant proteins, respectively. Overexpressing the CA or DN mutants may lead to the uncovering of their functional significance. Studies based on the localization and expression of CA and DN variants of plant Rab GTPases in plants, for example *Arabidopsis* Rab1b (*At*RabD2a), ARA-6 (*At*RabF1), ARA-7 (*At*RabF2b) and *At*Rab4b, and tobacco (*Nicotiana tabacum*) Rab2, which are homologous to yeast and mammalian counterparts, have shown that the Rab regulatory pathway is conserved in eukaryotes (Batoko et al. 2000, Grebe et al. 2003, Ueda et al. 2004).

The cycle of Rabs is coordinated with the cycle of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE proteins) involved in the membrane docking and fusion during vesicle trafficking. Thanks to GTP hydrolysis (Zerial and McBride 2001), a syntaxin, which is a key element of the SNARE complex on the target membrane, can bind the SNARE on the vesicle to determine docking. Many syntaxins have been localized on all endomembranes and many have been also located on the PM. Some of these syntaxins have specific functions such as phragmoplast formation (Batoko and Moore 2001, Heese et al. 2001). The roles of the others have to be fully defined. Out of five syntaxins present on the PM (SYP121–125, Uemura et al. 2004), two have been better characterized: SYP121 and SYP122.

SYP121 is involved in ABA-related secretion (Leyman et al. 1999, Leyman et al. 2000) as well as in non-host pathogen resistance (Assaad et al. 2004); SYP122 seems to have a more general function in secretion, including a role in cell wall deposition (Assaad et al. 2004), but appears also to be involved in some pathogen-related processes (Nühse et al. 2003). Since syntaxins are tail-anchored proteins inserted into the target membrane post-translationally (Borgese et al. 2003), it is not clear whether their initial anchoring site coincides with the final target membrane; their sorting has not been systematically investigated.

Each step of the secretory pathway where membrane fusion takes place can potentially involve a specific SNARE complex with a specific syntaxin and, eventually, a specific Rab protein.

Many Rabs (26 out of 57) are classified as Rab11 homologs in *Arabidopsis*; thus a high level of specialization or redundancy may be expected in this group. As a result, the *Arabidopsis* Rabs have been reclassified as RabA, RabB, etc., and the RabA clade, which corresponds to Rab11, has several subgroups (RabA1, RabA2, etc.; Rutherford and Moore 2002, Vernoud et al. 2003). In animal and yeast cells, some Rab11 GTPases play a role in membrane recycling from the endosomes to the PM, and in transport of receptor proteins between endosomes, the *trans*-Golgi network (TGN) and the PM (Ullrich et al. 1996, Schlierf et al. 2000, Wilcke et al. 2000, Band et al. 2002, Hales et al. 2002, Volpicelli et al. 2002). They have also been associated with exocytosis (Benli et al. 1996, Chen et al. 1998, Cheng et al. 2002, Ortiz et al. 2002).

In plants, the green fluorescent protein (GFP)-tagged Rab11 homologs from pea (*Pisum sativum*), Pra-2 and Pra-3, have been localized to Golgi bodies and endosomes, respectively (Inaba et al. 2002), when expressed in tobacco cells. Cytoimmunodetection of the *Arabidopsis* Rab11, ARA4 (*At*RabA5c), using a specific monoclonal antibody, revealed localization to Golgi vesicles (Ueda et al. 1996). In another study, the *Arabidopsis* Rab11 homolog, *At*RabA4b, co-fractionated with a non-TGN membrane fraction (Preuss et al. 2004). In ripening fruit, Rab11 is reported to be important for the secretion of cell wall-modifying enzymes (Zainal et al. 1996, Lu et al. 2001). The tomato *Le*Rab11 GTPase, like mammalian GTPases, could be involved in the exocytic or endocytic pathway (Somsel and Wandinger-Ness 2000, Lu et al. 2001). Rab11 proteins have also been shown to be involved in the biosynthesis of brassinosteroids and in light signal transduction (Yoshida et al. 1993, Nagano et al. 1995, Kang et al. 2001).

We studied *Le*Rab11a in *Nicotiana tabacum*. *Nt*Rab11 (accession gi|3024504) and *Le*Rab11a share 66% identity at the amino acidic level and possess the same active site. We analyzed the subcellular localization of *Le*Rab11a wild type and DN mutants in tobacco cells by co-expression of GFP/red fluorescent protein (RFP)–Rab11 with various known markers for endosomes, vacuoles, the *cis*-Golgi network and the TGN. The role of Rab11 in secretion was analyzed with a reporter protein, a secreted rat β-glucuronidase, secRGUS (Di Sansebastiano et al. 2007). Finally, the involvement of the syntaxins localized at the plasma membrane, SYP121 and SYP122, in the pathway regulated by this Rab11 was analyzed. The results indicate that *Le*Rab11a is involved in a pathway regulated by SYP122 but not by SYP121.

Results

Development of dominant negative mutants

To study the role of *Le*Rab11a in exocytosis, a DN mutant was generated by mutagenizing the

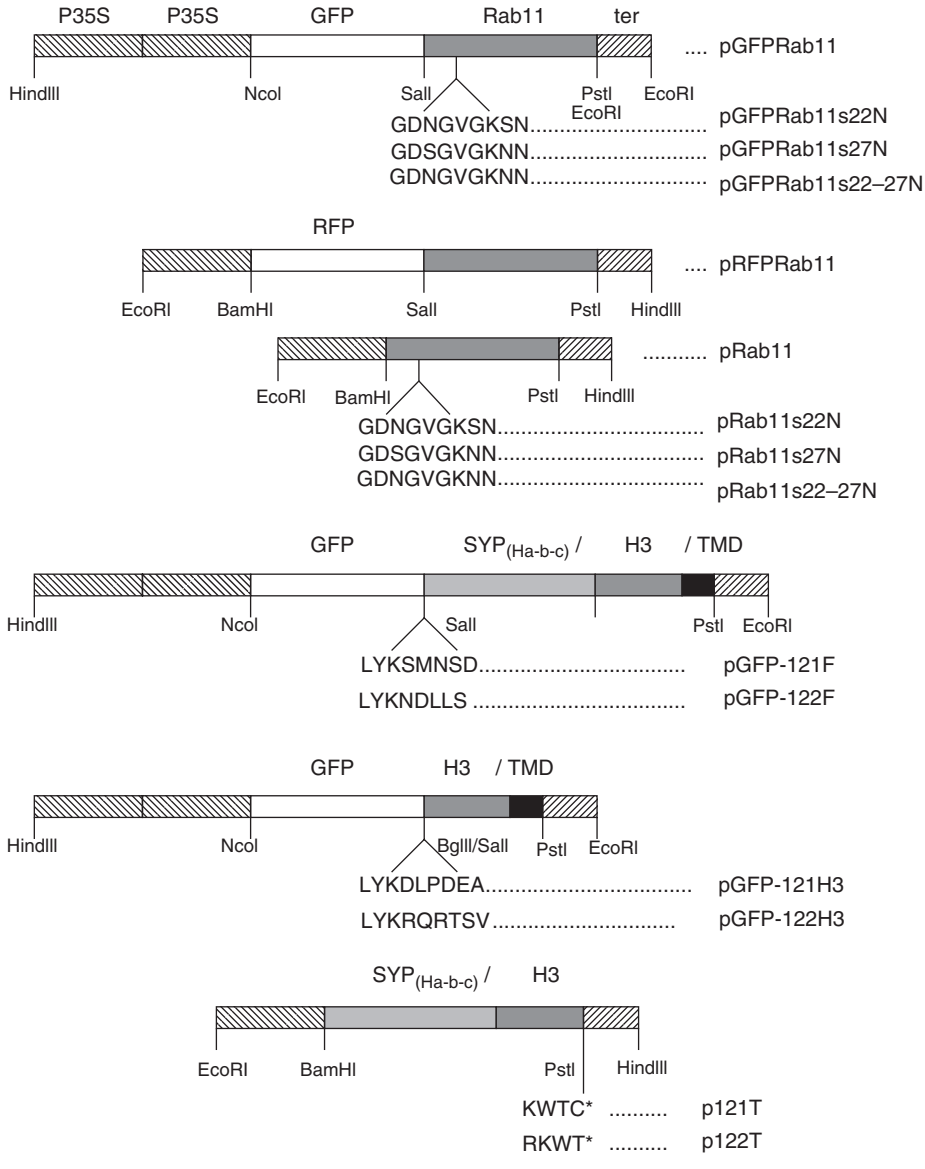

Fig. 1 Schematic representation of DNA constructs. The fragments shown are integrated between *Eco*RI and *Hin*dIII restriction sites in a pUC19-derived vector.

GTP-binding site. In Rab proteins, this site consists of three conserved domains toward the N-terminus of the protein with the amino acid sequence GXXXVGKS/T followed by the sequences DTAGQE and EXSA (where X is any amino acid) (Terryn et al. 1993, Haizel et al. 1995). Functional studies of Rab proteins often use mutagenesis of this site to block the protein in a GDP-bound state that is inactive (Zheng et al. 2005). To create a DN mutant of *Le*Rab11a and investigate its role in exocytosis, we mutagenized the first domain of its GTP-binding site (P-loop). In *Le*Rab11a, this domain contains a second serine (GDSGVGKS) that was recently shown to function in the binding of Mg^{2+} to regulate nucleotide dissociation (Scapin et al. 2006).

Three mutants of the *Le*Rab11a cDNA were generated: Rab11S22N, Rab11S27N and Rab11S22/27N. The same three mutations were also transferred to a GFP–Rab11 construct (Fig. 1). The constructs were expressed transiently in tobacco protoplasts, either alone or together with secRGUS that allowed the level of secretion to be monitored.

Single mutations had different effects. In a GTP-binding assay, mutation S27N prevented GTP binding, in contrast to mutation S22N that seemed to increase binding, possibly by slowing the rate of GTP dissociation. The doubly mutated protein Rab11S22/27N did not bind GTP (Fig. 2A) and it is inferred that this molecule is inactive. All of the chimeric proteins associated with the membrane fraction (Fig. 2C) as well as with the soluble fraction (Fig. 2D). Rab proteins cycle between a membrane-anchored state and a cytosolic state, and mutations in the GTP-binding site are not expected to modify membrane anchoring.

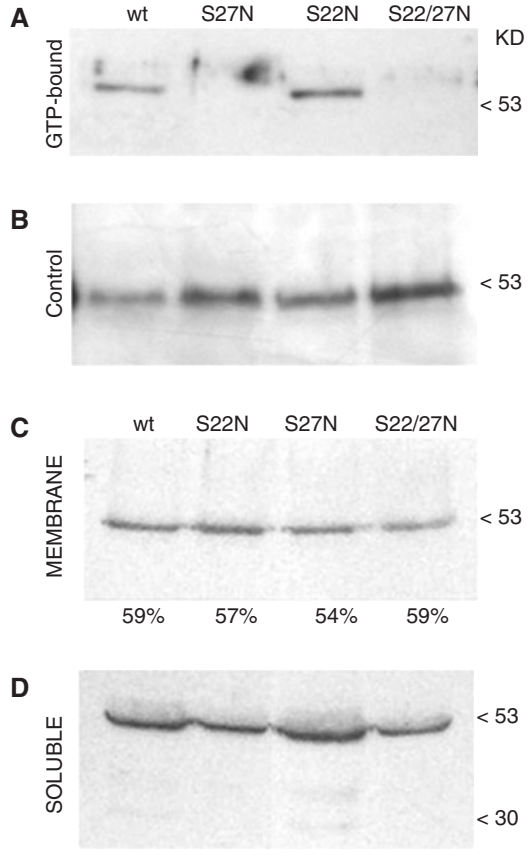

Fig. 2 Immunoblot analysis of GFP–Rab11 mutants. Wild-type and mutated proteins were detected with anti-GFP serum. (A) Soluble proteins from protoplast extracts were precipitated with GTP-conjugated agarose beads; (B) the presence of the protein was shown in total extracts from all samples. After fractionation, GFP–Rab11 forms can be found in both the membranous fraction (C) and soluble fraction (D). The proportion of membrane association increased for the double mutant S22/27N. The percentage membrane association is indicated (C). The variation was not statistically significant.

When experiments with different DN constructs were compared, the distribution of the proteins between the membrane and cytosolic fractions was not significantly different (Fig. 2C).

The effect of the expression of the chimeric proteins on the secretory pathway was analyzed by measuring the secretion of the secRGUS marker. This secretion was normalized to the quantity of total proteins and corrected by taking into account the level of an intracellular protein, α-mannosidase, in the medium (Di Sansebastiano et al. 2007). Whereas the other constructs had a moderate effect, the double mutant reduced secretion of secRGUS by 51% when compared with the control (Table 2). Co-expression of the double mutant with the wild-type form of Rab11, either with or without the GFP tag, compensated at least partially for this effect. The secretion of the marker was variable when the different mutants were expressed, and a *t*-test analysis confirmed statistical significance of inhibition. The double mutant, either alone [Rab11S22/27N: $t(16) = 20.4$; $P < 0.000$] or fused to GFP [GFP–Rab11S22/27N: $t(11) = 7.2$; $P = 0.000$], showed a much greater difference from the controls (secRGUS alone or co-expressed with Rab11 or GFP–Rab11) compared with the single mutants, out of which the most significant effect was due to GFP–Rab11S27N [$t(10) = 6.2$; $P = 0.000$]. On the basis of these data we considered Rab11S22/27N to be the best DN mutant to be used in further analysis.

Effect of Rab11S22/27N on other markers

The specificity of the inhibitory effect on exocytosis of the double mutant was supported by the weak or absent effects on markers targeted by different sorting pathways. A vacuolar variant of the RGUS enzyme, RGUS-Chi (Di Sansebastiano et al. 2007), was co-expressed and its distribution remained intracellular (not shown). Since the vacuole in which the enzyme was accumulated could not be visualized and intracellular mis-targeting could not be visualized, three more visual markers were used for co-expression with Rab11S22/27N: ERD2–yellow fluorescent protein (YFP) (Brandizzi et al. 2002), AleuGFP (Di Sansebastiano et al. 2001) and secGFP (Leucci et al. 2007).

The distribution pattern of ERD2–YFP remained unchanged (Fig. 3A, B). The distribution of AleuGFP in pre-vacuolar compartments (PVCs) and large central vacuoles appeared similar to that in control cells (Fig. 3C, D). In contrast, the distribution of secGFP changed when Rab11S22/27N was co-expressed. The percentage of fluorescent cells after 20 h of transient expression doubled (indicating the retention of GFP), compared with control situations, and the distribution clearly showed discrete accumulation sites (Fig. 3F). It is known that secGFP can be mis-targeted to some kind of vacuolar compartment because of a cryptic signal (Zheng et al. 2005), but the differences in patterns between DN-expressing cells (Fig. 3F) and controls (Fig. 3E) was clear. Since Rab11S22/27N expression could not be visualized, it was expressed in parallel in a control sample co-transformed with secRGUS; Rab11S22/27N expression was considered acceptable when a >30% inhibition of secretion was observed (not shown).

Localization of GFP–Rab11 to the trans-Golgi network

GFP–Rab11 was seen as small mobile dots, but some labeling also often appeared in association with the ER and in the cytosol, probably due to overexpression of the construct (Fig. 4A). The different patterns observed at the same time in the same protoplast population are shown in Fig. 4A.

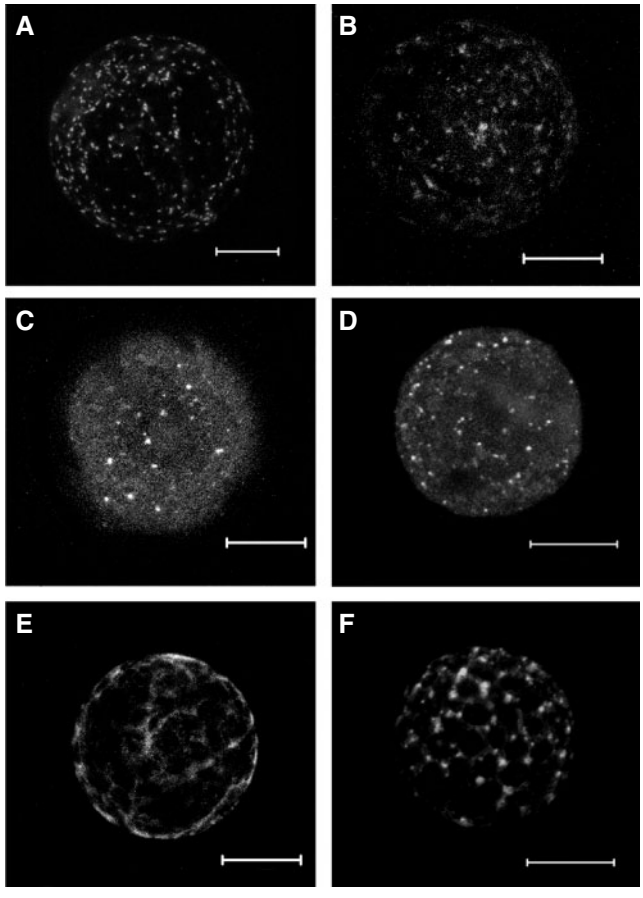

Fig. 3 Fluorescence pattern of GFP and YFP chimeras in control conditions or co-expressed with Rab11S22/27N. (A) Control ERD2–YFP; (B) ERD2–YFP co-expressed with the Rab11 mutant; (C) control AleuGFP; (D) AleuGFP co-expressed with the Rab11 mutant; (E) control secGFP; (F) secGFP co-expressed with the Rab11 mutant. All images are confocal projections of half of the cell. Proteins were expressed for 20 h before imaging. Scale bar = 20 μm.

The small dots were extremely mobile, as previously observed for Golgi streaming (Nebenfür et al. 1999). The fluorescent pattern of the GFP–Rab11 mutants did not show any statistically significant difference in the frequency of dots, ER or cytosolic distribution. Expression of GFP–Rab11 in the presence of the endocytotic marker FM4-64 showed no full co-localization of the two markers (Supplementary Fig. S1A–C); even after the arrival of FM4-64 in the ER it was only partial (Fig. 4B–D).

To characterize better the small structures labeled with GFP–Rab11, a new chimeric Rab11 (RFP–Rab11) was produced by fusing the RFP coding sequence (Campbell et al. 2002) to Rab11. This allowed comparison of the localization of RFP–Rab11 with that of three more markers: ERD2–YFP (Brandizzi et al. 2002), a marker for *cis*-Golgi compartments, AleuGFP (Di Sansebastiano et al. 2001), a marker of the PVC and of the acidic vacuole, and Venus–Syp61, a marker of the TGN (Uemura et al. 2004).

As shown in Fig. 4E–G, ERD2–YFP did not fully co-localize with RFP–Rab11. Both were localized in highly mobile dots and sometimes overlapped, but in most cases were separate. They moved independently and then appeared dissociated (Fig. 4H, I). AleuGFP showed no co-localization with RFP–Rab11 (Fig. 4L–N).

When RFP–Rab11 and Venus–Syp61 were co-expressed, complete co-localization was observed (Fig. 4O–Q). Extremely mobile small dots were labeled with both fluorescent proteins. These structures moved too fast to be captured in two-channel scan images, since fluorescence in the second channel always appeared shifted (Supplementary Fig. S1D–F). Overexpression rapidly induced the appearance of abnormally large structures where both fluorescent molecules co-localized (Fig. 4Q–Q). Such large structures were also observed in control conditions when Venus–SYP61 was expressed alone (Supplementary Fig. S2). It was also observed that Venus–SYP61-labeled structures were rapidly labeled by FM4-64 within 10 min of dye uptake. The dye and the protein were not fully co localized (Supplementary Fig. S1G–I, L–N); this was possibly due to a visual artifact because of the high mobility of the compartments known to be TGN or, alternatively, to a complex relationship between TGN and the early endosome (EE). The relationship between the TGN and the EE is not clear (Dettmer et al. 2006). Certainly membrane can move from the EE to the TGN very quickly, as evidenced by the transport of FM4-64 to the cell plate earlier than to the ER (Supplementary Fig. S1O–Q).

Rab11 regulates SYP122-dependent vesicle traffic

The relationship between the effect of the Rab11 double mutant and full-length or DN variants of two different plasma membrane syntaxins was investigated.

GFP was fused to the N-terminus of each of the two syntaxins: SYP121, which is associated with ABA-dependent secretion (Leyman et al. 1999), and SYP122, which may have a general role in secretion (Assaad et al. 2004). Soluble DN mutants, 121T and 122T, were also obtained by deleting the transmembrane domain (TMD) of these syntaxins (Geelen et al. 2002, Di Sansebastiano et al. 2006) (for a list of constructs, see Fig. 1).

The full-length forms GFP–121F and GFP–122F were localized at the PM (Fig. 5A, B) and their expression had no negative effect [GFP–121F, $t(7) = 1.3$; $P = 0.22$; GFP–122F, $f(7) = 2.1$; $P = 0.07$] on the secretion of secRGUS (Table 2). However, co-expression of the soluble forms 121T or 122T with the full-length GFP–121F form induced the appearance (23% with 121T and 20% with 122T, Fig. 5C, E) of

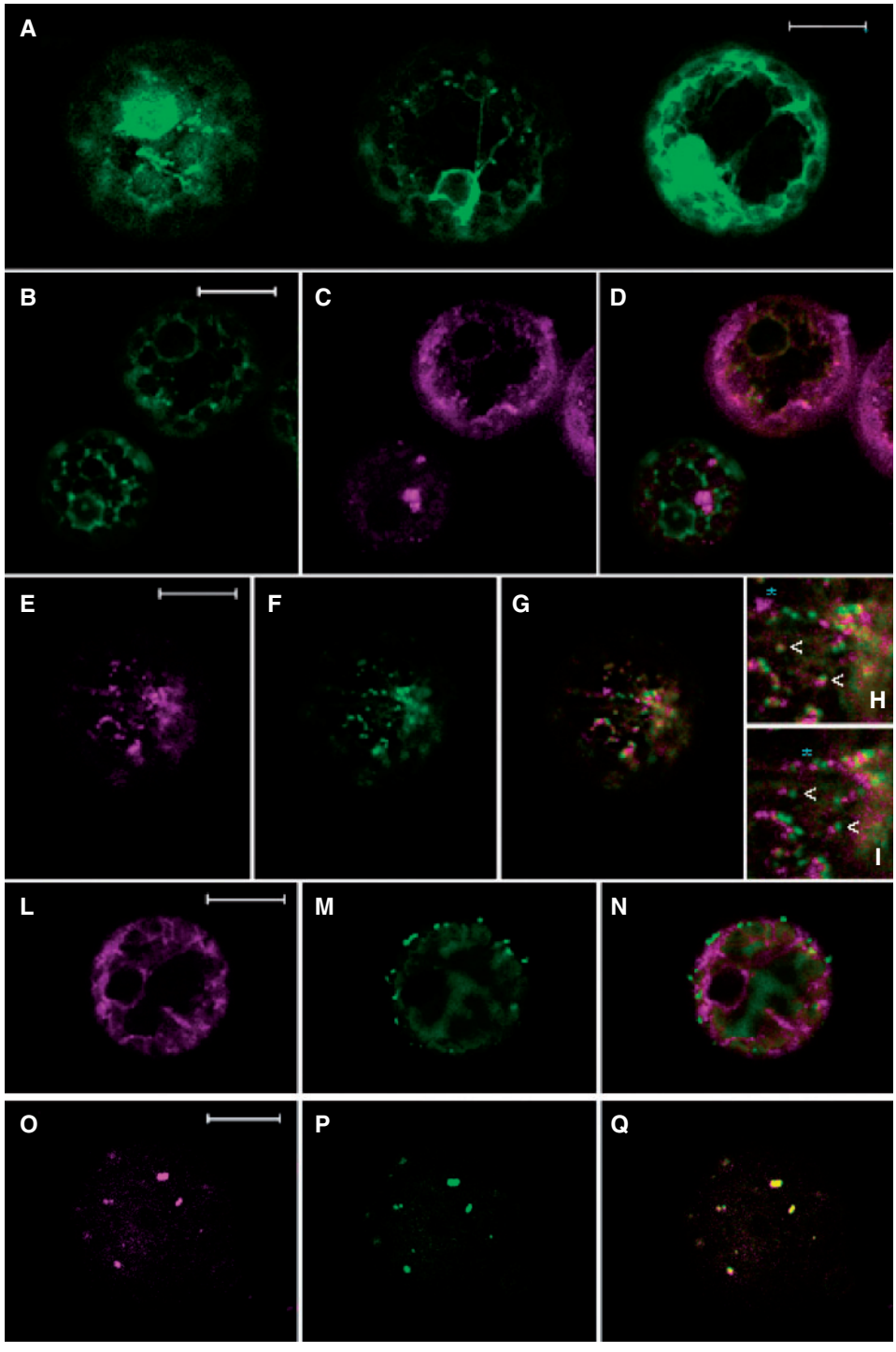

Fig. 4 Fluorescence patterns of Rab11 variants fused to a GFP or RFP tag expressed in protoplasts. (A) Three different patterns of GFP–Rab11 fluorescence, representative of the possible variability; (B) GFP–Rab11 fluorescence in a cell also stained by FM4-64; (C) FM4-64 staining of the same cells expressing GFP–Rab11 after 1 h; (D) merged image of GFP–Rab11 and FM4-64 fluorescence; (E) RFP–Rab11 fluorescence; (F) ERD2–YFP fluorescence in the same cell; (G) merged image of RFP–Rab11 and ERD2–YFP; (H) enlargement of the image in G; the star indicates a structure labeled by RFP–Rab11 only and arrows indicate structures where ERD2–YFP and RFP–Rab11 are associated; (I) the same enlargement as in H, 2 s later. The structure indicated by the star moves independently and the structures indicated by arrows dissociate; (L) RFP–Rab11 fluorescence; (M) AleuGFP fluorescence in the same cell; (N) merged image of RFP–Rab11 and AleuGFP; (O) RFP–Rab11 fluorescence; (P) Venus–Syp61 fluorescence in the same cell; (Q) merged image of RFP–Rab11 and Venus–Syp61. Scale bar = 20 μm.

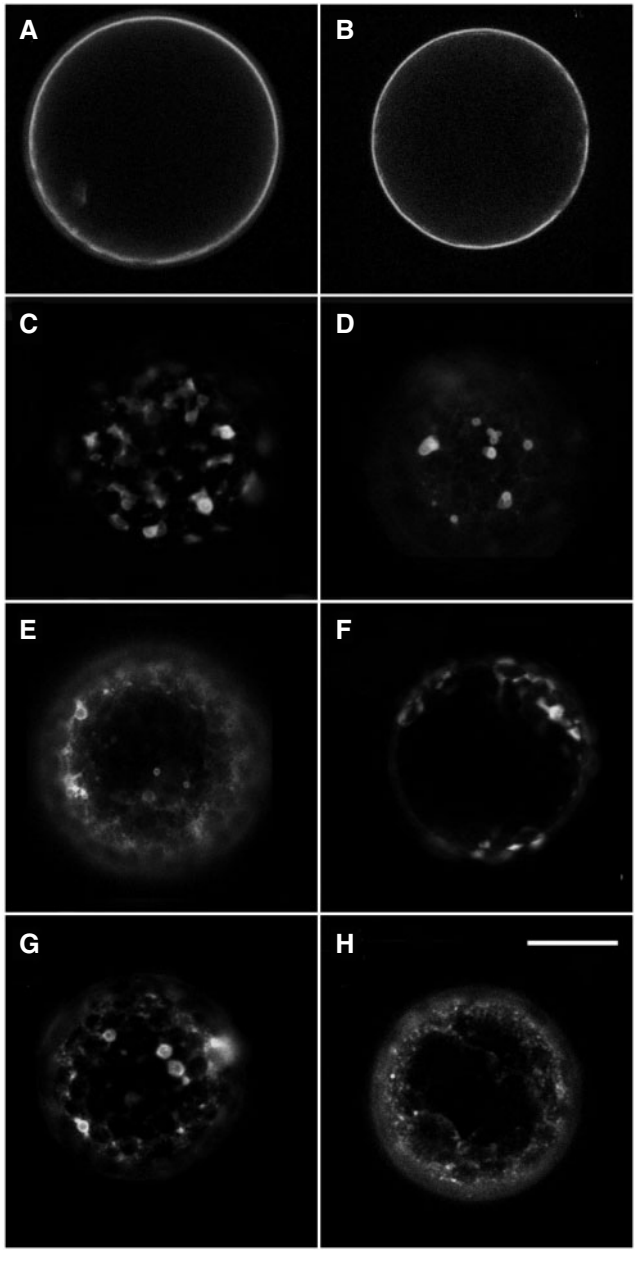

Fig. 5 Fluorescence patterns of GFP-tagged syntaxins. The percentage indicates the relative number of cells in the total population of transformed protoplasts showing the represented pattern. (A) GFP–121F (99 ± 1%); (B) GFP–122F (95 ± 5%); (C) GFP–121F co-expressed with 121T (23%); (D) GFP–122F co-expressed with 121T (37%); (E) GFP–121F co-expressed with 122T (20%); (F) GFP–122F co-expressed with 122T (26%); (G) GFP–121F co-expressed with Rab11S22/27N (30%); (H) GFP-122F co-expressed with Rab11S22/27N (32% of cells show this pattern; there are also an additional 16% of cells with endosomes). Data are derived from three independent experiments with no less than 300 cells counted. Scale bar = 20 μm.

structures identified as enlarged endosomes by FM4-64 co-localization (Fig. 6). Co-expression of 121T or 122T with the full-length GFP–122F induced an increased number of such structures (from 15% up to 37% with 121T and up to 26% with 122T, Fig. 5D, F). When the Rab11S22/27N DN mutant was co-expressed with either GFP–121F or GFP–122F (Fig. 5G, H), only GFP–122F appeared partially blocked in internal compartments including the ER (Fig. 5H), while GFP–121F localized in endosomes similar to those produced by 121T or 122T.

The accumulation of GFP–121F in aberrant endosomes when the soluble mutant (121T) was co-expressed has been reported previously (Di Sansebastiano et al. 2006). The behavior of GFP–122F was shown here to be identical. When co-expressed with its mutant 122T (Fig. 6A–F) or 121T (Fig. 6G–I), the observed distribution of GFP fluorescence always co-localized with FM4-64 staining. No differences could be evidenced with GFP–121F (not shown).

When Rab11S22/27N was co-expressed with GFP–121F the GFP fluorescence pattern was similar and continued to co-localize with FM4-64 entirely (not shown). In contrast, when the Rab11 double mutant was co-expressed with GFP–122F, dotted and ER structures were persistent after 20–24 h of expression; GFP labeling and FM4-64 staining did not merge exactly. Small structures were differently labeled up to 1 h after FM4-64 application (Fig. 6L–N); larger structures showed both types of fluorescence, especially when large abnormal endosomes were observed (Fig. 6O–Q).

Using secRGUS, we can monitor all exocytosis pathways because this marker is secreted by default. We believe that an increase in secRGUS exocytosis inhibition can be interpreted as additional effects on different secretory pathways. The co-expression of the two syntaxins DN 121T and 122T induces an increase in secRGUS inhibition (Table 3), indicating that it affects two distinct pathways. The same synergistic effect is reproduced when 121T is co-expressed with Rab11 DN (Table 3).

The analysis of variance (ANOVA) was significant [$F(20) = 11.65$; $P = 0.000$], and post hoc analysis showed that the probability value obtained comparing 121T and 122T co-expression with all other situations was always significant ($P < 0.05$), except for the situation where 121T and Rab11S22/27N are co-expressed ($P = 0.09$).

A new experiment was designed to obtain more information, coupling secRGUS measurements and evaluation of fluorescent patterns. For this purpose, a second type of DN mutant was developed for each of SYP121 and SYP122, allowing the visual control of expression during secRGUS assays. These mutants, GFP–121H3 (Di Sansebastiano et al. 2006) and GFP–122H3, consisted of a chimera of N-terminal GFP and the H3 domain followed by

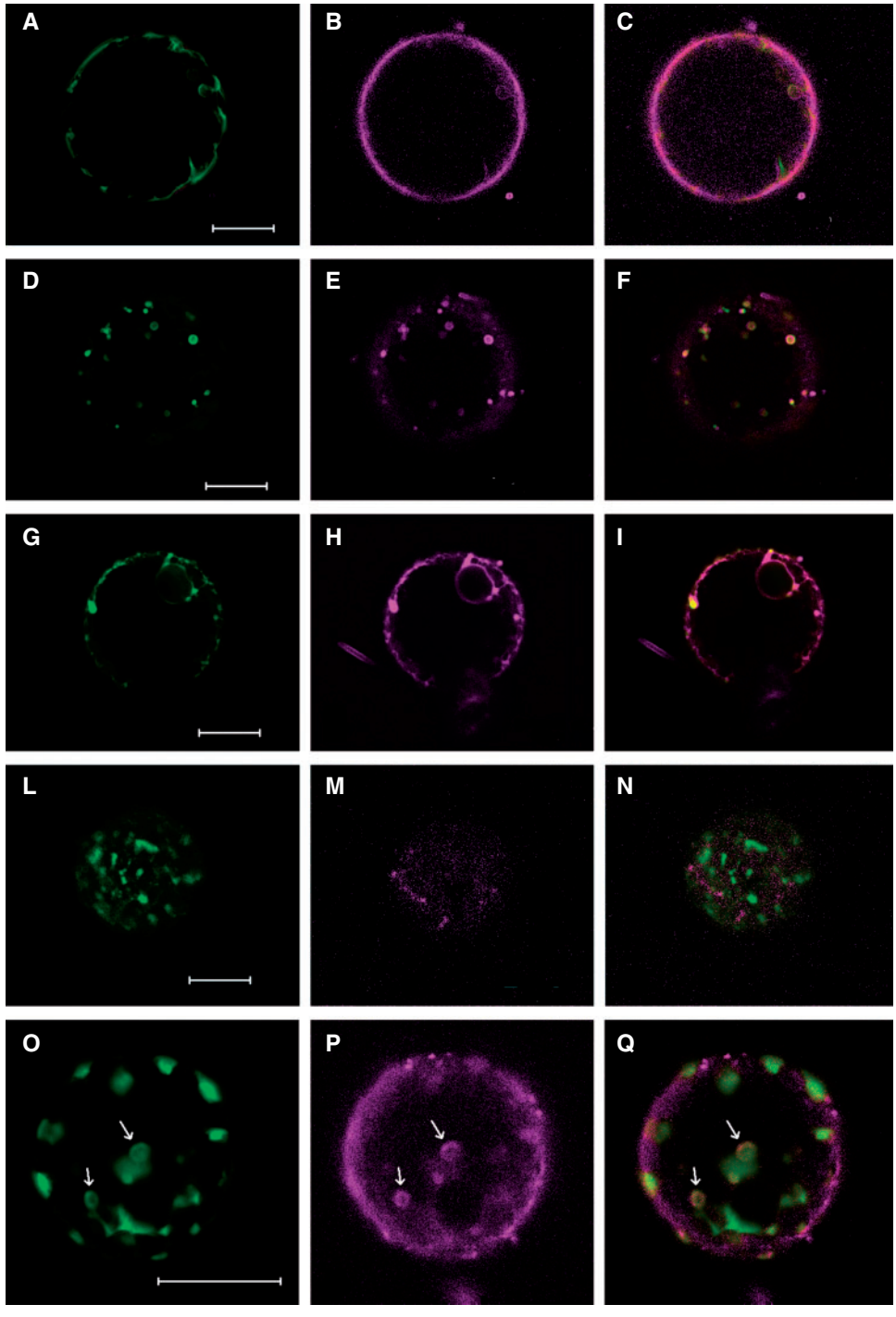

Fig. 6 FM4-64 staining of GFP–122F-expressing protoplasts. The first column shows GFP fluorescence, the second column shows the FM4-64 fluorescence, and the third column shows the two merged. (A–C) GFP–122F co-expressed with 122T. Markers co-localize in all membranous structures; the large ring-like structure indicated as aberrant endosomes appears occasionally to be connected to the PM. (D–F) GFP–122F co-expressed with 122T. The markers co-localize perfectly when fluorescence is restricted to large endosomes. Their formation and persistence suggest that they are the final destination of GFP–122F. (G–I) GFP–122F co-expressed with 121T. The markers also co-localize perfectly in structures different from large ring-like endosomes. Patterns from A to I co-exist in the same population of transformed protoplasts; they are also always observed when GFP–121F is co-expressed with any of the soluble mutants 121T or 122T. (L–N) GFP–122F co-expressed with Rab11S22/27N; endosomes and small GFP bodies did not co-localize. This pattern was not common to other combinations of constructs. (O–Q) GFP–122F co-expressed with Rab11S22/27N; co-localization with FM4-64 was observed when large endosomes formed (arrows). Scale bar = 20 μm.

the TMD of each syntaxin at the C-terminus. Alone, these constructs had a similar DN effect but, when each was co-expressed with Rab11S22/27N, the effects were different (Table 2). The inhibitory effects on secRGUS secretion of GFP–121H3 and Rab11S22/27N were additive (21% stronger reduction of secretion than Rab11S22/27N alone), while those of GFP–122H3 and Rab11S22/27N were not. ANOVA was significant [$F(9) = 81.64$; $P = 0.00$], and post hoc analysis confirmed the significance of these observations. The effect of GFP–121H3 co-expression with Rab11S22/27N showed statistically significant differences from all other situations ($P < 0.01$). GFP–122H3 co-expressed with Rab11S22/27N was not statistically different from Rab11S22/27N ($P = 0.1$) or GFP–122H3 ($P = 0.49$) expressed alone. In this experiment, we also measured the regular arrival of GFP–121/122H3 protein in the aberrant endosomes that characterized their pattern after 24 h expression. Rab11S22/27N specifically reduced the occurrence of endosomes labeled with GFP–122H3 by 47% ($53 \pm 9\%$ of control; $n = 3$) but, on the contrary, had no relevant effect on the occurrence of endosomes labeled with GFP–121H3 ($85 \pm 5\%$ of control; $n = 3$) (Supplementary Fig. S3).

Discussion

In this study, the involvement of *Le*Rab11a in secretion was examined. The *LeRab11a* cDNA sequence was derived from plasmid clone pNY650, isolated by Lu and co-workers (2001) from an early ripening tomato fruit phage library (Picton et al. 1993) using the mango *MiRab11a* cDNA (Zainal et al. 1996) as a probe. The most similar protein in the SWISSPROT database (94.5% identity) was the *Nicotiana plumbaginifolia* Rab GTPase, Np-Ypt3 (Dallman et al. 1992). The most similar (78.7%) of the *Arabidopsis* sequences was Ara-2 (Anai et al. 1991), now renamed *At*RabA1a under the systematic nomenclature of Periera-Leal et al. (2001) and Rutherford and Moore (2002), and this match was closely followed by *At*RabA1b. The functions determined for distinct animal and yeast Rab GTPases in that subfamily involved TGN–post-Golgi vesicle trafficking. Ara-4 or *At*Rab11f (now called *At*RabA5c) was localized on Golgi-derived vesicles (Ueda et al. 1996), and two other Rab11 GTPases, *Nt*Rab11b and *At*RabA4b, have been implicated in secretion of cell wall material to the apoplast in pollen tubes (de Graaf et al. 2005) and root hairs (Preuss et al. 2004), respectively.

With our study we support the thesis that *Le*Rab11a not only regulated secretion as shown by the expression of inactive forms of the protein, but this secretion involved the syntaxin SYP122 and not its close homolog SYP121. Secretion was monitored by the amount of a marker protein, secRGUS, found in the medium. This amount was corrected for leakage of intracellular proteins by the measurement of endogenous α-mannosidase in the medium. Functional studies of the GTP-binding site by mutations have revealed that a serine included in the first of the three domains of this site is important for GTP binding. In *Le*Rab11a there are two serines in this domain (Lu et al. 2001). In this study, we mutagenized these serines separately and together, giving rise to three mutants of the Rab protein (see Fig. 1), and analyzed them for effects on secretion of the marker protein secRGUS (Di Sansebastiano et al. 2007) and on membrane traffic mediated by the syntaxins, SYP121 or SYP122. Alteration of the GTP-binding site is not expected to influence membrane binding directly (Zuk and Elferink 1999, Zuk and Elferink 2000). In fact, the double mutant Rab11S22/27N exhibited a stronger association with membranes, which may be due to the S22N mutation (Fig. 2), but quantification of such variation showed no statistical significance. Since Rab11 alternates between a cytosolic and a membrane-associated form, it is possible that this cycling is affected by the S22N mutation, leading to a more persistent association of the mutated proteins with membranes. Secretion of the marker secRGUS was significantly reduced by expression of single mutant proteins (10–18%) but, when the double mutant was expressed, secretion was reduced by 33–51% (Tables 1–3), producing a more significant effect (as confirmed by ANOVA). This indicates that this Rab is involved in secretion and that both serines are important for its function.

Table 1 Percentage secretion of secRGUS measured in protoplasts co-expressing Rab11-derived constructs and controls

Construct co-expressed with secRGUS	Secretion ± SD; *n* value; *P*-value (*t*-test)
None (control)	$96 \pm 5\%$; $n = 9$
Rab11	$97 \pm 3\%$; $n = 5$; $P = 0.728$
Rab11S22N	$82 \pm 3\%$; $n = 3$; $P = 0.015$
Rab11S27N	$90 \pm 15\%$; $n = 3$; $P = 0.279$
Rab11S22/27N	$49 \pm 3\%$; $n = 9$; $P = 0.000$
Rab11S22/27N + GFP–Rab11	$80 \pm 9\%$; $n = 3$; $P = 0.002$
GFP	$92 \pm 6\%$; $n = 3$; $P = 0.127$
GFP–Rab11	$95 \pm 9\%$; $n = 8$; $P = 0.658$
GFP–Rab11S22N	$72 \pm 12\%$; $n = 4$; $P = 0.000$
GFP–Rab11S27N	$69 \pm 11\%$; $n = 3$; $P = 0.000$
GFP–Rab11S22/27N	$56 \pm 14\%$; $n = 4$; $P = 0.000$
GFP–Rab11S22/27N + Rab11	$78 \pm 7\%$; $n = 3$; $P = 0.000$

It was recently shown (Scapin et al. 2006) that the first serine (S22) in the domain is necessary for binding Mg^{2+} ions, and dissociation from GDP. It is expected that its mutation should alter the GDP dissociation rate while the mutation of the second serine (S27) should have a drastic effect on binding of GTP. We showed that the S27N mutation prevented GTP binding, but did not drastically modify the secretion of secRGUS, while the S22N mutation did not modify GTP binding and had little effect on secRGUS secretion (Fig. 2 and Table 1). Only the double mutation had a significant effect on secretion when ANOVA was performed. The biochemical characteristics of such a mutant include those of both single mutations. The difficulty in obtaining a DN mutant with a single mutation has been described for other GTP-binding proteins (van den Berghe et al. 1999). Thus, in this study, the double mutant Rab11S22/27N was used as a DN mutant to study the role of Rab11 in secretion.

Table 2 Percentage secretion of secRGUS measured in protoplasts co-expressing Rab11 and syntaxin constructs and controls

Construct co-expressed with secRGUS	Secretion ± SD; *n* value; *P*-value (*t*-test)
None (control)	100%; $n=6$
Rab11S22/27N	67 ± 4%; $n=4$; $P=0.000$
GFP–121F	98 ± 4%; $n=3$; $P=0.227$
121T	53 ± 5%; $n=3$; $P=0.000$
GFP–121H3	49 ± 6%; $n=5$; $P=0.000$
GFP–121H3 + Rab11S22/27N	38 ± 4%; $n=3$; $P=0.000$
GFP–122F	96 ± 5%; $n=3$; $P=0.072$
122T	56 ± 10%; $n=3$; $P=0.000$
GFP–122H3	56 ± 6%; $n=5$; $P=0.000$
GFP–122H3 + Rab11S22/27N	60 ± 1%; $n=3$; $P=0.000$

Rab11S22/27N was also combined with GFP–121H3 (increasing secretion inhibition by 21%) and GFP–122H3 (Supplementary Fig. 3).
n indicates the number of independent tests.

Rab11S22/27N had a much stronger effect on sorting of secGFP to the PM than on sorting of ERD2–YFP from the ER to the *cis*-Golgi or of AleuGFP from the Golgi to the PVC and the central vacuole (Fig. 3). This is consistent with the involvement of Rab11 in an anterograde post-Golgi transport to the PM. Rab11S22/27N certainly induced secondary effects that were revealed by the loss of motility of Golgi bodies for reasons that are unclear and deserve to be investigated further.

Mammalian Rab11-like GTPases mediate membrane trafficking steps involved in the recycling of membrane proteins between the endosomes and the PM (Ullrich et al. 1996) and secretion of newly synthesized proteins (Chen et al. 1998, Chen and Wandinger-Ness 2001). In this study, we co-expressed GFP–Rab11 or RFP–Rab11 with a number of intracellular markers and located the tagged protein on the TGN (Fig. 4). GFP–Rab11 did not co-localize with the endosomal marker FM4-64 (Uemura et al. 2004) within the first hour of staining, showing that Rab11 is not located on membranes of the endocytic pathway. The red variant RFP–Rab11 did not co-localize with the PVC marker AleuGFP (Di Sansebastiano et al. 2001) (Fig. 4L–N), indicating that Rab11 is not involved in the sorting pathway to the lytic vacuole.

ERD2–YFP (a marker for *cis*-Golgi compartments) and RFP–Rab11 labeled different regions of the same structures that moved independently but also transiently co-localized (Fig. 4H, I). This pattern is consistent with the presence of Rab11 on the Golgi and TGN elements where vesicles destined for the PM bud off.

RFP–Rab11 co-localized with Venus–Syp61 (a marker of the TGN) (Uemura et al. 2004) in mobile dotted structures. Overexpression of Venus–Syp61 very rapidly induced the appearance of abnormally large structures in

Table 3 Percentage secretion of secRGUS measured in protoplasts co-expressing Rab11 and native and soluble DN syntaxin mutants

Construct co-expressed with secRGUS	121F	121T	122F	122T	Rab11	Rab11-S22/27N
–	90 ± 10%; $n=3$	61 ± 9%; $n=3$	87 ± 7%; $n=3$	65 ± 6%; $n=5$	86 ± 9%; $n=3$	61 ± 6%; $n=3$
121F		94 ± 2%; $n=3$	92 ± 8%; $n=3$	87 ± 5%; $n=3$	87 ± 14%; $n=3$	68 ± 11%; $n=4$
121T			92 ± 2%; $n=3$	43 ± 4%; $n=3$	72 ± 5%; $n=4$	56 ± 17%; $n=3$
122F				90 ± 4%; $n=3$	95 ± 5%; $n=4$	68 ± 4%; $n=3$
122T					72 ± 2%; $n=3$	67 ± 16%; $n=3$
Rab11						79 ± 8%; $n=3$

Data derive from three totally independent experiments, different from those reported in the other tables. SecRGUS secretion inhibition increased when 121T + 122T or 121T + Rab11S22/27N were combined.

which it co-localized with GFP/RFP–Rab11 (Fig. 4O–Q). GFP/RFP-tagged Rab11 was functional since it complemented the DN mutant effect (Fig. 3). Immunolocalization of the related *P. sativum* Rab GTPase, Pra3, also showed co-localization with a TGN marker protein, AtVTI11 (Zheng et al. 1999, Inaba et al. 2002). Recently the same localization was shown for *Os*Rab11 in *Arabidopsis* protoplasts by Heo and co-workers (2005). These results indicate a role for Rab11 in anterograde transport to the PM rather than endosomes or vacuoles, and possibly in exocytosis as attributed to the role of the homologous Rab GTPases Ypt31/32 and Rab11a from yeast and mammals, respectively (Chen et al. 2001, Chen et al. 2005).

It was recently hypothesized that the TGN may function as an endocytic compartment in plants (Dettmer et al. 2006), which is not consistent with our finding that Rab11a chimeras do not show complete co-localization with FM4-64. FM4-64-labeled compartments not labeled by GFP–Rab11 may be late endosomes, and we cannot exclude that the observed sites for co-localization may correspond to EEs. In fact, the dye internalization was very rapid, and the pattern, taken into consideration in our observations, appearing constant from 30 to 60 min after loading, already included all sorts of endomembranes except probably the TGN and tonoplast (Bolte et al. 2004). At earlier stages, when EEs may be visible, the strong labeling of the PM was limiting our imaging possibilities. In our opinion, TGN/EE compartments are closely related but not necessarily identical, as demonstrated by the brefeldin A (BFA) effect. The effect of this drug is to induce early endosomal compartments to accumulate in the core of BFA compartments (Geldner et al. 2001), whereas *trans*-Golgi markers tend to be found mainly in the periphery of BFA compartments (Wee et al. 1998, Grebe et al. 2003). The VHA-a1–GFP-labeled compartments shown by Dettmer and co-workers (2006) to co-localize with both FM4-64 and SYP41 (which is known to co-localize with SYP61) may indicate that the VHA-a1 ATPase was important in both compartments. The TGN and EE are certainly strictly related and they may coincide in some cells, but we consider it premature, at this stage, to generalize, as done by Lam and co-workers (2007), in predicting that the partially coated reticulum (PCR)/TGN/EE are in fact the same compartment. When we observe a movement of FM4-64-labeled membrane from the PM to the TGN, it could simply be a sign of the connection between the EE and TGN and reflect the mainstream of membrane flux. This mainstream is expected to be different in different cell types such as BY2 or protoplasts and also to change depending on the stage of the cell cycle, as demonstrated by FM4-64 incorporation into the mitotic cell plate.

GFP/RFP–Rab11 localized to the same compartment as the mutated GFP–Rab11S22N, GFP–Rab11S27N, and GFP–Rab11S22/27N, indicating that these mutations did not alter the localization of the protein.

Since the DN effect on various markers and in vivo localization supported the idea of Rab11 involvement in TGN to PM traffic, we assayed whether Rab11 was involved in the pathway of secretion implicating the SNAREs SYP121 or SYP122. The full-length fusions GFP–121F and GFP–122F were localized at the PM (Fig. 5A, B). When the soluble DN mutants of the same syntaxins (121T and 122T) were expressed together with the unmodified form GFP–121F or GFP–122F, the formation of GFP-labeled endosomes was induced (Fig. 5C–F). These endosomes appeared or increased in number whichever combination of unmodified and modified forms of the syntaxins were used. This pattern may be due to an indirect effect on specific interactors of these SNAREs, leading to the formation of large compartments where the proteins, even if functional, accumulate due to a defect in recycling from the endosomes to the PM. Since both syntaxins have been shown to play a role in the anterograde sorting from the Golgi to the PM, this pattern may be a secondary effect of membrane traffic alteration or, alternatively, the indication of a second function for both these syntaxins in endocytosis. The effect observed with the co-expression of the unmodified GFP–121F or GFP–122F and Rab11S22/27N was different. GFP–122F was only seen to be retained in ER-like structures. The accumulation of GFP–122F in the ER (shown in Figs. 5H and 6L–N) may have been due to a defect in the transport of the protein to the PM, after its synthesis and tail anchoring to the ER membrane (Di Sansebastiano et al. 2006). This effect was specific for GFP–122F and was not observed with GFP–121F. Thirty-two percent of transformed cells showed this pattern without any visible large endosome. An additional 16% showed large endosomes that co-localized with FM4-64, but the GFP–122F distribution in the ER and unidentified compartments remained, as shown in Fig. 6O–Q. In other words, the newly synthesized SYP122 needs Rab11a to get to the PM, otherwise it backs up into the ER, whereas SYP121 has no such requirement.

The use of SYP soluble mutants was important to show that the variation of the pattern of a functional GFP chimera may not be a definitive result; when more mutants induce the same effect, it could be unspecific and a different pattern can be more informative.

Furthermore, in the present study, the effect of co-expression of two DN mutants was introduced. This experimental setting is based on the idea that when a DN mutant blocks the correct function of a sorting pathway, it is reasonable to expect no changes due to expression of other DN mutants from proteins involved in the same pathway. On the contrary, the expression of DN mutants

of proteins functioning in a different pathway should produce a variation in the effect.

This hypothesis was confirmed; the co-expression of the two syntaxins DN 121T and 122T produced greater inhibition of secRGUS secretion than either alone (Table 3) because they affected two distinct pathways. The same synergistic effect was reproduced when 121T was co-expressed with Rab11 DN (Table 3). At the same time, in these experimental conditions, the full length of both SYPs was able to 'compensate' the DN effect of either soluble mutant. This compensatory effect was expected in consideration of the previous studies showing the lack of phenotype in knockout plants for the single SYP genes; a dwarfed and necrotic phenotype can be observed only in a double mutant (Assaad et al. 2004). In this regard, it is essential to remember that in a knockout mutant the protein is missing and its cofactors are available; in the case of DN expression, the cofactors are blocked by the competitive binding with the mutant. The real relationship between these two syntaxins remains of course to be investigated.

From a strictly statistical point of view, the situation described in Table 3 needs to be consolidated because the variance between the effect caused by different combinations of mutants (especially 121T/Rab11S22/27N vs. 122T/Rab11S22/27N) is significant but not very high. In this work, we supported our conclusion from an interpretation of Table 3 data derived from a completely independent set of experiments.

To support this result, a new experiment was designed to obtain more information, coupling evaluation of syntaxin GFP-tagged DN mutants by secRGUS measurements and fluorescent patterns (Di Sansebastiano et al. 2006).

The GFP-tagged mutants of the syntaxins SYP121 and SYP122 (GFP–121H3 and GFP–122H3) contain the signature sequence of the syntaxin, included in the H3 domain at the C-terminus, which is required for the interaction with the partners of the SNARE complex. The N-terminal part represents the regulatory domain required for suppressing the activity of the protein during its sorting (Leyman et al. 1999). With the deletion of this N-terminal peptide, the mutants lose this regulation and thus should be able to interact with other regulatory elements at various steps of their sorting (Di Sansebastiano et al. 2006). By observing their fluorescence in transiently transformed protoplasts, the transformation efficiency and expression level can be monitored visually. Co-expression of Rab11S22/27N may have an additional inhibitory effect upon secRGUS secretion. However, if the pathway affected is the same, the reduction of secretion should not be modified. Our experiments allowed us to conclude that Rab11 is a regulatory element of the membrane trafficking driven by SYP122 and not by SYP121. Using the SYP122 mutant GFP–122H3 and/or the Rab11 double mutant, no additive effect was observed. On the contrary, the effect on secretion of GFP–121H3 was clearly increased by Rab11S22/27N, probably because each interfered with a different pathway of secretion and secRGUS transport was reduced to a much larger extent when both were expressed together. SecRGUS was shown again to be a good marker for monitoring different transport pathways at once. In fact this enzyme, like other widely used reporter proteins, secGFP (Leucci et al. 2007) or α-amylase (Phillipson et al. 2001), is secreted by default probably by different vesicle pools.

Moreover, in this experiment, we also had the possibility to measure the regular arrival of GFP–121/122H3 protein in the aberrant endosomes. Rab11S22/27N specifically reduced the occurrence of endosomes labeled with GFP–122H3 by 47% but, in contrast, had no significant effect on the occurrence of endosomes labeled with GFP–121H3 (see Supplementary Fig. S3). This observation was perfectly complementary to data on secRGUS secretion: if Rab11 were specifically required for SYP122 sorting, then only GFP–122H3 sorting would be altered by a reduction of endosomes appearing. This observation also gives additional information about sorting determinants in syntaxins. Functional specificity seems to be due to the regulatory N-terminal sequences (Tyrrell et al. 2007), but sorting specificity due to C-terminal sequences may also be relevant (Di Sansebastiano et al. 2006). This last experiment was consistent with the previous observation that the Rab DN mutant also induced the appearance of endosomes labeled by GFP–121F. In fact we deduced that aberrant endosomes are a non-specific effect of SNARE recycling after normal arrival at the PM. An interference with sorting to the target membrane would, as observed for GFP–122F, trap more protein in ER-related endomembranes.

Many plant proteins with the potential to regulate exocytosis have been identified by molecular analysis; Rab11 is one such molecule.

Specific interactions between SNAREs, regulated by specific Rab proteins, are a central event of vesicular traffic and drive vesicle fusion to target membranes. The study of SNAREs, especially with biochemical approaches, presents great difficulties because the specificity found in function and localization does not correspond to an equivalent difference in individual chemical and physical characteristics in vitro. All SNAREs share, to a certain extent, non-specific affinity for each other in vitro.

Three different syntaxins have been localized and characterized on the plant plasma membrane, and are candidates to have a role in the last steps of exocytosis: SYP121 (Leyman et al. 2000), SYP111/KNOLLE (Assaad et al. 2001) and SYP122 (Assaad et al. 2004). Of these,

SYP111 has been clearly shown to be involved in cell division and phragmoplast formation, SYP121 is probably involved in responses to ABA (Geelen et al. 2002), but less is known about SYP122 though it appears to be involved in constitutive secretion and also in pathogen-related responses (Nühse et al. 2003, Assaad et al. 2004).

The protein partners of these syntaxins in SNARE complexes are still not fully known. However, the interaction of the same SNAP (SNAP33) has been shown both with SYP111 (Heese et al. 2001) and with SYP121 (Kargul et al. 2001, Collins et al. 2003). Nevertheless, SYP111 should be involved uniquely in cell plate formation and it is known not to be interchangeable with SYP121 (Tyrrell et al. 2007). Specificity is also found in the function of SYP121 and its closest homolog SYP122. It is true that the initial production of knockout mutants revealed that they have overlapping functions (Assaad et al. 2004), but further studies of plant–pathogen interactions showed that the molecular functions by which SYP121 affects penetration resistance and negatively regulates other defenses are different, as only the latter functions are shared with SYP122 (Zhang et al. 2007).

Rab11 proteins, as well as syntaxins, may play different roles depending on the tissue, but the specific interaction between such cell regulatory elements should be maintained in vivo, even in a heterologous environment.

In conclusion, here we established a new experimental system by co-expression of the DN mutant of *Le*Rab11a with GFP-tagged DN mutants of SNAREs.

The same approach can be used for other proteins of the secretory pathway. Thus 'mapping' the regulatory activity of different elements on specific pathways can be performed. Using this approach, we 'mapped' *Le*Rab11a activity on a pathway to the PM involving SYP122 rather than SYP121, although it is entirely possible that other members of the Rab11 clade may function elsewhere.

The possibility that SYP121 and SYP122 drive independent secretory events, clearly evidenced by recent studies (Zhang et al. 2007), is supported by the *Le*Rab11a differential effect.

Materials and Methods

Constructs

Rab11 constructs were obtained by cloning the *Le*Rab11a cDNA (AJ245570) as a *Bam*HI/*Pst*I fragment in pGY, a pUC-derived vector containing the cauliflower mosaic virus (CaMV) 35S promoter and *nos* terminator (Di Sansebastiano et al. 1998). Restriction sites were inserted by amplifying the cDNA (Lu et al. 2001) by PCR with Rab01 forward (*gcaaa agcgg atcca gtttt gaaga tggca*) and Rab02 reverse (*taaag cttga gctat tgtct tactg cagcc*) primers.

GFP–Rab11 was obtained by inserting the Rab11 cDNA in a GFP-containing pBI-derived vector (Di Sansebastiano et al. 2006) as a *Sal*I/*Pst*I fragment. Restriction sites were inserted by amplifying the cDNA by PCR with Rab13 forward (*gcaga tatgt gtcga cgatg gcaggt*) and Rab02 reverse primers.

To construct RFP–Rab11, RFP was substituted for GFP in GFP–Rab11 as a *Bam*HI/*Sal*I restriction fragment. Restriction sites were inserted by amplifying the protein with RFP01 forward (*aggat cccta tggcc tcctc cgagg acgtc atcaa*) and RFP02 reverse (*aatgt cgacg cgccg gtgga gtggc ggccct*) primers.

Mutations were inserted with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Ca, USA) using the following primers: S22N_RabR (*gatta gattt accca cacca ttatc acctat*)/S22N_Rab (*gtgtt gatag gtgat aatgg tgtgg gtaaa tctaa tctgc*) to insert the mutation S22N; S27N_RabR (*aaagc agatt agatt taccc acacc actat caccta*)/S27N_RabF (*caagc ttgtg ttgat aggtg ataat ggtgt gggtaa*) to insert the mutation S27N; and S(22–27)NF (*gatag gtgat aatgg tgtgg gtaaa aataa tctgc*)/S(22–27)NR (*cctgg aaagc agatt atttt taccc acacc attat cacc*) to insert both mutations.

SYP121-derived constructs were described previously (Di Sansebastiano et al. 2006). GFP–121F was obtained by inserting SYP121 (Leyman et al. 1999) in a GFP-containing pBI-derived vector (Di Sansebastiano et al. 2006) as a *Sal*I/*Pst*I fragment. Sites were inserted by amplifying the protein with STXFSal forward (*gtcga ccatg aatga tctat tttca ggatc*) and STX1 reverse (*gcctg cagtc atttt ttcca tggc*) primers. GFP–121H3 was obtained by inserting an N-terminal fragment of SYP121 as a *Bgl*II/*Pst*I fragment. Sites were inserted by amplifying the protein with 2CtSP forward (*ttata ccgtc acagg agatc ttcc*) and STX1 reverse primers. 121T was generated by cloning a *Bam*HI/*Pst*I PCR product into the expression vector. The primers STX3 forward (*gcgga tccat gaatg atcta tttt*) and STX2 reverse (*gcctg cagtt aacaa gtcca tttt*) were used.

GFP–122F was obtained by inserting SYP122 (accession No. AJ245407.1; GI: 5701796) in a GFP-containing pBI-derived vector (Di Sansebastiano et al. 2006) as a *Sal*I/*Pst*I fragment. Sites were inserted by PCR with 122FOR forward (*gaagat gtcgac agcca tgaac gatc*) and 122REV reverse (*atgct catgc atctg cagag ggaac ct*) primers. GFP–122H3 was obtained by inserting an N-terminal fragment of SYP122 as a *Sal*I/*Pst*I fragment. Restriction sites were inserted by PCR with the primers 122H3 forward (*tcgtc gtcga cggac cggca aagaa ctt*) and 122REV reverse. 122T was generated by cloning a *Bam*HI/*Pst*I PCR product into the expression vector. The primers 122BAM forward (*tcgag gatcc cttaa gccat gaacg atct*) and 122T reverse (*agcct gcagc aaaat ggcaa atcaa gtcca*) were used.

Protoplast transient expression

Nicotiana tabacum cv. SR1 protoplasts were isolated following the protocol of Maliga and co-workers (1976), cultured and rinsed using the indicated media, and transformed by polyethylene glycol (PEG, Fluka AG, Buchs, CH)-mediated direct gene transfer essentially as described (Freydl et al. 1995, Di Sansebastiano et al. 1998). A 10 μg aliquot of plasmid was used for the transformation of about 600 000 protoplasts. At 2 h after addition of PEG and plasmid, the protoplasts were rinsed to remove the PEG, resuspended in 2 ml of culture medium and incubated at 26°C in the dark.

Transformation efficiency depends on the amount of supercoiled plasmid DNA so it can vary independently from the quantity of DNA. In the case of non-GFP-tagged DN mutants, where no visual screening was possible, 20 μg of DNA were used for each construct to guarantee overloading. The transformation efficiency of the reported experiments was always >40%. All analyses were performed 24 h after transformation.

FM4-64 dye staining

For staining protoplasts, the dye FM4-64 (Molecular Probes, Leiden, The Netherlands) was used at a concentration of 100 μM, from a stock (1 mM) in 0.4 M mannitol. Within the first 10 min, the dye stains only the PM of protoplasts, then is rapidly internalized. Images were produced as specified. The pattern was apparently stable from 30 min up to 60 min after staining, and a timing difference did not appear relevant.

Confocal microscopy

Protoplasts transiently expressing fluorescent constructs were observed by fluorescence microscopy in their culture medium at different times after transformation. They were examined with a confocal laser microscope (LSM Pascal Zeiss). GFP, YFP and Venus were detected with the filter set for fluorescein isothiocyanate (FITC; 505–530 nm), RFP with a 560–615 nm filter set, while chlorophyll epifluorescence was detected with the filter set for trimethylrhodamine isothiocyanate (TRITC; >650 nm) and eliminated. An excitation wavelength of 488 nm was used.

To detect FM4-64 fluorescence, the He–Ne laser was used to produce a 543 nm excitation, and the emission was recorded with the 560–615 nm filter set.

Protein extraction from protoplasts and enzymatic tests

Protoplasts were harvested by a 5 min centrifugation at $65 \times g$, after addition of about 4 vols. of W5 to the incubation medium (final volume 10 ml). An aliquot of supernatant was saved and stored at –20°C (extracellular fraction); the cell pellet was resuspended in 1 ml of 0.1 M Na-acetate pH 5 and lysed by three cycles of freezing (in liquid nitrogen) and thawing. The soluble proteins were separated from insoluble residues by centrifugation for 5 min at $10\,000 \times g$ (intracellular fraction). This extract and the medium saved after harvesting the cells were both directly used to measure enzymatic activity of secRGUS (Di Sansebastiano et al. 2007) and α-mannosidase (the constitutive enzyme used as internal control). Measurements were made in an RF-5301 Shimadzu PC spectrofluorophotometer.

The reaction substrates were 4-methyl-umbelliferyl-β-D-glucuronide (Sigma, Steinheim, Germany) and 4-methyl-umbelliferyl-α-D-mannoside (Sigma) to measure secRGUS and α-mannosidase activity, respectively. Assays were normalized by comparing secRGUS activity with the internal control (α-mannosidase); both samples were excited at 370 nm and fluorescence measured at 480 nm.

The secRGUS percentage of secretion was calculated as the rate of extracellular activity over total (extracellular and intracellular) activity, after considering the 10-fold dilution of the incubation medium and the secretion of the marker mannosidase as an index of contamination.

Within each experiment, the secRGUS secretion in control conditions, always above 80%, was normalized to 100% to make all experiments comparable.

The statistical significance of the effect of DN mutants was tested by *t*-test analysis, and ANOVA to discriminate between the combined effects of two DN mutants was first validated by univariate test of significance. The Student–Newman–Keuls test was applied for ANOVA post hoc comparison.

Data reported in each table derive from independent groups of experiments performed independently.

When control is not equal to 100% it is due to variability derived from the presence of multiple control samples in a single experiment. In some cases, a secretion >100% can be observed; this derives from the correction of the data in consideration of the contamination of the intracellular marker mannosidase. In other words, if contamination evidenced in the control sample was higher than in co-transformations where secretion was already very efficient, the correction factor increased this last value >100%. We preferred to keep these values as such, not to alter the statistical evaluation.

Protein extraction for SDS–PAGE

Protoplasts were harvested by a 5 min centrifugation at $65 \times g$ and resuspended in the extraction buffer [1% Tris-buffered saline (TBS) supplemented with the proteinase inhibitor cocktail 'Complete' by Roche]. Protoplasts were lysed by three consecutive freezing–thawing cycles. Lysed cells were centrifuged for 30 min at $14\,000 \times g$. The supernatant was considered to contain the soluble protein fraction; the pellet was resuspended in the extraction buffer supplemented with 2% SDS and left at room temperature for 10 min to solubilize membrane proteins. Insoluble aggregates in the membrane fraction were removed with a short centrifugation at $10\,000 \times g$. The 'soluble protein' and 'membrane-bound protein' fractions were precipitated with 2 vols. of acetone. After centrifugation at $15\,000 \times g$ for 30 min, pellets were resuspended in volumes proportional to the original sample for gel analysis.

Binding of GFP–Rab11 variants to GTP-agarose

Protoplasts were harvested by a 5 min centrifugation at $65 \times g$ and resuspended in the 'binding' buffer (20 mM HEPES pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 5× proteinase inhibitor cocktail 'Complete' by Roche). Protoplasts were lysed by three consecutive freezing–thawing cycles. Lysed cells were centrifuged for 30 min at $14\,000 \times g$, 1 ml of the supernatant was used to estimate total proteins, and total proteins of an aliquot were extracted for immunoblot analysis. The extract was then incubated with 100 μl of GTP-agarose suspension (Sigma G9768, St. Louis, USA) for 1 h with agitation at 4°C. The agarose beads were pelleted by centrifugation, washed once in 'binding buffer' and resuspended in 40 μl of SDS–PAGE sample buffer. GTP-bound proteins were analyzed by immunoblotting as described below.

SDS–PAGE and immunolabeling

The proteins were separated in polyacrylamide gels with SDS (4% stacking gel, 15% separation gel; Laemmli and Favre 1973) in the minigel system 'Mini-Protean II Dual Slab Gel System' from Bio-Rad. Then they were electrophoretically transferred on a nitrocellulose membrane (Hybond-C Extra, Amersham, Little Chalfont, UK) that was incubated overnight in 100 ml of 5% milk–TBS (20 mM Tris–HCl pH 7.5; 500 mM NaCl; 5% w/v milk powder) to saturate the nitrocellulose membrane with proteins and anti-GFP (Molecular Probes A6455, Leiden, NL) primary antibodies; anti-rabbit secondary antibodies coupled to peroxidase (Sigma) were used.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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