Selective targeting of plasma membrane and tonoplast traffic by inhibitory (dominant-negative) SNARE fragments

Matthew Tyrrell, Prisca Campanoni, Jens-Uwe Sutter, Réjane Pratelli†, Manuel Paneque‡, Sergei Sokolovski and Michael R. Blatt*
Laboratory of Plant Physiology and Biophysics, IBLS, Plant Sciences, Bower Building, University of Glasgow, Glasgow G12 8QQ, UK

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*For correspondence (fax +44 141 330 4447; e-mail m.blatt@bio.gla.ac.uk).
†Present address: Institute of Cellular and Molecular Botany, University of Bonn, Kirschallee 1, D53115 Bonn, Germany.
‡Present address: Facultad de Ciencias Agronómicas, Universidad de Chile, Casilla 1004, Santiago, Chile.

Summary

Vesicle traffic underpins cell homeostasis, growth and development in plants, and is facilitated by a superfamily of proteins known as SNAREs [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors] that interact to draw vesicle and target membrane surfaces together for fusion. Structural homologies, biochemical and genetic analyses have yielded information about the localization and possible roles of these proteins. However, remarkably little evidence is yet available that speaks directly to the functional specificities of these proteins in selected trafficking pathways in vivo. Previously, we found that expressing a cytosolic (so-called Sp2) fragment of one plasma membrane SNARE from tobacco and Arabidopsis had severe effects on growth, tissue development and secretory traffic to the plasma membrane. We have explored this dominant-negative approach further to examine the specificity and overlaps in Sp2 activity by generating a toolbox of truncated SNARE constructs and antibodies for transient expression and analysis. Using a quantitative ratiometric approach with secreted green fluorescent protein (secGFP), we report here that traffic to the plasma membrane is suppressed selectively by Sp2 fragments of plasma membrane SNAREs AtSYP121 and AtSYP122, but not of the closely related SNARE AtSYP111 nor of the SNARE AtSYP21 that resides at the pre-vacuolar compartment (PVC). By contrast, traffic of the YFP-tagged aquaporin fusion protein TIP1;1–YFP to the tonoplast was blocked (leading to its accumulation in the PVC) when co-expressed with the Sp2 fragment of AtSYP21, but not when co-expressed with that of AtSYP121. Export of secGFP was also sensitive to the Sp2 fragment of the novel, plant-specific SNARE AtSYP71 that was recently found to be present in detergent-resistant, plasma membrane fractions. Co-incubation analyses of the plasma membrane SNAREs with the regulatory subdomain included within the Sp2 fragments showed activity in destabilizing protein complexes, but only with the complementary SNAREs. We conclude that the Sp2 fragment action accurately reflects the known specificity and targeting of these SNAREs, implies functional overlaps that are of potential physiological interest, and underscores the use of a dominant-negative strategy in functional studies of a major subfamily of SNAREs in plants.

Keywords: membrane vesicle traffic, tonoplast intrinsic protein, secreted GFP, endoplasmic reticulum, Nicotiana tabacum, Arabidopsis thaliana.

Introduction

Eukaryotic cells maintain traffic of vesicles to shuttle membrane material, proteins and soluble cargo between endomembrane compartments, the plasma membrane and the extracellular space. Vesicles are formed by budding and constriction at the formative membrane surface, and their delivery is achieved by fusion and intercalation with the lipid bilayer of the target membrane (Pratelli et al., 2004; Surpin and Raikhel, 2004). These processes sustain membrane turnover and thereby contribute to cellular homeostasis, differentiation and growth. Vesicle traffic contributes to
neurotransmitter release and nervous signal transmission across the synaptic junctions of nerves, to cell-wall delivery and budding in yeast (Chen and Scheller, 2001; Jahn et al., 2003), and to cell polarity, growth and development in plants (Blatt and Thiel, 2003; Hurst et al., 2004).

Central to the process of vesicle fusion is a family of membrane-trafficking proteins, SNAREs [soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors], that are conserved among all eukaryotes (Jahn et al., 2003; Pratelli et al., 2004; Surpin and Raikhel, 2004). Complementary SNAREs, identified by their core residues [either arginine (R) or glutamine (Q)], are localized to different membrane compartments, and interact to form a tetrameric bundle of coiled helices that draws the membrane surfaces together and facilitates fusion. In reconstituted membrane preparations, this complex forms a minimal set of proteins required for fusion (Hu et al., 2003; Parlati et al., 1999; Weber et al., 1998). In vivo, other cytoplasmic factors (including the N-ethylmaleimide-sensitive factor, Sec1, and its homologues) control interaction between the SNARE elements (Jahn et al., 2003; Pratelli et al., 2004; Surpin and Raikhel, 2004; Sutter et al., 2006a) Nonetheless, the combinatorial specificity of SNARE interactions is thought to contribute significantly to membrane recognition and vesicle targeting (Paumet et al., 2004; Varlamov et al., 2004).

Of the core SNAREs in plants, the syntaxins (members of the Q-SNARE subclass; Sutter et al., 2006a) are the best characterized to date, and, functionally, most intriguing. In Arabidopsis, the count of syntaxin-like SNAREs far outnumbers the identifiable membrane compartments. One subclade of three members (AtSYP71, AtSYP72 and AtSYP73) shows no obvious homologies to any grouping among the yeast and mammalian SNAREs, but includes at least one member that is found at the plasma membrane (Alexandersson et al., 2004; Marmagne et al., 2004). There are hints, too, of a functional diversity that extends beyond the canonical roles in membrane targeting and vesicle fusion (Sutter et al., 2006a). Finally, a growing body of kinetic and physiological data further underscores the complexity of vesicle traffic, at least at the plasma membrane where physical access is possible in vivo (Homann and Tester, 1997; Sutter et al., 2000, 2006a,b).

By contrast, information remains scarce regarding the partitioning of specific membrane proteins to the plasma membrane, far less on roles for SNAREs or their specificity in these processes. SNARE-related vesicle traffic has been implicated in the spatial distribution of the auxin efflux carrier Pin1 and its sensitivity to the ARF-GEF inhibitor brefeldin A (Geldner et al., 2001; Steinmann et al., 1999) that disrupts Golgi structure and trafficking (Nebenfuhr et al., 2002). There is genetic evidence for vesicle traffic associated with one subclade of Q-SNAREs during cytokinesis and cell division in Arabidopsis (Muller et al., 2003; Volker et al., 2001; Waizenegger et al., 2000). Additionally, sequence homologies to mammalian and yeast SNAREs (and, in several instances, also immunohistochemical localization studies) suggest important operational similarities. Finally, over-expression of at least one SNARE, AtSYP21 (AtPep12) which is associated with the pre-vacuolar compartment (PVC) (Surpin and Raikhel, 2004; Sutter et al., 2006a), has been found to affect cargo export to the vacuole (Foresti et al., 2006). However, virtually no evidence is yet available that can speak directly to the functional specificity in vivo between the various SNARE proteins in selected trafficking pathways.

Previously, we identified NtSYP121 (=NtSyr1) and AtSYP121 (=AtSyr1) from tobacco and Arabidopsis, respectively, as plasma membrane Q-SNAREs that are associated with abscisic acid and drought stress (Leyman et al., 1999, 2000). We found that disrupting NtSYP121 function by expressing a dominant-negative, cytosolic (so-called) Sp2 fragment in vivo had severe effects on growth, tissue development and traffic to the plasma membrane (Geelen et al., 2002). These observations suggested an approach to determining the specificity of SNARE function in vivo through selective interference with SNARE-dependent trafficking steps. Here we explore this approach further and outline a method for fluorescence ratiometric analysis to quantify trafficking dynamics using confocal imaging of a secretory marker green fluorescent protein (secGFP). Additionally, we examine traffic of the aquaporin TIP1;1 to the tonoplast membrane, and compare the effects of co-expression with Sp2 fragments of selected SNAREs on the traffic of secGFP export and TIP1;1. We report here that secGFP export to the plasma membrane is suppressed selectively by Sp2 fragments of plasma membrane SNAREs, but not of the closely related SNARE AtSYP111 nor of the PVC SNARE AtSYP21. Conversely, traffic of the YFP-tagged TIP1;1 to the tonoplast is blocked at the PVC when co-expressed with the Sp2 fragment of AtSYP21, but not when co-expressed with that of AtSYP121. These, and additional results provide evidence for selective interference of the Sp2 fragments through interactions that accurately reflect the known specificity and targeting of the corresponding SNAREs, and to point to functional overlaps that are of potential physiological interest. They also underscore the utility of a dominant-negative strategy used previously in functional studies of SNAREs in plants.

Results

Coding sequences for Sp2 fragments were generated to express the cytosolic domains of each of five Arabidopsis syntaxin-like Q-SNAREs, terminating between the syntaxin signature (H3) sequence and the C-terminal trans-membrane anchor of the native protein. For the plasma membrane SNAREs AtSYP121, AtSYP122 and AtSYP71, and for the pre-vacuolar SNARE AtSYP21, the coding sequence was...
terminated at the single, conserved cysteine (Figure 1); for the phragmoplast-associated SNARE AtSYP111, the coding sequence was terminated 19 residues earlier to give a soluble product with a hydrophilic C-terminus. Each of these sequences was subcloned between the tandem 35S promoter and nopaline synthase terminator of the binary vector pPTKan (Allen et al., 2000) to generate corresponding expression cassettes for Agrobacterium tumifaciens-mediated transfection of tobacco. Shorter segments of the same coding sequences were also generated that included the Ha, Hb and Hc coil domains in each case, but omitted the highly conserved H3 coil domain (Figure 1). His6-tagged and purified proteins from these constructs were used to generate polyclonal antibodies and were tested against the purified epitopes and Arabidopsis protein extracts (Figure 2).

Sp2 fragment of AtSYP121 suppresses secGFP marker export

We used a GFP variant, secGFP, that normally is secreted to the apoplast (Batoko et al., 2000; Geelen et al., 2002) as a marker for traffic to the plasma membrane. When secreted, secGFP does not build up in a fluorescent form, but it accumulates and is visualized readily within the secretory pathway, notably within the endoplasmic reticulum, when transport to the apoplast is inhibited. Tobacco leaves were infiltrated with Agrobacterium to co-express the secGFP construct with each of the Sp2 fragments, and, as a control, on its own. secGFP accumulation was monitored by confocal laser scanning microscopy of epidermal cells on the lower leaf surface. As an internal control for the efficacy of expression, we included a second marker, YFP-HDEL, which is normally retained within the endoplasmic reticulum. Imaging with YFP-HDEL also provided a convenient reference to ensure the image plane was positioned for maximum signal within the epidermis, especially at lower magnifications, giving images spread over several hundred epidermal cells.

**Figure 1.** Schematic of AtSYP121 constructs.
The relative positions of the Ha, Hb and Hc domains, the syntaxin signature sequence (H3) domain and the trans-membrane (TM) domain are shown, and final amino acid residues indicated for the Sp2 and Sp3 fragments.

![Image](Image 197x171 to 238x181)

![Image](Image 197x214 to 238x224)

![Figure 2](21 31 41 51 71 112 122 wt)

![Figure 3](21 31 41 51 71 112 122 wt)

**Figure 2.** Antibodies raised to Sp3 fragments show specificity between selected Arabidopsis SNAREs (see also Figure 3). Western blot analysis using polyclonal antibodies raised to AtSYP121-Sp3 (αAtSYP121) and AtSYP71-Sp3 (αAtSYP71) fragments to probe His6-purified Sp2 fragments of the SNAREs AtSYP21, AtSYP31, AtSYP41, AtSYP51, AtSYP71, AtSYP111, AtSYP112 and AtSYP122 (left panels) and total protein extracts from Arabidopsis leaves (right panels). Aliquots of 10 µg Arabidopsis protein or 0.2 µl purified Sp2 fraction were loaded per lane and probed as described in Experimental procedures. The upper left panel was deliberately over-exposed to reveal a very low level of cross-reactivity with AtSYP21-Sp2 and a second band associated with the AtSYP122-Sp2 fraction. The additional band in the AtSYP121-Sp2 fraction is consistent with the occurrence of a second Met (start codon) at residue 32. Both antibodies recognized a single band in the leaf extract close to the predicted molecular weights for AtSYP121 (34.7 kDa) and AtSYP71 (30.0 kDa). Similar results were obtained with αAtSYP21, αAtSYP111 and αAtSYP122 antibodies (not shown).

Figure 3 shows the results from one experiment with and without co-expression of the Sp2 fragment of AtSYP121 (AtSYP121-Sp2) obtained 3 days after infiltration (compare Figure 9 of Geelen et al., 2002). Similar results were obtained in each of ten other experiments. Expressing the two marker constructs alone yielded a strong YFP fluorescence signal within the epidermis, but little evidence of any fluorescence from the secGFP marker (Figure 3a,b). By contrast, co-expression of the markers together with the AtSYP121-Sp2 fragment yielded significant GFP fluorescence within the epidermis (Figure 3c,d). Examined under high magnification, the YFP marker was seen to distribute within a reticulate network around the cell periphery and was included within a nuclear ring (Figure 3e), characteristic of marker retention within the endoplasmic reticulum; close inspection showed the GFP signal to co-localize with the YFP fluorescence. It also appeared within highly mobile puncta (not shown) reminiscent of the Golgi (Boevink et al., 1998).
The Sp2 fragment AtSYP121-Sp2 suppresses export of the secreted cargo marker secGFP.

Laser-scanning confocal images of tobacco leaf epidermis collected 3 days after co-transfection with secGFP and the endoplasmic reticulum marker YFP-HDEL alone (a,b) or together with AtSYP121-Sp2 (c–e), showing (from left to right) the merged image, YFP fluorescence and GFP fluorescence. The images clearly show the enhanced GFP fluorescence associated with its retention within the cell by expression of the Sp2 fragment. Low-magnification images (a,c) were used for statistical analysis as described in the text. (e) High-magnification image showing the co-localization of the fluorophores in a reticulate network at the cell periphery, in the nuclear ring (n) and within transvacuolar strands (s) of one cell. Scale bars = 400 μm (a,c) 100 μm (b,d) and 10 μm (e).

Western blot analysis of total leaf protein (10 μg per lane) from sections of the same tobacco leaf transfected with secGFP and YFP-HDEL only (XFP) and together with AtSYP121-Sp2 (XFP + Sp2). Blots were probed with αAtSYP122 (left) or αAtSYP121 (right). Note the presence of different cross-reactive bands with each of the two polyclonal antibodies, consistent with the presence of close homologues in tobacco to the Arabidopsis Q-SNAREs AtSYP121 and AtSYP122 (see also Leyman et al., 1999; Kargul et al., 2001).

Use of the fluorescence ratio $f_{475-515}/f_{535-590}$ to quantify secretory block

We carried out experiments infiltrating tobacco leaves with Agrobacterium suspensions of sufficient density for each construct to ensure ≥ 90% transfection efficiency (cf. Figure 3). As a result, by far the greatest source of fluorescence variability over the leaf was expected to arise from surface undulations, as regions of leaf surfaces often fell outside the plane of focus of the microscope at low magnification. Including the YFP-HDEL construct as a reference served to identify areas within the focal plane, allowing us to discount other regions within images. Thus we expected roughly 80% of epidermal cells within the focal plane to show a strong GFP signal against the background of YFP fluorescence, assuming transfections were randomly distributed and a 10% drop-out rate for each of the constructs on a cell-by-cell basis. However, at low magnification, a greater variability in the two fluorescence signals was clearly evident when co-expressing the marker constructs with AtSYP121 Sp2, even from cells within the focal plane (Figure 3c,d). Analysis of variance in the GFP fluorescence between experiments, as well as between different leaf sections within one experiment, showed a substantial difference in signals obtained (Table 1).

One potential source of variability in this case might be associated with stochastic differences in transgene expression (Elowitz et al., 2002; Paulsson and Ehrenberg, 2001), and we sought therefore to test this possibility. We carried out experiments with GFP and YFP fluorophores, both carrying the C-terminal HDEL sequence for retention in the endoplasmic reticulum, co-infiltrating tobacco with Agrobacterium carrying the constructs as separate plasmids and using a single construct carrying GFP-HDEL and YFP-HDEL cassettes in tandem on one plasmid (Samalova et al., 2006). Significantly, in each of four independent experiments, we observed that the two fluorophores gave similar variations in relative fluorescence regardless of the

Table 1: Comparison of the mean (±SE) coefficients of variance (SD/mean, expressed as percentages.) of GFP fluorescence calculated from the absolute $f_{475-515}$ signals and from the fluorescence ratios $f_{475-515}/f_{535-590}$ for n representative experiments expressing secGFP with AtSYP121-Sp2 and YFP-HDEL, and expressing GFP-HDEL with YFP-HDEL together from separate plasmids and from the tandem plasmid

<table>
<thead>
<tr>
<th>Construct</th>
<th>$f_{475-515}$</th>
<th>$f_{475-515}/f_{535-590}$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>secGFP/AtSYP121-Sp2/YFP-HDEL</td>
<td>28 ± 10</td>
<td>11 ± 4</td>
<td>5</td>
</tr>
<tr>
<td>GFP-HDEL, YFP-HDEL (separate)</td>
<td>30 ± 12</td>
<td>16 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>GFP-HDEL, YFP-HDEL (tandem)</td>
<td>31 ± 10</td>
<td>14 ± 6</td>
<td>4</td>
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transfection strategy, whether co-expressed from separate plasmids or as tandem cassettes on a single plasmid. Figure 4(a) shows images from one of these sets of experiments (see Table 1 for a comparison of coefficients of variance). Visible throughout the frames are many cells that fluoresced in both YFP and GFP channels, although individual epidermal cells showed preferential expression of YFP or GFP fluorescence. Because cells transfected with the tandem construct must carry the coding sequences for both markers, it follows that their transcription, translation and processing exhibit a degree of stochastic behaviour from one cell to the next.

We found that calculation of the ratio of fluorescence signals, i.e. of GFP relative to a retained YFP standard, greatly reduced the differences between experiments and within any one set of measurements. To quantify GFP and YFP fluorescence and determine the extent of fluorescence variability, we analysed images collected using a 10x objective in six regions selected randomly within infiltrated leaf segments for each construct or set of constructs using standardized excitation and collection settings on the confocal microscope. As before, the focal plane in each case was adjusted to give the maximum YFP fluorescence signal through the epidermal plane. Measurements were corrected for fluorescence background by comparison with images collected from non-transfected leaves. For statistical analysis, each image was segmented to give nine subframes from which the fluorescence signals for both markers were determined. Fluorescence ratios \( f_{475-515}/f_{535-590} \) were calculated, and the ratios were binned before plotting.

Figure 4(b) shows the distribution of \( f_{475-515}/f_{535-590} \) ratios for GFP-HDEL and YFP-HDEL expressed from separate constructs and from the tandem cassette construct. For the situation in which the level of GFP fluorescence was a constant proportion of YFP fluorescence – the ideal, at least when expressed after transfections using the tandem cassette construct. The \( f_{475-515}/f_{535-590} \) distribution was expected to yield a constant independent, for example, of the copy number of plasmids transfecting the cells. So, in principle, we would anticipate a single, sharp peak in the \( f_{475-515}/f_{535-590} \) distribution in this case. In fact, transfections with the tandem cassette construct showed an appreciable spread in the \( f_{475-515}/f_{535-590} \) ratio distribution, consistent with the pattern of fluorescence variation determined visually, and similar to the \( f_{475-515}/f_{535-590} \) ratios obtained using the separate plasmid constructs (Figure 4b). In each case, \( f_{475-515}/f_{535-590} \) ratios were well-fitted to normal (Gaussian) distributions of the form

\[
f_{475-515}/f_{535-590} = A \cdot \exp \left[ -\frac{0.5(F_x - F_0)^2}{b} \right]
\]

where \( A \) is the peak amplitude at the mean fluorescence ratio \( F_0 \), \( F_x \) is the fluorescence at any given ratio \( x \), and \( b \) is the coefficient of spread and reflects the variability in \( f_{475-515}/f_{535-590} \) about the mean ratio \( F_0 \). Fittings yielded ratio means of 1.12 ± 0.02 and 2.38 ± 0.03, and coefficients of spread of 0.18 ± 0.02 and 0.23 ± 0.03 for experiments using separate plasmid constructs and the tandem construct, respectively (see legend to Figure 4b). The higher ratio obtained in the latter instance is an intrinsic feature of expression from the tandem construct (Samalova et al., 2006). Nonetheless, in both cases, the distribution of ratios characterized by the coefficient of spread, \( b \), was similar (see also Table 1). Thus, we suggest that stochastic variations in fluorophore expression predominate over any cell-to-cell variability associated with transfer of individual plasmids.

For comparison, the same analytical method was applied to experiments using the secGFP and YFP-HDEL constructs with and without the addition of AtSYP121-Sp2 (Figure 3), making use of co-transfections with GFP-HDEL and YFP-HDEL as a measure of maximum retention. The results of such analysis are shown in Figure 5 and highlight a number of features. The first, and most obvious, is that co-transfection of the marker constructs together with AtSYP121-Sp2 resulted in a large
displacement of the mean $f_{475-515}/f_{535-590}$ ratio relative to the control without the Sp2 fragment. In the absence of the Sp2 fragment, the low $f_{475-515}/f_{535-590}$ ratio after background correction was consistent with the weak fluorescence yield expected of secGFP in the apoplast (and fractionally in the vacuole, see Zheng et al., 2005) and the occasional retention of secGFP in a small percentage of cells (see Figure 3a,b). Expressing AtSYP121-Sp2 increased the mean $f_{475-515}/f_{535-590}$ ratio by 1.85 ± 0.02-fold in this experiment (see also legend to Figure 6, Table 2), as expected from visual analysis of the secGFP retention (Figure 3c,d). Over all 11 experiments with this Sp2 fragment, a cumulative mean increase of 1.7 ± 0.2-fold was observed. The results suggest a strong effect of the Sp2 fragment, although the mean $f_{475-515}/f_{535-590}$ ratios with AtSYP121-Sp2 appeared intermediate to values obtained with separate constructs including the GFP-HDEL marker as a measure for full retention in the endoplasmic reticulum (Figure 4b and legend to Figure 6). Such a direct comparison assumes an equivalence in the

**Table 2** Fitted parameters for fluorescence ratio distributions of Figures 5 and 6

<table>
<thead>
<tr>
<th></th>
<th>$F_0$</th>
<th>$b$</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.330 ± 0.004</td>
<td>0.041 ± 0.003</td>
</tr>
<tr>
<td>+Sp2 fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtSYP121</td>
<td>0.61 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>AtSYP122</td>
<td>0.71 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>AtSYP71</td>
<td>0.473 ± 0.008</td>
<td>0.045 ± 0.005</td>
</tr>
<tr>
<td>AtSYP111</td>
<td>0.332 ± 0.002</td>
<td>0.031 ± 0.003</td>
</tr>
<tr>
<td>AtSYP21</td>
<td>0.35 ± 0.01</td>
<td>0.040 ± 0.005</td>
</tr>
<tr>
<td>GFP-HDEL</td>
<td>1.05 ± 0.02</td>
<td>0.15 ± 0.02</td>
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**Figure 5.** AtSYP121-Sp2 suppresses export of the secreted cargo marker secGFP.

Comparison of the $f_{475-515}/f_{535-590}$ fluorescence ratio distributions from one of 11 independent experiments with tobacco leaves co-transfected with secGFP and YFP-HDEL only (control, dark grey bars) and with AtSYP121-Sp2 (+Sp2, light grey bars). Bars for +Sp2 are right-shifted 0.005 units for clarity. Data shown are for the experiment in Figure 3. Solid lines are fittings to a normal distribution function [equation (1)], and fitting parameters are shown in Table 2. Expressing AtSYP121-Sp2 increased the mean $f_{475-515}/f_{535-590}$ ratio by 1.7 ± 0.2-fold ($n = 11$).

**Figure 6.** AtSYP122-Sp2 and AtSYP71-Sp2, but not the Sp2 fragments of AtSYP111 and AtSYP21, suppress export of the secreted cargo marker secGFP.

Comparison of the $f_{475-515}/f_{535-590}$ fluorescence ratio distributions from representative experiments with tobacco leaves co-transfected with secGFP and YFP-HDEL only (dark grey bars, control) and together with (a) AtSYP122-Sp2 ($n = 11$), (b) AtSYP71-Sp2 ($n = 10$) or (c) AtSYP111-Sp2 and AtSYP21 ($n = 8$) (light grey bars, +Sp2). Bars for +Sp2 are right-shifted 0.005 units, and data for the retention standard (GFP-HDEL) are omitted for clarity. Solid lines are fittings to a normal distribution function [equation (1)]. For reference, the fitting for AtSYP121-Sp2 is included from Figure 5 in each case (dashed curve). Expressing AtSYP122-Sp2 and AtSYP71-Sp2 increased the mean $f_{475-515}/f_{535-590}$ by 1.82 ± 0.09 and 1.27 ± 0.06-fold relative to the control. Expressing AtSYP111-Sp2 and AtSYP21-Sp2 had no significant effect on the mean $f_{475-515}/f_{535-590}$ ratio. Solid lines in (c) correspond to the control and AtSYP21-Sp2 (data omitted for clarity); the light grey bars correspond to AtSYP111-Sp2. Fitting in the latter case was virtually identical to the control (below). Fitting parameters for the data are shown in Table 2.

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mean levels of expression of the two GFP markers, but it is
consistent with the effects of the same Sp2 fragment
on export of the plasma membrane protein KAT1 (Sutter
et al., 2006b) (see below).

A second feature of note is that values for the coefficient of
spread, b, showed a significant increase compared with the
control (see legend to Figure 5). Some increase was expec-
ted as a result of AtSYP121-Sp2 addition, which, like that of
the markers, might show a range of expression levels and
thus affect the distribution of fluorescence ratios. Nonethe-
less, the spread of fluorescence ratios, as characterized by
the b values, was significantly less with secGFP and
AtSYP121-Sp2 when compared with that for GFP-HDEL.
For the experiments summarized in Figures 3 and 5, this
value was 0.10 ± 0.02, roughly 63% of the values obtained
on co-expressing GFP-HDEL with YFP-HDEL (see also legend
to Figure 6). A similar conclusion may be drawn from a
comparison of the coefficients of variance (Table 1). Thus,
on the basis of a straight numerical comparison with the
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Secretry block is specific to Sp2 fragments of the target
membrane SNAREs

To date, all evidence indicates that Sp2 fragments of the
plasma membrane SNAREs NtSYP121 and AtSYP121 sup-
press traffic at a late step in export to the plasma membrane
(see Figure 3) (Geelen et al., 2002; Sutter et al., 2006b).
Nonetheless, these data do not preclude parallel actions of
the dominant-negative proteins at analogous sites within
the plant cell, and the observation raises a fundamental
question about the specificity of Sp2 action in vivo. To
explore this question, we made use of the secGFP assay and
population distribution analysis described above, and
quantified f475–515/f535–590 ratios to compare the efficacies in
secretory block of Sp2 fragments derived from two addi-
tional Q-SNAREs related to AtSYP121, a Q-SNARE associ-
ated with vacuolar trafficking, and one additional Q-SNARE,
AtSYP71, belonging to a largely uncharacterized clade of
proteins. AtSYP122 is the closest homologue of AtSYP121,
and the functions of these two plasma membrane
Q-SNAREs in vegetative growth overlap (Assaad et al.,
2004). AtSYP111 (=Knolle) is also related to AtSYP121, but is
known to contribute to phragmoplast formation, a role that
is not interchangeable with that of AtSYP121 (Lauber et al.,
1997; Muller et al., 2003). AtSYP21 (=Pep12) is localized to
the PVC, and contributes to vacuolar traffic from the Golgi
apparatus and trans-Golgi network (Conceicao et al., 1997;
Foresti et al., 2006; Surpin and Raikhel, 2004). Finally,
AtSYP71 is one of three members in Arabidopsis of a novel
clade of Q-SNAREs unique to plants and protists (Sanders-
foot et al., 2000; Sutter et al., 2006a); it has been suggested
to function in the endoplasmic reticulum (Yoshizawa et al.,
2006), but is found in proteomic screens of plasma
membrane, lipid-raft proteins (Alexandersson et al., 2004;
Marmagne et al., 2004), and a homologue appears similarly
in tobacco (Mongrand et al., 2004).

Figure 6 summarizes the results of analyses from one set
of experiments, carried out in parallel with that of Figures 3
and 5, with and without co-expression of the Sp2 fragments
of each of these Q-SNAREs (AtSYP122-Sp2, AtSYP71-Sp2,
AtSYP111-Sp2 and AtSYP21-Sp2) 3 days after infiltration.
Confocal images were collected and quantified as described
above, and Sp2 expression was confirmed by Western blot
analysis (not shown). In every case, expressing the two
marker constructs alone yielded a strong YFP fluorescence
signal within the endoplasmic reticulum of the epidermal
cells, but little evidence of any appreciable retention of the
secGFP marker. Expression of the markers together with the
AtSYP122-Sp2 fragment yielded a strong GFP fluorescence
signal within the epidermis, which, like the effect of
AtSYP121-Sp2, was confined primarily within the endoplas-
mic reticulum (not shown). Similar results were obtained
when expressing AtSYP71-Sp2, but co-expression with
AtSYP111-Sp2 and with AtSYP21-Sp2 was not observed to
lead to a visible change in GFP fluorescence within the epidermis. Calculating the $f_{475-515}/f_{535-590}$ ratios for AtSYP122-Sp2 and AtSYP71-Sp2 (Figure 6a,b) showed an increase in the $f_{475-515}/f_{535-590}$ distribution means for AtSYP122-Sp2 and AtSYP71-Sp2 to values of $0.71 \pm 0.01$ and $0.473 \pm 0.008$, respectively; means for all experiments yielded significant increases of $1.98 \pm 0.11$-fold for AtSYP122-Sp2 and $1.39 \pm 0.06$-fold for AtSYP71-Sp2 relative to the controls (see also legend to Figure 6). By contrast, we found that the Sp2 fragments of AtSYP111 and AtSYP21 were ineffective in blocking secGFP export. Analyses of the $f_{475-515}/f_{535-590}$ ratios from two experiments are included in Figure 6(c), and clearly show a close overlay of the $f_{475-515}/f_{535-590}$ distributions for AtSYP111-Sp2 and AtSYP21-Sp2 with the control.

Although AtSYP111 is known not to be interchangeable with AtSYP121 (Lauber et al., 1997; Muller et al., 2003), assessing the efficacy of AtSYP111-Sp2 in blocking cell-plate formation is not straightforward. However, traffic to the vacuole is readily verified and a number of vacuolar markers are available, including synthetic cargo and intrinsic membrane proteins (Foresti et al., 2006; Ma et al., 2004; Reisen et al., 2003). To test the effects of Sp2 fragments on vacuolar traffic, we expressed the Arabidopsis tonoplast intrinsic protein TIP1;1 (yTIP) as a YFP fusion alone and together with either the Sp2 fragment of the PVC SNARE AtSYP21 or with the Sp2 fragment of the plasma membrane SNARE AtSYP121, in each case verifying expression of the latter by Western blot analysis (not shown). Figure 7 shows representative images collected from one experiment, with similar results obtained in an additional two independent experiments. Expression of TIP1;1–YFP on its own yielded a characteristic labelling of the tonoplast (Ma et al., 2004; Reisen et al., 2003), showing invaginations and clefts around the perimeter when expressed in tobacco epidermal cells (Figure 7a). Co-expression with AtSYP121-Sp2 yielded a very similar pattern of labelling (Figure 7), indicating that the plasma membrane-associated Sp2 did not interfere with TIP1;1 traffic to the tonoplast. When co-expressed with AtSYP21-Sp2, however, labelling of the tonoplast was virtually absent. Instead, the epidermal cells showed prominent YFP fluorescence within a large number of mobile internal structures with variable diameters of 1–4 μm (Figure 7b) that co-localized with the PVC marker BP80-GFP (Figure 7d). Finally, quantitative analysis of TIP1;1–YFP sequestration confirmed an overwhelming shift in labelling to these organelles in the presence of AtSYP21-Sp2, but not...

![Figure 7](image-url)
when TIP1;1-YFP was co-expressed with AtSYP121-Sp2 (Figure 7e). Thus, we conclude that the Sp2 fragments of all three plasma membrane SNAREs are effective in suppressing secGFP traffic to the plasma membrane, and this action is not shared by Sp2 fragments of SNAREs that normally function elsewhere in the plant cell. Conversely, the Sp2 fragment of PVC-associated AtSYP21, but not the Sp2 fragment of the plasma membrane SNARE AtSYP121, effectively targets vacuolar traffic of at least one major intrinsic protein.

The Sp2 N-terminus harbours a capacity for interaction specificity

Although the data above speak to a degree of functional specificity between Sp2 fragments of SNAREs active at the plasma membrane and those associated with endomembrane vesicle traffic, they offer little insight into possible mechanisms of Sp2 action. Because Q-SNAREs associate with complementary R-SNAREs and are also capable of forming other homo- and heteromeric complexes (Antonin et al., 2002b; Fasshauer et al., 1997a; Lerman et al., 2000), several competitive (dominant-negative) interactions of the Sp2 fragments could account for the secretory block observed in vivo. Protein–protein interactions might draw on the highly conserved H3 (syntaxin signature sequence) domain or the N-terminal domain (referred to hereafter as the Sp3 fragment) that includes the Ha, Hb and Hc coils and is thought to interact with the H3 domain, thereby controlling assembly of the SNARE complex (Jahn et al., 2003; Sutter et al., 2006a; Ungar and Hughson, 2003). Because inter-protein interactions between H3 coils are well-documented but less is known of contributions from other domains, we were particularly interested to know whether the Sp3 domain might contribute to the functionality and specificity of the secretory block that we observed.

To test for possible interactions with the SNAREs, we carried out sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (SDS–PAGE) and Western blot analysis of the Q-SNAREs AtSYP121, AtSYP122 and AtSYP71 after co-incubation with Sp3 fragments for each of the SNAREs. Previous observations (B. Gehl and M.R. Blatt, unpublished data) showed that the Arabidopsis plasma membrane Q-SNAREs will interact with themselves in detergent-resistant multimeric complexes, at least when heterologously expressed. We took advantage of this property to examine the stability of the SNARE complexes in the presence of the Sp3 fragments. Constructs of AtSYP121, AtSYP122 and AtSYP71 in baculovirus were used to transfect Sf9 insect cell cultures. Each construct included an N-terminal sequence extension encoding the Flag epitope (MDYKDDDDK). Lysates of the transfected cells were incubated without or with Sp3 fragments for each of the SNAREs that had been obtained by expression in Escherichia coli and His<sub>6</sub>-based purification (see Experimental procedures) before solubilization, separation by SDS–PAGE and Western blot analysis. Figure 8 summarizes the results of incubations with the Sp3 fragment of AtSYP122 probed with anti-Flag antibody. For each of the paired lanes, the Flag antibody recognized the monomeric Q-SNAREs (around 37 kDa for AtSYP121 and AtSYP122, and approximately 34 kDa for AtSYP71) as well as a small number of higher-molecular weight bands that were roughly double the apparent molecular weight of the monomers (arrows), consistent with the formation of detergent-resistant homodimers. Little difference in the pattern of high-molecular weight bands was visible for AtSYP121 and AtSYP71, either with or without the addition of the Sp3 fragment of AtSYP122. However, almost complete loss of the higher-molecular weight bands resulted when this Sp3 fragment was incubated with the lysate of cells expressing AtSYP122. Analogous effects were observed for each of the plasma membrane SNARE fragments (not shown), indicating that each Sp3 fragment was effective in destabilizing complexes, but only those incorporating the corresponding full-length SNARE. Although these experiments do not allow us to identify binding interactions with the Sp3 fragments, they do indicate that the N-terminal domain of each Sp2 fragment is itself capable of selectively affecting SNARE complexes generated by the corresponding Q-SNARE.

Discussion

The multiplicity of SNARE proteins in plants raises a number of challenges for their analysis. Past studies have yielded a mixed picture, indicating a degree of redundancy for several Arabidopsis SNAREs, while for others demonstrating an
absolute requirement for their presence during the life cycle of the plant and suggesting a complete absence of functional overlap (for reviews see Jurgens, 2004; Sutter et al., 2006a). To date, the most common approaches have drawn on mutational and complementation screens and genetic ablations that have provided characterizable developmental (Heese et al., 2001; Lukowitz et al., 1996) or cellular (Sato et al., 1997; Yano et al., 2003; Zhu et al., 2002) phenotypes. In other instances, however, genetic analysis has yielded little difference from the wild-type (Assaad et al., 2004) or conversely have shown such mutations to be lethal (Sanderfoot et al., 2001b). Thus, deriving information about the function of specific proteins can be difficult, and functional causality often must be inferred indirectly from changes in long-term developmental or structural defects.

As an alternative strategy, we have explored the use of dominant-negative (so-called Sp2) fragments of SNAREs to target and disrupt membrane traffic in vivo. This approach relies on the availability of a suitable marker for vesicle traffic, and assumes that the protein fragments interfere selectively in SNARE protein interactions. To test this assumption, we examined SNARE-mediated traffic to the plasma membrane and tonoplast using Sp2 fragments of a selection of Q-SNAREs, most with known functions and localizations in vivo. These studies draw on use of a fluorescent marker, and, for secretory traffic to the plasma membrane, on statistical analysis of fluorescence ratiometry of confocal images. They yield several important observations. (1) Traffic of secGFP to the plasma membrane was suppressed by Sp2 fragments of the predominant plasma membrane Q-SNAREs AtSYP121 and AtSYP122, as has been reported for the tobacco NtSYP121 Sp2 fragment (Geelen et al., 2002) and for traffic of the Arabidopsis KAT1 K+ channel (Sutter et al., 2006b). Similarly, expressing the Sp2 fragment of the novel Q-SNARE AtSYP71 led to a block of secretion, consistent with its recent localization to the plasma membrane in Arabidopsis (Alexandersson et al., 2004; Marmagne et al., 2004) and that of its homologue in tobacco (Mongrand et al., 2004). (2) By contrast, export of the secretory marker was not affected by the Sp2 fragment of the Q-SNARE AtSYP111 (=Knolle) that is related to AtSYP121 and AtSYP122 but is functional at the cell plate (Lauber et al., 1997; Muller et al., 2003) nor by the Sp2 fragment of the pre-vacuolar SNARE AtSYP21. (3) Conversely vacuolar traffic of TIP1;1-YFP was suppressed by the Sp2 fragment of AtSYP21, but not by that of AtSYP121, leading to its accumulation in enlarged PVC structures. Finally, (4) our results showed that N-terminal fragments of AtSYP121, AtSYP122 and AtSYP71 interfered with the stability of SNARE protein complexes in vitro, but only of those that included the corresponding full-length Q-SNARE. These results provide unequivocal evidence for a selectivity among the dominant-negative fragments that accurately reflects the known specificity and targeting of the SNAREs, and they imply functional overlaps between SNARE functionalities that are of potential physiological interest. They also underscore the utility of a dominant-negative strategy for functional studies of SNAREs in plants.

Membrane targeting with Sp2 fragments

Significantly, we found that the effects of the different Sp2 fragments functionally recapitulate the specificities previously inferred from genetic mutant and rescue studies. Among the 24 Arabidopsis syntaxin-like Q-SNAREs, the coding sequences for no less than nine different proteins have been classified within the SYP1 clade that shows close homology to the plasma membrane SNAREs of yeast and mammals (Pratelli et al., 2004; Sanderfoot et al., 2000; Sutter et al., 2006a). Of these, AtSYP121 (=AtSyr1) and AtSYP122 (=AtSyr4) have both been shown to localize to the plasma membrane (Collins et al., 2003; Leyman et al., 1999; Uemura et al., 2004). Although each of these SNAREs has been associated with a distinct pathogen defence response (Collins et al., 2003; Nuhse et al., 2003), basal expression characteristics and cell-wall depositions (Assaad et al., 2004), genetic ablations of either of these SNAREs generates plants that appear little different from the wild-type. Assaad et al. (2004) reported severe effects on growth and necrosis only in the atsyp121 atsyp122 double knockout, suggesting that these two gene products are important for normal vegetative growth but that their activities largely overlap in vivo. By contrast, the plasma membrane-like Q-SNARE, AtSYP111, appears to be finely tuned for cell-plate formation during cell division. The atsyp111 knockout was previously shown to be embryo-lethal (Lauber et al., 1997; Lukowitz et al., 1996) and was not rescued by AtSYP121, even when expressed under control of the AtSYP111 regulatory sequences (Muller et al., 2003).

Consistent with these inferred roles, our analysis shows that the dominant-negative Sp2 fragments of AtSYP121 and AtSYP122 are competent to suppress the export of a secreted marker cargo, secGFP. In each case, we found that co-expression of the Sp2 fragments with secGFP led to retention of the cargo within the endomembrane secretory pathway, as evident from the accumulation of the GFP fluorescence signal (Figures 3, 5 and 6). These findings parallel a previous study of secretory traffic mediated by the homologous tobacco SNARE, NtSYP121 (=NtSyr1), which similarly showed a block of secGFP traffic by the corresponding Sp2 fragment (Geelen et al., 2002). They also complement recent evidence that the Sp2 fragments of both NtSYP121 and AtSYP121 selectively suppress delivery to the plasma membrane of an integral membrane protein, the KAT1 K+ channel from Arabidopsis (Sutter et al., 2006b). AtSYP71-Sp2 was also effective in suppressing secGFP export, albeit to a lesser degree. The observation implies a role late in traffic to the plasma membrane that is consistent
with its occurrence in plasma membrane lipid rafts (Andersonson et al., 2004; Marmagne et al., 2004; Mongrand et al., 2004). The fact that Sp2 fragments of either AtSYP121, AtSYP122 or AtSYP71 were sufficient to affect secretion, although the functions of at least two of the SNAREs are largely redundant, implies an overlap in their actions (see below).

By comparison, the corresponding fragment of AtSYP111 had no effect on secGFP secretion (Figure 6). This observation is consistent with the lack of complementarity inferred from genetic studies (Muller et al., 2003), and is also supported by recent evidence suggesting that AtSYP111 may co-localize with AtSYP121 at the plasma membrane although it appears to be functional only at the cell plate in dividing cells (Dhonukshe et al., 2006). Analysis of secGFP traffic with the Sp2 fragment of AtSYP21 yielded much the same conclusion, again consistent with the specificity in action of the Sp2 fragments of the plasma membrane Q-SNAREs. AtSYP21 was identified by functional complementation in yeast (Bassham et al., 1995), and has been shown to localize to the (pre-)vacuolar compartment in Arabidopsis (Conceicao et al., 1997; Sanderfoot et al., 1998).

The converse experiment, using the aquaporin fusion protein TIP1;1–YFP as a marker, demonstrated that its traffic to the vacuole is severely impaired by AtSYP21-Sp2, but not by AtSYP121-Sp2 (Figure 7). These observations are significant for three reasons. Firstly, they confirm the efficacy of the Sp2 fragment of the (pre-)vacuolar SNARE and the complementary lack of effect of the plasma membrane Sp2 fragment. Thus, they underscore the functional selectivities between the different Sp2 fragments. Secondly, they provide an important control for our use of Agrobacterium-based transformation. This method is well-established as a tool for analyzing membrane trafficking and related activities in vivo (Batoko et al., 2000; Boevink et al., 1998; Foresti et al., 2006; Geelen et al., 2002; Reisen et al., 2003; Runions et al., 2006; Samalova et al., 2006; daSilva et al., 2004; Sutter et al., 2006a,b), and it makes use of pathogenically disarmed Agrobacterium. Nonetheless, we note that alterations in secretory traffic are generally associated with pathogenesis in Arabidopsis and other plants (Assaad et al., 2004; Collins et al., 2003; Dangl and Jones, 2001), and, by inference, with Agrobacterium infestations. Thus, the fact that transfections with AtSYP21-Sp2, although active in situ, did not influence secGFP export (Figure 6), discounts any appreciable effect per se of the bacteria on secretory traffic.

Finally, the observations suggest that traffic to the vacuole differs substantially between different markers, and this conclusion offers another perspective on previous observations by Oufattole et al. (2005) of parallel, but non-overlapping pathways to the vacuole. We note that Foresti et al. (2006) found traffic through the PVC was largely insensitive to a cytotoxic fragment of AtSYP21 that was similar to AtSYP21-Sp2 in assays using the fusion cargo Amy-spo; however, Amy-spo traffic was blocked by over-expressing the full-length AtSYP21 protein. Intriguingly, AtSYP21 over-expression had little effect on traffic of BobTIP26-1, the Brassica homologue of TIP1;1 from Arabidopsis, and led only to a weak retention in the PVC. Our findings complement these results, demonstrating the profound sensitivity of TIP1;1 traffic to AtSYP21-Sp2. The sensitivity of vacuolar traffic to the full-length protein, rather than the Sp2 fragment, appears to reflect its position as a focal interactant between different sets of SNARE partners (Sanderfoot et al., 2001a), and, consequently, as a balance point between pathways to and from the PVC for the soluble cargo (Foresti et al., 2006). This pattern of action mirrors the effects of over-expressing another SNARE, Sed5p, which also forms multiple SNARE complexes and contributes selectively to traffic both in and out of the Golgi in yeast (Hardwick and Pelham, 1992; Hardwick et al., 1992). Thus, a simple explanation for the two sets of observations is that traffic of the aquaporins is closely dependent on traffic mediated by a PVC-associated SNARE homologous to AtSYP21, while traffic of the soluble cargo is strongly affected by manipulations that draw components other than this SNARE homologue out of the Golgi–PVC–vacuole circuit. Clearly, it will be of interest to follow up these observations in comparative studies with other vacuolar markers.

It is important to bear in mind that these analyses were carried out in tobacco, but using inhibitory (dominant-negative) Sp2 fragments constructed to Arabidopsis SNAREs. Thus, their interpretation is based on the assumptions of close parallels in SNARE family function, sequence and hence complementarity in protein interactions between tobacco and Arabidopsis. In fact, there is good reason to anticipate major overlaps in this respect among plant species. Analyses of SNARE clade structure between Arabidopsis, rice and the unicellular alga Cyanidioschyzon merolae, for example, highlight a remarkable consistency in association between the eight major subclades (Yoshizawa et al., 2006), and direct sequence comparisons show that most of the Arabidopsis Q-SNAREs have counterparts in rice, many with sequence identities of 75% or greater (Sutter et al., 2006a), implicating an evolutionary conservation that pre-dates the divergence of monocotyledonous and dicotyledonous plants. Whether a similar, near-one-to-one correspondence of sequence homologies applies to tobacco remains to be seen, but is certainly plausible. We have noted that NtSYP121 and AtSYP121 share extensive sequence identity (Leyman et al., 1999) and functional attributes (see also Sutter et al., 2006b), and that antibodies to AtSYP121 and AtSYP122 cross-react with distinct protein bands from tobacco extracts (see Figure 3); NtSYP121 interacts with the Arabidopsis Q-SNARE AtSNAP33 and tobacco harbours a native protein of similar molecular weight that is recognized by the αAtSNAP33 antibody (Kargul et al., 2001). Finally, tobacco also includes...
at least one homologue of the SYP7 subclade that is unique to plants and protists, and is similarly localized to plasma membrane lipid rafts (Mongrand et al., 2004). In short, we may view the likelihood that the Arabidopsis Sp2 fragments target the functionality of the corresponding tobacco SNAREs with a degree of cautious expectation.

Quantifying the secretory block

Establishing a quantifiable measure for secGFP retention in these studies presented a significant challenge and deserves comment. We found that by far the greatest variability in transgene expression was associated with the leaf tissue itself, and was especially notable on a cell-by-cell basis. Even under conditions giving > 90% transfection of epidermal cells, the fluorescence signal from the retained GFP-HDEL marker varied appreciably between experiments and even between leaves from the same tobacco plant. This variability was greatly reduced (as evidenced by an approximately two-to threefold reduction in the coefficients of variance; Table 1) when we compared the fluorescence ratios, \( F_{475-515}/F_{535-590} \), and was verified in control experiments co-expressing GFP-HDEL and YFP-HDEL in which both markers were expected to be retained within the endoplasmic reticulum. The resulting \( F_{475-515}/F_{535-590} \) values showed normal distributions (Figure 4), consistent with a stochastic pattern in the levels of expression between genes within a population of cells (Elowitz et al., 2002). That similar, or even lower, coefficients of variance and spread in the \( F_{475-515}/F_{535-590} \) distribution resulted when the fluorescent markers were introduced by co-transfection within a single T-DNA plasmid, is also a strong indicator that co-transfections favoured the transfer of multiple plasmids in any one cell. Finally, we found that secretory block resulted in a pronounced displacement in the mean \( F_{475-515}/F_{535-590} \) ratio to higher values, with independent measurements normally distributed about this mean. Such a displacement would be expected if the primary effect was on GFP export rather than more global changes, for example in marker transgene expression (Paulsson and Ehrenberg, 2001), and this conclusion was supported by (albeit necessarily limited) cell-by-cell analysis for retention of the secGFP fluorescence signal.

Distribution analysis provided an effective measure for distinguishing normal secretory activity from its suppression or block within cell populations, with the advantage that it accommodated changes in the spread of fluorescence ratio distributions associated with additional construct expression. Because independent fluorescence ratio distributions were inherently Gaussian, the characteristics of individual distributions were subsumed within a single set of two parameters \((F_0, b)\) defined through non-linear least-squares fittings. Thus, validating the effects of secretory block was reduced to a simple comparison of the differences between parameter sets obtained for each of the different Sp2 fragments to determine the overlap and peak-to-peak separations. Baselines for the control (secGFP) and full retention / secretory block (GFP-HDEL) indicated a dynamic range of \( F_{475-515}/F_{535-590} \) values (between approximately 0.2 and 1.2) that yielded quantifiable differences among the actions of Sp2 fragments of the various plasma membrane Q-SNAREs, and indicated an incomplete block of secretory traffic with AtSYP121-Sp2, consistent with previous observations (Sutter et al., 2006b; see also Geelen et al., 2002).

Samalova et al. (2006) recently applied a ratiometric method for quantifying secretory traffic. Although their approach did not include a comparable analysis of population distributions, they reached much the same conclusion. In fact, they achieved further reductions in the stochasticity of expression through the use of polypeptide synthesis incorporating the foot-and-mouth disease virus 2A sequence for post-translational protein cleavage. Translation of both markers initially as a single polypeptide greatly reduced the variability in cellular expression and yielded considerably greater sensitivity for quantifying secretory traffic on a cell-by-cell basis. Nonetheless, the dynamic range and resolution achieved by this method is roughly equivalent to that of co-expression (compare Figures 4–6) when applied to cell populations, primarily because an appreciable fraction of polypeptide incorporating GFP-related fluorophores is retained uncleaved \textit{in vivo}, and thus yields a significant baseline signal even under control conditions. Thus, we conclude that the advantages of polypeptide synthesis are largely outweighed in population analyses by the associated background fluorescence.

Sp2 fragments uncover SNARE function by competition

How might the Sp2 fragments interfere with traffic to the plasma membrane? The N-terminal region of Q-SNAREs, such as mammalian syntaxin 1A and the yeast plasma membrane homologues Sso1 and Sso2, is recognized as forming an independently folded regulatory domain that includes the first three coiled-coil (Ha, Hb, Hc) sequences (Hong, 2005; Jahn et al., 2003). This regulatory domain interacts with the core SNARE coil (the so-called ‘syntaxin signature sequence’ or H3 domain) to form a closed state that affects its accessibility and hence to SNARE complex assembly (Margittai et al., 2003; Munson and Hughson, 2002; Ungar and Hughson, 2003). Energy minimization analysis has predicted a similar folding for the N-terminus of at least one plant SNARE homologue (Blatt et al., 1999). The Sp2 fragments of each of the Arabidopsis SNAREs that we used included the highly conserved core SNARE coil as well as the three N-terminal Ha, Hb and Hc domains (see Figure 1). Thus, one possible explanation for the block of traffic is that, by introducing an excess of soluble protein that includes these domains, the native SNAREs might be trapped in an equivalent closed conformation through direct binding
with the Sp2 fragment, thereby preventing further SNARE interactions.

At least two further explanations for Sp2 action are conceivable. One possibility is that the core SNARE coil of the Sp2 fragment may interact with complementary domains of other Q- and R-SNAREs to form a ternary SNARE complex that is non-functional in the absence a membrane anchor on the Sp2 fragment. Such partial functionality underlies the block of SNARE function in neurotransmitter release by Clostridium botulinum (BotN/X) toxins that cleave the soluble domains of several neuronal SNAREs in vivo; BotN/X toxin cleavage products as well as synthetic SNARE fragments have been used to probe component activities in SNARE complex formation (O'Connor et al., 1997; Ferrer-Montiel et al., 1998; Scales et al., 2000; Sun et al., 2003; Rickman et al., 2004). Indeed, Scales et al. (2000) showed a block of noradrenaline secretion in a PC12 cell line that was selective among soluble domains of neuronal syntaxins analogous to our Sp2 fragments. Finally, Q-SNAREs also assemble as homo-multimers (Qa and Qa) (Lerman et al., 2000; Misura et al., 2001) and hetero-multimers (Qa:Qb:Qc) (Fasshauer et al., 1997b), at least in vitro if not in vivo (Antonin et al., 2002b). Thus, it is possible that the Sp2 fragments may ‘titrate out’ functional Q-SNAREs by forming similar homo- and heteromultimers – or, alternatively, interfere in regulatory functions that depend on such complexes – even without contributions from the complementary SNAREs of the canonical complex.

The latter explanation is consistent with our co-incubation analyses. We found that the expressed and His-purified Sp3 (Ha, Hb, Hc) fragments are able to interfere in the formation of SNARE complexes, and that this activity showed specificity for the corresponding full-length SNARE. For example, only higher-molecular weight complexes of AtSYP122, potentially including SNARE homodimers, were suppressed when incubated with the Sp3 fragment of AtSYP122 in cell extracts including each of the plasma membrane SNAREs (Figure 8). The fact that these proteins pair and bind, even after solubilization, underscores a stability of these complexes that accords with previous analyses of these extra-canonical SNARE interactions (Antonin et al., 2002a; Lerman et al., 2000). Of course, the ability of these protein domains to interact in vitro does not confirm a similar behaviour in vivo, nor does it preclude other interactions that might compete for SNARE complex elements and thereby suppress vesicle traffic. Nonetheless, domains within the Sp2 fragments clearly must encode sufficient structural information that selectivity in SNARE pairing is retained in vitro and specificity in block of traffic is maintained in vivo.

Redundancy and overlap in Sp2 action

Finally, the traffic block by the Sp2 fragments of the two SNARE homologues AtSYP121 and AtSYP122 merits review. We noted previously that these two SNAREs are functionally redundant, at least to the extent that growth of plants carrying single gene knockouts is not significantly compromised (Assaad et al., 2004). Presumably, the general vesicle trafficking functions in each case are interchangeable; so, by inference, the strict functional selectivity shown by the two different Sp2 fragments should not have affected secGFP export. Alternatively, if the function of one of the two SNAREs was specific for vesicles carrying the secGFP cargo, then we might have anticipated a selective block by the corresponding Sp2 fragment, but not by both. By analogy, our recent studies of AtSYP121 and NtSYP121 (Sutter et al., 2006b) have shown that plasma membrane traffic of the KAT1 K+ channel is uniquely suppressed by the Sp2 fragments of these SNAREs when compared with traffic of the H+-ATPase. The obvious, but non-trivial explanation is that interactions in vivo with the Sp2 fragments of AtSYP121 and AtSYP122 must include common protein elements additional to those associated with the regulatory fragment interactions (Figure 8) to explain the cross-interactivity in secGFP traffic block. Much the same explanation applies for traffic suppression by AtSYP71-Sp2. Indeed, as a Qc-SNARE, AtSYP71 interactors may include the Qa-SNAREs AtSYP121 and AtSYP122 (Sutter et al., 2006a). The implicit cross-reactivity of these Sp2 fragments also has important practical implications, because the quasi-equivalence in activities apparently circumvents difficulties of redundancy in function between closely related SNAREs. An analogy may be drawn to suppression by RNAi of gene subfamilies (Novina and Sharp, 2004; Tomari and Zamore, 2005): Whereas the use of Sp2 fragments may not necessarily identify the exclusive activity of an individual gene product, the approach is likely to confirm its inclusion within a particular cellular pathway and its role in the plant cell.

Conclusions

We have found that the cytosolic domains, so-called Sp2 fragments, of plasma membrane and PVC Q-SNAREs from Arabidopsis behave in vivo as functional dominant-negative inhibitors to suppress export of the secretory cargo marker, secGFP. Comparisons using quantitative fluorescence ratio image analysis show that secretory inhibition is effected by Sp2 fragments corresponding to the known plasma membrane Q-SNAREs AtSYP121 and AtSYP122, and by the Qc-SNARE AtSYP71 recently identified in a proteomic screen of plasma membrane proteins. Secretory traffic was unaffected by Sp2 fragments of the closely related Q-SNARE AtSYP111, or by AtSYP21 which functions in membrane traffic between the Golgi apparatus and the vacuole. By contrast, the Sp2 fragment of AtSYP21 was effective in blocking traffic of the tonoplast marker TIP1;1–YFP, whereas the Sp2 fragment of AtSYP121 was not. These observations, and supporting in vitro co-incubation experiments,
demonstrate a selectivity in action that reflects the known physiological characteristics of these vesicle-trafficking proteins. They also underscore the utility of this approach in functional studies of SNARE proteins.

Experimental procedures

Molecular cloning and constructs

Full-length clones of AtSYP111, AtSYP121, AtSYP122, AtSYP21 and AtSYP71 were PCR-amplified from cDNA clones kindly provided by A. Sanderfoot (Department of Plant Biology, University of Minnesota, Minneapolis) and from RAFL clones (Seki et al., 2002) of Arabidopsis thaliana. These clones were also used as templates to generate Sp2 and Sp3 constructs, using the primers listed in Appendix S1 to introduce a stop codon before the C-terminal transmembrane domain (Sp2) or the syntaxin signature sequence (Sp3) as indicated in Figure 1. Full-length, Sp2 and Sp3 sequences were cloned between the 35S promoter and Nos terminator of the E. coli vector pBSKS+, and the resulting cassette were used as templates for re-amplification with alternative restriction sites (see Appendix S1 for primers) and subcloned using the SacI or BamHI and PstI sites into the binary vector pPTKan (Allen et al., 2000). For expression in E. coli, the Sp2 and Sp3 sequences were subcloned into pQE80 and pQE82i (Qiagen; www.qiagen.com/), using refilled BamHI and HindIII restriction sites. The proteins were expressed in E. coli strain M15 and induced for 4 h with 1 mM IPTG as described previously (Leyman et al., 1999). The binary vectors psecGFP and pGFP-HDEL, pYFP-HDEL and the tandem construct pGFP-YFPHDEL carrying the fluorophore coding sequences terminated by the HDEL endoplasmic reticulum retention sequence were obtained from Dr lan, Moore Department of Plant Sciences, (University of Oxford, UK). The tonoplast marker pTIP1;1-YFP was obtained from Lorenzo Frigerio (Department of Biology, University of Warwick, UK) and the PVC marker pPB80-GFP was obtained from Juergen Denecke (Department of Biology, University of Leeds, UK). All constructs were checked by sequencing.

For Spodoptera frugiperda (SF) insect cell expression, full-length PCR products for AtSYP121, AtSYP122 and AtSYP71 were amplified and cloned using EcoRI and NotI sites into the baculovirus expression vector pVLFlag-His which was generated by insertion of the Flag-His6 coding sequence 5¢-ATGGATTATAAAGATGGACAGA-TGACAGCTCATCATCATCATCAT-3¢ (MDYKDDDDKHHHHHH) upstream between the BamHI and EcoRI sites of pVL1393 (Pharmline). These clones were transformed into E. coli, and purified plasmid DNAs were used to produce recombinant baculovirus by co-transfection together with the impaired Baculogold virus (Pharmline) into SF9 insect cells.

Plant growth and transient expression

Wild-type tobacco (Nicotiana tabacum) plants were grown in pre-sterilized soil at 26°C and 60% relative humidity under a 16 h day/night cycle (200 μmol m⁻² PAR). Plants with 3–4 fully expanded leaves after 4–6 weeks’ growth were selected for infiltration. Plant materials were introduced by heat shock into Agrobacterium tumefaciens GV3101 (Koncz and Schell, 1986). Transient transfections were performed as described previously (Geelen et al., 2002) and were carried out with Agrobacterium suspended in an infiltration buffer of 10 mM MgCl₂ and 100 μM acetosyringone at final OD₆₀₀ of 0.03 or 0.1 for fluorophore expression and at OD₆₀₀ of 0.1 or 0.3 for SNARE expression.

Confocal microscopy

Expression of fluorescence in epidermal cells of the lower epidermis was assessed 2-4 days post-infiltration as described previously (Geelen et al., 2002; Sutter et al., 2006b). Confocal images were obtained on a Zeiss CLSM510 microscope using 10× and 20× Planapar objectives for low-resolution analysis and either 40× water-immersion or 63× oil-immersion objectives (Zeiss) for high-resolution image collection (http://www.zeiss.com/). GFP fluorescence was excited using the 458 nm line from a 30 mW argon laser set at 6.1 A, un-attenuated and with a scan speed of 1.60 μsec per pixel. Fluorescent light was collected after passage through an HFT458/ 514 dichroic lens, and an NFT515 dichroic lens was used to split the emitted light. Fluorescence was collected using separate tracks. In track 1, channel 2 was set with a 166 μm pinhole and 475–525 nm bandpass filter for GFP detection (effective bandpass, 475–515 nm). For chloroplast detection, channel 3 was set with a 188 μm pinhole and 560–615 nm or 560 nm longpass filter to detect chloroplast fluorescence (effective bandpass, 560–615 nm or > 560 nm). Detector gains were set at 750 V (GFP) and 650–700 V (chloroplasts). In track 2, YFP fluorescence was excited using 514 nm light from the argon laser attenuated to 10%, and fluorescent light was collected after passage through HFT458/514 and NFT515 dichroic lenses and a 535–580 nm interference filter. Bright-field images were collected using the transmitted light detector. Detector gains were set at 750 V (YFP) and 200–230 V (bright-field).

Image quantification and distribution analysis

Standardized imaging parameters were set to avoid saturation in the brightest cells, and amplifier offsets were set to minimize pixels with a value of 0 in the vacuoles of the dimmest samples in an initial survey of representative transfected leaf tissues. At least six images of each sample were collected using the 10× objective from areas selected at random within the tissue, and the focal planes were adjusted within the epidermal layer to give maximum fluorescence signal in the YFP channel. Average GFP and YFP pixel intensities were measured using the histogram function of the Zeiss am software, version 3.2. Background fluorescence was similarly estimated from six images of non-infiltrated areas of leaf tissue. For quantification of fluorescence intensities, images were segmented to give nine subframes (1 mm² per subframe) from which the fluorescence signals for each marker were summed, and the fluorescence ratio, f₉75-515/f₅35-590, was calculated. Means ± SD for the f₉75-515/f₅35-590 ratios were determined, and outliers (> ± 2× SD, generally corresponding to out-of-focus regions) were eliminated before binning and plotting. From each experiment, f₉75-515/f₅35-590 ratio distributions were fitted independently to a Gaussian function [equation (1)] by non-linear least-squares fitting using a Marquardt-Levenberg algorithm (Marquardt, 1983). The resulting parameter sets were used to determine the resolution between individual f₉75-515/f₅35-590 means as described above.

Antibodies, Sp3 co-incubations and immunodetection

Antibodies were generated against the Sp3 fragment proteins. His₆-tagged Sp2 and Sp3 fragments were purified by Ni-affinity chromatography as described previously (Leyman et al., 1999), and anti-Sp3 sera were collected from white rabbits (Diagnostics Scotland) after verifying the absence of cross-reactivity with pre-immune sera (not shown). Antisera were purified by depletion against agarose bound with E. coli proteins (Sigma; http://www.sigmaaldrich.com/), and were titred and tested for specificity
against Sp2 and Sp3 fragments expressed in E. coli and after expression in plants (see Figures 2 and 3).

Leaf total protein was extracted by grinding leaf tissue frozen in liquid N₂ and resuspending at 1:1 v/v in denaturing buffer containing 60 mM Tris–HCl, pH 6.8, 2% sodium dodecylsulfate, 8 M urea, 5% β-mercaptoethanol, 30% glycerol, 1 mM PMSF, complete EDTA-free protease inhibitor (Roche, Basel) and 0.01% bromophenol blue. Samples were heated to 95°C for 5 min, and centrifuged at 16 000 g for 10 min to remove debris. Protein was quantified by Bradford or Amidoblack assay (Bio-Rad; http://www.bio-rad.com/), and calibrated against bovine serum albumin.

Protein for co-incubation analysis was extracted from SF9 insect cell cultures 60 h after transfection with recombinant baculovirus carrying the various SNARE constructs, by centrifugation at 800 g for 10 min at 4°C. Cells were resuspended in solubilization buffer (130 mM NaCl, 10 mM HEPES, pH 7.4, and centrifuging a second time at 16 000 g for 30 min at 4°C. Cells were resuspended in solubilization buffer (130 mM NaCl, 10 mM HEPES, pH 7.4, 1% w/v Triton X-100, 1 mM CaCl₂, 1 mM PMSF and complete EDTA-free protease inhibitor), sonicated for 6 sec, and incubated with gentle shaking for 16 h at 4°C. Solubilized cell lysates were centrifuged at 16 000 g for 30 min at 4°C, and the solubilized proteins were collected in the supernatant and quantified as above. The solubilized proteins were incubated for 48 h at 4°C with His₆-purified Sp3 fragments at a rate of 5 µg solubilized protein to 0.5 µg Sp3 fragment in 10 volumes of solubilization buffer, and were analysed by Western blot after 1 h incubation in denaturing buffer.

For Western blot analysis, aliquots corresponding to 10 µg (0.1 µg for His₆-purified Sp2 fractions) of protein/sample were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Bio-Rad). Nitrocellulose membranes were blocked with 5% non-fat milk in Tris-buffered saline solution containing 0.1% Tween (TBST) at 4°C overnight, incubated at room temperature for 2 h with primary antibody in TBST and 5% non-fat milk, washed three times for 15 min with TBST, and incubated at room temperature for 1 h with a 1:10 000 dilution of goat anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (Sigma-Aldrich) in TBST and 5% non-fat milk. Filters were then washed in TBST four times for 15 min, and the bound antibodies were detected using ECL chemiluminescent substrates (Amersham Biosciences; http://www5.amershambiosciences.com/). Polyclonal rabbit anti-Sp3 primary antibodies were used at dilutions of 2000- to 5000-fold. Polyclonal rabbit anti-Flag antibodies (Sigma) were used at a dilution of 1:5000.

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Supplementary material
The following supplementary material is available for this article online:

Appendix S1. Table of SNARE Sp2 and Sp3 primers.
This material is available as part of the online article from http://www.blackwell-synergy.com

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