Binding of SEC11 Indicates Its Role in SNARE Recycling after Vesicle Fusion and Identifies Two Pathways for Vesicular Traffic to the Plasma Membrane

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SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins drive vesicle fusion in all eukaryotes and contribute to homeostasis, pathogen defense, cell expansion, and growth in plants. Two homologous SNAREs, SYP121 (=SYR1/PEN1) and SYP122, dominate secretory traffic to the Arabidopsis thaliana plasma membrane. Although these proteins overlap functionally, differences between SYP121 and SYP122 have surfaced, suggesting that they mark two discrete pathways for vesicular traffic. The SNAREs share primary cognate partners, which has made separating their respective control mechanisms difficult. Here, we show that the regulatory protein SEC11 (=KEULE) binds selectively with SYP121 to affect secretory traffic mediated by this SNARE. SEC11 rescued traffic block by dominant-negative (inhibitory) fragments of both SNAREs, but only in plants expressing the native SYP121. Traffic and its rescue were sensitive to mutations affecting SEC11 interaction with the N terminus of SYP121. Furthermore, the domain of SEC11 that bound the SYP121 N terminus was itself able to block secretory traffic in the wild type and syp122 but not in syp121 mutant Arabidopsis. Thus, SEC11 binds and selectively regulates secretory traffic mediated by SYP121 and is important for recycling of the SNARE and its cognate partners.

INTRODUCTION

SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) comprise a highly conserved superfamily of proteins that mediate in the traffic of vesicles between endosomal compartments as well as in secretory traffic to the plasma membrane in all eukaryotic cells. SNAREs drive the fusion of vesicle and target membranes and enable the transfer of soluble and membrane-associated cargos in a highly regulated manner (Pratelli et al., 2004; Jahn and Scheller, 2006; Lipka et al., 2007; Bassham and Blatt, 2008). In plant cells, SNARE-mediated vesicle traffic delivers wall materials and membrane to the cell surface, making essential contributions to homeostasis, cell expansion, and growth (Blatt, 2000; Shope et al., 2003; Campanoni and Blatt, 2007). It also plays important roles in resistance to pathogens (Kaide et al., 2007; Zhang et al., 2007) and responses to abiotic stress (Grefen and Blatt, 2008; Eisenach et al., 2012). Cognate SNAREs localize to vesicle and target membranes, and during the SNARE cycle, their assembly in a stable heteromeric core complex draws vesicle and target membranes together for fusion of the two bilayers. Each SNARE in the complex contributes one or more of a set of highly conserved motifs, classified according to their structure and the amino acid (Q [Gln] or R [Arg]) present at the center of the motif (Fasshauer et al., 1998; Bock et al., 2001). Qa-SNAREs generally localize to the target membrane along with Qb- and Qc-SNAREs, or with Qb-cSNAREs that are often referred to as SNAP25-related (synaptosome-associated protein of 25 kD) proteins; R-SNAREs are present on the vesicle membrane and are also known as VAMPs (vesicle-associated membrane proteins).

Plants display the widest spatiotemporal and developmental distribution of SNAREs among eukaryotes (Sanderfoot et al., 2000; Pratelli et al., 2004), reflecting the need for specialized functions throughout the life cycle of the plant (Bassham and Blatt, 2008; Grefen and Blatt, 2008). The Qa-SNAREs of the plasma membrane are a case in point. In Arabidopsis thaliana, two of these SNAREs, SYP121 (=SYR1/PEN1) and SYP122, show a high degree of structural homology and overlap in function (Assaad et al., 2004; Tyrrell et al., 2007; Bassham and Blatt, 2008; Rehman et al., 2008). Single mutations of these Qa-SNAREs have little effect on plant growth under many conditions, and reduced growth is normally evident only in the syp121/syp122 double mutant (Assaad et al., 2004; Zhang et al., 2007). Both SYP121 and SYP122 are expressed throughout the development of the plant and in most, if not all, tissues (Uemura et al., 2004; Enami et al., 2009). The Qa-SNAREs also share the cognate partner proteins SNAP33, VAMP721, and VAMP722 (Kwon et al., 2008; Rehman et al., 2008; Karnik et al., 2013b), indicating substantial mechanistic overlaps in driving vesicle fusion. Nonetheless, a number of functional differences between SYP121 and SYP122 have surfaced. SYP121 has unique roles in responses to drought and the water stress hormone abscisic acid (Leyman et al., 1999; Sutter et al., 2007; Eisenach et al., 2012), and it facilitates targeted vesicle traffic for defense against pathogen attack (Kwon et al., 2008). Furthermore, SYP121 uniquely interacts with K+ channels to facilitate the uptake
of the solute for cell expansion and plant growth (Honsbein et al., 2009, 2011; Grefen et al., 2010a). These observations raise the most fundamental questions about the nature of the regulation that could give rise to their distinctive roles in vesicle traffic and its control, and they highlight our relative ignorance generally about the vesicle-trafficking pathways to the plasma membrane mediated by SYP121 and SYP122.

Clues to the molecular mechanics regulating SYP121 have come from recent studies of SEC11 (=KEULE), a member of the Sec1/Munc18 (SM) protein family (Südhof and Rothman, 2009; Hughson, 2013) that, in animals and yeast, regulate Qa-SNARE availability for SNARE complex assembly and stabilize the complex during vesicle fusion. SEC11 was originally identified as a binding partner of the Qa-SNARE SYP111 (=KNOlle) that is expressed and functions only during cytokinesis (Waizenegger et al., 2000; Assaad et al., 2001; Park et al., 2012). Nonetheless, SEC11 is expressed throughout the vegetative plant and not just during cell division. Kamik et al. (2013b) demonstrated that SEC11 will bind with SYP121 and regulate its activity at the plasma membrane. Significantly, SEC11 binding to the N terminus of SYP121 affected binary interaction with the Qa-SNARE but not its assembly in the SNARE core complex. These findings highlighted a role for SEC11 unlike that of SM proteins in animals, and they offered a strategy with which to explore potential differences in traffic control mediated through SYP121 and SYP122.

Here, we set out this strategy using SEC11 and its N-terminal fragment that binds SYP121 to probe secretory traffic in Arabidopsis. We report that SEC11 interacted selectively with SYP121 over SYP122 in vitro and when expressed in yeast and that it rescued secretory traffic block in the sp122 mutants, but not in the sp121, mutant in vivo. The N-terminal fragment of SEC11 itself inhibited secretion, but only in plants expressing SYP121. These and additional results underscore the specificity of SEC11 in regulating traffic to the plasma membrane mediated by SYP121 and, most importantly, they point to a role for SEC11 in recycling of the SNARE complex after vesicle fusion.

RESULTS

Qa-SNARE Fragments SYP121\(^{1-546}\) and SYP122\(^{1-288}\) Block Secretory Traffic

The cytosolic fragments of the Qa-SNAREs SYP121\(^{1-546}\) (=SYP121-Sp2) and SYP122\(^{1-288}\) (=SYP122-Sp2) were shown previously to block secretion at a stage late in transit to the plasma membrane in tobacco (Nicotiana tabacum) and Arabidopsis (Geelen et al., 2002; Sutter et al., 2006; Tyrell et al., 2007; Besserer et al., 2012; Kamik et al., 2013b). Secretory block was proposed (Figure 1A) to arise by the titration of cognate SNARE partners, thus accounting for the lack of selectivity between the cytosolic fragments (Tyrell et al., 2007), but this interpretation was not addressed directly using the complementary mutant backgrounds. We expressed the fluorescent secretory marker secreted yellow fluorescent protein (secYFP) and the endoplasmic reticulum marker GFP-HDEL with and without SYP121\(^{1-546}\) (Figure 1B) and SYP122\(^{1-288}\) using the tetracistronic vector pTecG-2in1-CC (Figure 1C) (Kamik et al., 2013b) to transiently transform Arabidopsis Columbia-0 (wild type), sp121-1 (sp121), and sp122-1 (sp122) mutant seedlings (Zhang et al., 2007; Honsbein et al., 2009; Grefen et al., 2010b). secYFP is exported from the cell and normally yields little or no fluorescence once in the apoplast. Thus, only secYFP retained in the cell fluoresces strongly. Coexpression of the endoplasmic reticulum marker GFP-HDEL allowed transformations to be verified on a cell-by-cell basis and enabled the quantification of secYFP retention as the ratio of yellow fluorescent protein (YFP) to green fluorescent protein (GFP) fluorescence in the tissue.

Cocultivation with Agrobacterium tumefaciens normally gives a strong transformation of the root epidermis (Honsbein et al., 2009; Grefen et al., 2010b; Karnik et al., 2013b). Therefore, we quantified secYFP traffic by confocal microscopy of seedling roots, using standardized excitation and emission settings, and analyzed 3D projections for YFP and GFP fluorescence after subtracting background fluorescence signals recorded from untransformed seedlings. Figure 2A shows images from one set of transformations and includes immunoblot analysis for the expression of the Qa-SNARE fragments. Figure 2B summarizes the results from all experiments (n = 15 for each data set). In

![Figure 1. Schematics of SYP121, Its Cytosolic Fragments, and Their Proposed Block of Secretory Traffic.](image)
In every case, seedlings transformed with secYFP and GFP-HDEL alone showed a low ratio of YFP to GFP fluorescence, typically around 0.5 or less, regardless of the genetic background of the seedlings. A small increase in the mean YFP:GFP ratio was observed in measurements from the syp121 and syp122 mutants when compared with the wild-type seedlings, but the difference was not significant. Coexpression with SYP121\(^{DC}\) and SYP122\(^{DC}\) yielded substantial increases in the YFP:GFP ratio, indicating the retention of secYFP, again regardless of the genetic background. We noted a small difference between the mutant backgrounds only when comparing the YFP:GFP ratios on SYP122\(^{DC}\) coexpression, suggesting that this Qa-SNARE fragment was more effective in blocking secYFP export in the syp121 mutant. Again, the difference between the genetic backgrounds was not very significant, and SYP121\(^{DC}\) was equally effective in retaining secYFP in all three Arabidopsis lines. These findings support the interpretation of a common pool of interacting partners shared by the two Qa-SNAREs.

**SEC11 Rescues Secretory Block in syp122 but Not in syp121 Mutant Plants**

Although SEC11 rescues traffic block by SYP121\(^{DC}\) in tobacco (Kamnik et al., 2013b), these earlier findings do not distinguish between two general mechanisms. Expressing SEC11 might rescue traffic by binding with and sequestering the SYP121\(^{DC}\) fragment, preventing its actions either in the cytosol or at the plasma membrane; alternatively, SEC11 rescue might reflect a genuine facilitation of vesicle fusion through its interaction with the native SYP121 in Arabidopsis or its homolog at the plasma membrane in tobacco. To distinguish between these two possibilities, we used the pTecG-2in1-CC vector to coexpress secYFP and GFP-HDEL with SEC11 alone and together with the two Qa-SNARE fragments. Again, experiments were performed in Arabidopsis wild-type, syp121, and syp122 seedlings, and image data were quantified using the YFP:GFP fluorescence ratio. Figure 3A includes the results from one set of transformations along with immunoblot analysis for the expression of the Qa-SNARE fragments and SEC11. The results of all of the experiments are summarized in Figure 3B. (These and the subsequent studies were run in parallel with the data of Figure 2. The latter are included here and in the subsequent figures for visual comparison.) We found that expressing SEC11 on its own marker GFP-HDEL rendered as 3D projections, along with the corresponding, mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type, syp121, and syp122 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL alone (control) and together with SYP121\(^{DC}\) and SYP122\(^{DC}\) (indicated at right). Immunoblot analysis verifying the expression of SYP121\(^{DC}\) and SYP122\(^{DC}\) using anti-myc antibody is shown at right. Bar = 50 μm.

**Figure 2.** SYP121\(^{DC}\) and SYP122\(^{DC}\) Fragments Block Secretion in Wild-Type, syp121, and syp122 Arabidopsis.  
(A) Representative confocal images (left to right) of fluorescence from the secretory marker secYFP and the retained (endoplasmic reticulum) GFP-HDEL rendered as 3D projections, along with the corresponding, mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type, syp121, and syp122 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL alone (control) and together with SYP121\(^{DC}\) and SYP122\(^{DC}\) (indicated at right). Immunoblot analysis verifying the expression of SYP121\(^{DC}\) and SYP122\(^{DC}\) using anti-myc antibody is shown at right. Bar = 50 μm.  
(B) secYFP:GFP-HDEL fluorescence ratios as means ± se of >15 independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from 3D projections of root images after correcting for the background fluorescence similarly recorded from untransformed seedlings of the same age. Significance is indicated by lettering at P < 0.05.
had no measurable effect on secYFP traffic when assayed in this fashion (Karnik et al., 2013b). Coexpressing SEC11 with SYP121\textsuperscript{AC} and SYP122\textsuperscript{AC} rescued traffic block by the Qa-SNARE fragments in the wild-type and syp122 mutant seedlings. In the syp121 mutant, however, SEC11 coexpression was without effect in reducing the YFP:GFP ratios when compared with those determined for seedlings expressing SYP121\textsuperscript{AC} or SYP122\textsuperscript{AC} alone. We concluded that SEC11 is able to rescue secYFP traffic, irrespective of the Qa-SNARE fragment, provided that the native SYP121 is expressed. In short, the results argue against SEC11 titration of SYP121\textsuperscript{AC} and SYP122\textsuperscript{AC} in the cytosol, instead implicating a direct and selective interaction with SYP121 at the plasma membrane.

To test SEC11 selectivity between the two full-length Qa-SNAREs, we used a modified version of the yeast mating-based split-ubiquitin (SUS) assay (Grefen et al., 2010a; Grefen and Blatt, 2012b; Grefen, 2014). SUS assays are useful to test interactions between membrane-anchored proteins. One requirement of the SUS approach is that the bait protein be membrane anchored so that the bait construct cannot migrate to the nucleus on its own to activate the reporter gene in the absence of prey interaction. The CytoSUS assay overcomes this limitation, incorporating, as an N-terminal anchor for a soluble bait construct, the integral membrane protein subunit of the yeast oligosaccharyltransferase complex of the endoplasmic reticulum lumen (OST4p) (Möckli et al., 2007). We designed a Gateway-compatible SUS bait vector with this construct, here designated mOST4, to give expression with SEC11. Diploid yeast expressing SYP121 or SYP122 as the prey protein with mOST4-SEC11 as the bait protein were obtained as before by mating and growth on synthetic medium lacking leucine, tryptophan, uracil, and methionine. Expression of the bait is under methionine promoter repression; therefore, methionine was used to test for interaction specificity. The results (Supplemental Figure 1) showed that growth on selective medium was retained in yeast expressing mOST4-SEC11 and SYP121, even in the presence of 50 and 500 μM methionine, but little or no growth was found with yeast expressing the bait with SYP122 under the same conditions.

**Figure 3.** SEC11 Rescues Secretory Block in Wild-Type and syp122, but Not in syp121, Arabidopsis.

(A) Representative confocal images (left to right) of fluorescence from the secretory marker secYFP and the retained (endoplasmic reticulum) marker GFP-HDEL rendered as 3D projections, along with the corresponding, mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type, syp121, and syp122 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL with SYP121\textsuperscript{AC} and SYP122\textsuperscript{AC} alone and together with SEC11 (indicated at right). Immunoblot analysis verifying the expression of SYP121\textsuperscript{AC}, SYP122\textsuperscript{AC}, and SEC11 using anti-myc and anti-HA antibodies, respectively, is shown at right. Controls with secYFP and GFP-HDEL alone and with SYP122\textsuperscript{AC} are found in Figure 2. Bar = 50 μm.

(B) secYFP:GFP-HDEL fluorescence ratios as means ± SE of more than five independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from 3D projections of root images after correcting for the background fluorescence similarly recorded from untransformed seedlings of the same age. Significance is indicated by lettering at P < 0.05. Data for SYP122\textsuperscript{AC} have been omitted from the graph for clarity (see Figure 2B).
These data are consistent with the findings of syp121-sensitive traffic rescue, confirming a selectivity of SEC11 for interaction with SYP121.

Traffic Rescue Is Not Dependent on SEC11 Binding with the N Terminus of SYP121

Phe-9 is highly conserved among many Qa-SNAREs of animals, yeast, and Drosophila melanogaster; it is critical for SM protein binding to regulate SNARE core complex assembly (Bracher and Weissenhorn, 2002; Burgoyne and Morgan, 2007; Südhof and Rothman, 2009), and it is present in SYP121 (Grefen et al., 2010a). Karnik et al. (2013b) found that the SYP121F9A mutation suppressed SEC11 binding to the SYP121 N terminus and eliminated the ability of SEC11 to stabilize the so-called “closed” conformation of SYP121 against assembly with its cognate SNARE partners in vitro. We used this knowledge to test whether SEC11 binding to this domain of SYP121 contributed to traffic rescue by the SM protein. We generated mutants of the Qa-SNARE soluble fragment incorporating the Phe substitution (SYP121DC,F9A) and, as a control, the equivalent substitution in the adjacent Ser residue (SYP121DC,S10A), using the pTecG-2in1-CC vector to transform wild-type and syp121 mutant Arabidopsis as before. The results (Supplemental Figure 2) showed that expressing the soluble fragments SYP121DC, SYP121DC,F9A, and SYP121DC,S10A each yielded a similar retention of secYFP and elevated the YFP:GFP ratio, both in wild-type and syp121 mutant seedlings, indicating that Phe-9 of the SYP121DC fragment does not contribute to traffic block.

We next assessed whether SEC11 was similarly effective in rescuing traffic blocked by the soluble SYP121DC,F9A fragment as it was when block was achieved with SYP121DC. We reasoned that if SEC11 binding to the Qa-SNARE fragment N terminus was important for its rescue of traffic, such as might result from stabilizing the closed state of SYP121DC, then secYFP traffic rescue should be lost when SEC11 was coexpressed with the SYP121DC,F9A mutant. Figure 4 summarizes experiments in which SYP121DC, SYP121DC,F9A, and SYP121DC,S10A were coexpressed with SEC11 in wild-type and syp121 mutant seedlings. In wild-type Arabidopsis, the fluorescence ratios on SEC11 coexpression with each of the soluble fragments SYP121DC, SYP121DC,F9A, and SYP121DC,S10A were reduced significantly, consistent with SEC11 rescue of secYFP secretion. In the syp121 mutant plants, secYFP retention and the fluorescence ratios were comparable to the signals when

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Figure 4. SEC11 Rescues Secretory Traffic Independent of Phe-9 Substitution in SYP121DC,F9A, but Only in the Presence of the Native SYP121.

(A) Representative confocal images (left to right) of fluorescence from the secretory marker secYFP and the retained (endoplasmic reticulum) marker GFP-HDEL rendered as 3D projections, along with the corresponding mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type and syp121 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL alone (control) and with each of the soluble fragments SYP121DC, SYP121DC,F9A, and SYP121DC,S10A together with SEC11 (indicated at right). Immunoblot analysis verifying the expression of the Qa-SNARE fragments and SEC11 using anti-myc and anti-HA antibodies, respectively, is shown at right. Controls with secYFP and GFP-HDEL with SYP121DC are found in Figure 2. Bar = 50 μm.

(B) secYFP:GFP-HDEL fluorescence ratios as means ± SEM of more than five independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from 3D projections of root images after correcting for the background fluorescence similarly recorded from untransformed seedlings of the same age. Significance is indicated by lettering at P < 0.05.
SYP121\textsuperscript{AC} was expressed alone, regardless of the mutation in the Qa-SNARE fragment. These data, like the results of Figure 3, demonstrate a requirement for the native SYP121. Additionally, they show that traffic rescue by the full-length SEC11 cannot be explained by its putative interaction with the N terminus of SYP121\textsuperscript{AC}.

**SEC11 Interaction with the N Terminus of SYP121 at the Plasma Membrane Is Important for Secretion and Traffic Rescue**

To test if SEC11 interaction with the native SYP121 N terminus contributes to the rescue of vesicle traffic, we examined secYFP retention in syp121 mutant Arabidopsis stably complemented to express the full-length mutant SYP121\textsuperscript{F9A} under the control of the constitutive Ubiquitin-10 promoter (syp121:SYP121\textsuperscript{F9A}) (Karnik et al., 2013b). Seedlings of syp121:SYP121\textsuperscript{F9A} plants were transiently transformed to quantify secYFP and GFP-HDEL fluorescence as before. For comparison, we include data for parallel transformations of wild-type and syp121 mutant plants in Figure 5. Image analysis showed enhanced secYFP fluorescence and roughly a 5-fold increase in the YFP:GFP fluorescence ratio in the syp121:SYP121\textsuperscript{F9A} seedlings expressing the full-length mutant SYP121\textsuperscript{F9A} when compared with the wild-type control, even when only the secYFP and GFP-HDEL constructs were expressed (Figures 5A and 5B, control). The elevated fluorescence ratio was statistically indistinguishable from the ratios observed on coexpressing the SYP121\textsuperscript{AC} fragment. Coexpression with SEC11 had no additional effect in elevating the fluorescence ratio in the syp121:SYP121\textsuperscript{F9A} complemented plants. These results indicated that disrupting SEC11 binding to the N terminus of the full-length Qa-SNARE alone was sufficient to suppress secYFP traffic.

SEC11, like other SM proteins (Südhof and Rothman, 2009; Hughson, 2013), is thought to form a clothespeg-like structure that clamps the SNARE complex once assembled within its major cleft, thereby stabilizing the complex for vesicle fusion. SEC11 binding with the SYP121 N terminus tethers the two proteins (Park et al., 2012). It favors a quasi-open conformation of SYP121 that is not fusion competent; only together with additional regulatory factors is SEC11 thought to convert SYP121 to an "open" conformation ready for SNARE complex assembly (Karnik et al., 2013b). SEC11 binding to the SYP121 N terminus is affected by mutations within a minor cleft thought to form at the surface of the N-terminal half of SEC11. Introducing the positively charged Arg residue in the SEC11\textsuperscript{L128R} mutant abolishes SEC11 binding with the SYP121 N terminus but does not affect SM protein binding to the SNARE complex assembled of SYP121, SNAP33, and VAMP721 (Karnik et al., 2013b). We hypothesized that if binding with SYP121 in the complex was important for traffic rescue at the plasma membrane, then expressing SEC11\textsuperscript{L128R}, which eliminates tethering but not binding to the SNARE complex, should nonetheless rescue secYFP traffic.

To test SEC11\textsuperscript{L128R} interaction with the full length SYP121, again we used the mOST4-anchored SEC11 in the SUS assay with the Qa-SNARE. Diploid yeast expressing SYP121 as the prey protein with either mOST4-SEC11 or mOST4-SEC11\textsuperscript{L128R} as the bait protein were obtained and grown as before (Grefen et al., 2010a; Grefen and Blatt, 2012a). Figure 6 shows that growth on selective medium was retained in yeast expressing mOST4-SEC11 and SYP121, even in the presence of 50 and 500 \textmu M methionine, concentrations that yielded little or no growth of diploid yeast expressing mOST4-SEC11\textsuperscript{L128R} and SYP121. These data indicated that the SEC11\textsuperscript{L128R} mutation suppresses its binding with the full-length SYP121 protein. Thus, the SEC11\textsuperscript{L128R} mutant appeared unable to bind the Qa-SNARE in binary complex, much as was deduced previously from in vitro pull-down assays with the soluble Qa-SNARE protein (Karnik et al., 2013b).

To test its effect on secYFP traffic, we used the pTecG-2in1-CC vector to express SEC11\textsuperscript{L128R} without and with SYP121\textsuperscript{AC} in wild-type, syp121, and syp122 Arabidopsis. Figure 7 includes one set of fluorescence images, and the analysis of all transformations includes data from the parallel control and assays with SYP121\textsuperscript{AC} (Figures 2 to 5). Comparison of the fluorescence images and ratios shows that SEC11\textsuperscript{L128R} was without significant effect on YFP retention when expressed on its own in wild-type and syp121 mutant plants. However, expressing SEC11\textsuperscript{L128R} alone in the syp122 mutant led to secYFP retention and a high mean fluorescence ratio, similar to that observed in seedlings expressing the SYP121\textsuperscript{AC} fragment. Furthermore, SEC11\textsuperscript{L128R} expression was ineffective in preventing secYFP retention, both in wild-type Arabidopsis and in the mutant lines coexpressing SYP121\textsuperscript{AC}. Thus, contrary to our expectation, the results suggested not only that association with the native SYP121 N terminus is important for SEC11 rescue of traffic but that SEC11\textsuperscript{L128R} itself suppresses secretory traffic when SYP121 provides the dominant pathway for secretion at the plasma membrane.

**The SEC11\textsuperscript{149} Peptide Blocks SYP121-Mediated Secretory Traffic**

Proteins of the SM family are characterized by common structural elements (Misura et al., 2000; Südhof and Rothman, 2009; Hu et al., 2011; Rizo and Südhof, 2012), including an N-terminal (so-called domain 1) and two additional domains, the last of these divided by a "hinge" that forms the apex of the clothespeg-like structure (Figure 8A). Studies of neuronal nSec1 in complex with the Qa-SNARE Syn1A have implicated domain 1 (residues 4 to 134 of nSec1) in binding with the closed conformation of the Qa-SNARE to sequester it in a fusion-incompetent state (Südhof and Rothman, 2009). Similar associations may also occur in yeast (Furgason et al., 2009) but have yet to be explored in plants. Following alignments with nSec1 and its homolog Munc18-1 (Figure 8B), we designed constructs to express a domain 1 peptide spanning residues 1 to 143 of SEC11 (SEC11\textsuperscript{1-143}) without and with the mutation of residue Leu-128 (SEC11\textsuperscript{1-143,L128R}). We also designed a construct to express a second peptide corresponding to domain 2 spanning residues 150 to 270 (SEC11\textsuperscript{150-270}).

To test if SEC11 binds SEC11\textsuperscript{1-149} in vitro, we used a SYP121\textsuperscript{AC} tagged with streptavidin at its C terminus and the SEC11, SEC11\textsuperscript{1-149}, and SEC11\textsuperscript{1-149,L128R} proteins tagged with GST at their N termini, much as described previously (Karnik et al., 2013b). For comparison, we also tested the binding with the cognate SNAREs VAMP721 and SNAP33 (Karnik et al., 2013b). Each of the proteins was expressed in _Escherichia coli_ and purified by affinity chromatography prior to assays. Incorporating the tags in this orientation avoided potential interference with the critical domains required for
protein interactions. The purified protein fusions were used in pull-down assays with affinity resin-bound SYP121\textsuperscript{AC} as the bait and the SM protein and peptides as the prey. The results (Figure 8C) showed that SNAP33, VAMP721, SEC11, and SEC11\textsubscript{149} all bound SYP121\textsuperscript{AC} with signals 4- to 5-fold above background, but SEC11\textsubscript{149,128R} association with SYP121\textsuperscript{AC} was similar to the background.

To test SEC11 peptide interaction with SYP121 in vivo, we transiently transformed Arabidopsis seedlings using the tricistronic vector pBiFCt-2in1-NC for ratiometric bimolecular fluorescence complementation (rBiFC) (Grefen and Blatt, 2012a; Karnik et al., 2013b; Blatt and Grefen, 2014; Lipka et al., 2014). The 2in1 rBiFC system incorporates a set of independent Gateway-compatible cassettes with coding sequences for the two halves of YFP, nYFP and cYFP, to generate fusion constructs for coexpression and testing of fluorescence complementation in the plant. The vector also includes a third cassette incorporating the coding sequence for a soluble red fluorescent protein (RFP) fluorescent marker that can be used, on a cell-by-cell basis, as a transformation control and ratiometric reference for quantification. Again, to avoid
potential interference of the rBiFC tags, we designed the constructs of SEC11 and its peptides as N-terminal fusions with cYFP, and we used SYP121\textsuperscript{AC} as a C-terminal fusion with nYFP. Figure 8D shows representative confocal images of Arabidopsis expressing SYP121\textsuperscript{D}nYFP alone as a control and with each of the cYFP-SEC11 fusion constructs. YFP and RFP fluorescence were quantified using the same standardized methods as were applied in the secYFP trafficking assays, and background fluorescence was subtracted before calculating the fluorescence ratios. We found (Figure 8E) that coexpressing SEC11 with SYP121\textsuperscript{D} led to almost a 5-fold increase in the YFP:RFP fluorescence ratio compared with the background control of SYP121\textsuperscript{D} expressed alone. Coexpression with SEC11\textsuperscript{D}149 gave a reduced fluorescence ratio roughly 3.5-fold over the control. However, a significant rBiFC signal above the control was not evident on coexpression with SEC11\textsuperscript{D}149,L128R or on expressing SEC11\textsuperscript{D}150-270 with SYP121\textsuperscript{D}. These results, and the biochemical data, demonstrate that the SEC11\textsuperscript{D}149 peptide retains the capacity to interact with the Qa-SNARE, and, like full-length SEC11, this association is eliminated by mutation of Leu-128.

We challenged secretory traffic with SEC11\textsuperscript{D}149 in vivo using the pTecG-2in1-CC vector in wild-type, syp121, and syp122 Arabidopsis. Consistent with its capacity for SYP121 binding, expressing SEC11\textsuperscript{D}149 (Figures 9A and 9B) led to a significant increase in secYFP retention, if marginally less than that observed with SYP121\textsuperscript{D} in the wild-type and syp122 plants. However, expressing SEC11\textsuperscript{D}149 in the syp121 mutant failed to promote secYFP retention above the background observed on expressing secYFP and GFP-HDEL alone. To test SEC11\textsuperscript{D}149 binding alternatively to SYP121\textsuperscript{D} in the syp121 mutant, we repeated these trials using the SYP122\textsuperscript{D}F9A fragments, neither of which binds SEC11\textsuperscript{D}149, in place of SYP121\textsuperscript{D} (Supplemental Figures 3 and 4). In this case, SEC11\textsuperscript{D}149 was unable to rescue traffic block by this Qa-SNARE fragment in the syp121 mutant and, indeed, enhanced block in the wild-type and syp122 seedlings that carry SYP121. We conclude that SEC11\textsuperscript{D}149 suppresses secretory traffic, analogous to the action of the Qa-SNARE fragment, but in a manner that associates strictly with the presence of the native SYP121 protein. Its rescue of traffic in the syp121 mutant background is similarly allied to its ability to bind with SYP121\textsuperscript{AC}, as we outline in Discussion.

The SEC11\textsuperscript{D}149 Peptide Suppresses Cell Expansion Dependent on SYP121

Finally, we generated independent, stable lines of Arabidopsis in all three genetic backgrounds (wild type, syp121, and syp122) to

Figure 7. SEC11\textsuperscript{L128R} Blocks Secretion in the syp122 Mutant.

(A) Representative confocal images (left to right) of fluorescence from the secretory marker secYFP and the retained (endoplasmic reticulum) marker GFP-HDEL rendered as 3D projections, along with the corresponding, mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type, syp121, and syp122 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL with SYP121\textsuperscript{D}, SEC11\textsuperscript{L128R}, and SYP121\textsuperscript{D} + SEC11\textsuperscript{L128R} (indicated at right). Controls for the syp122 mutant with secYFP and GFP-HDEL alone and with SYP121\textsuperscript{AC} are included for comparison; additional controls are found in Figures 2 and 3. Immunoblot analysis verifying the expression of SYP121\textsuperscript{AC} and SEC11\textsuperscript{L128R} using anti-myc and anti-HA antibodies, respectively, is shown at right. Bar = 50 μm.

(B) secYFP:GFP-HDEL fluorescence ratios as means ± se of three or more independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from 3D projections of root images after correcting for the background fluorescence similarly recorded from untransformed seedlings of the same age. Significance is indicated by lettering at P < 0.05.
Figure 8. The SEC11<sup>149</sup> Fragment Interacts with SYP121<sup>HC</sup>.

(A) and (B) Schematic of SEC11 domain topology, including major and minor clefts as indicated, and alignment with human Munc18-1 and neuronal Sec1 proteins (Misura et al., 2000). Fragments SEC11<sup>149</sup> and SEC11<sup>149-270</sup>, as well as the SEC11<sup>149, L128R</sup> mutant, were generated to express as shown. Leu-128 is critical for interaction with the N terminus of SYP121 (Karnik et al., 2013b).

(C) Silver-stained gel images (top) of proteins recovered in pull-down assays with SYP121<sup>HC</sup>-streptavidin as bait. Images of bait and equimolar prey inputs are shown below. Inputs and pull-down bands are GST-tagged preys (left to right) of VAMP721<sup>HC</sup>, SNAP33, SEC11, SEC11<sup>149</sup>, and SEC11<sup>149, L128R</sup>. Bound:bait ratios are plotted below derived from band intensities recorded using ImageJ (see Methods).
overexpress myc-tagged SEC11^{149} under dexamethasone (Dex)-inducible control (Aoyama and Chua, 1997) and to express SEC11-GFP constitutively. Dex induction was found to give maximum expression of the SEC11^{149} construct at concentrations above 1 μM and within 12 h of treatment; induction was stable for at least 48 h following a single treatment (Supplemental Figure 5). Plants derived from at least two independent transformed lines for each construct were analyzed for rosette size, fresh and dry weight, leaf number, and leaf development. In the absence of Dex induction, we found no significant differences between the untransformed and transgenic lines carrying the SEC11^{149} construct; lines in the syp121 background showed a marginal reduction in dry weight (Supplemental Figure 6), but the difference was not significant. A small but significant reduction was observed in leaf number in the syp122 mutant.

Repeated treatments with 10 μM Dex were performed over 3-week periods to induce and maintain transgene expression. We observed a moderate reduction in vegetative growth when SEC11^{149} was expressed in the wild-type background compared with the untransformed controls treated with Dex, and a similar, albeit much stronger effect was evident in the syp122 mutant background; by contrast, no differences in rosette size and fresh or dry weight were recovered with SEC11^{149} expression in the syp121 background, although immunoblot analysis confirmed expression of the transgene in each case (Figures 10A to 10D). Plants expressing SEC11^{149} in the wild-type and syp122 backgrounds yielded fewer leaves that were generally smaller (Figures 10D and 10E). We also analyzed the growth of lines constitutively expressing SEC11-GFP in the same genetic backgrounds. Vegetative growth and fresh and dry weights in this case were enhanced for all independent lines in the wild-type and syp122 backgrounds but not in the syp121 mutant background. This effect was most evident 3 to 4 weeks after germination (Figure 11); however, differences in rosette area and biomass were not significant 5 to 6 weeks after the onset of flowering, suggesting that SEC11 overexpression accelerated growth only early on prior to floral induction.

Because the epidermal layer of the leaf is a major determinant of leaf expansion (Walter et al., 2009), we quantified epidermal cell size and numbers in fully expanded leaves of untransformed plants and plants expressing the Dex-inducible SEC11^{149} construct, both after 3-week treatments with Dex. We found the syp122 mutant and to a lesser extent the wild-type backgrounds to show smaller mean epidermal cell areas when compared with their corresponding controls without Dex (Figures 12A and 12B). All of the lines developed a primary inflorescence after 5 to 6 weeks. In the syp122 background especially, SEC11^{149} expression was associated with curling of the leaf edges and reduced inflorescence size (Figures 12C and 12D). Even after 8 weeks, these plants showed a smaller inflorescence compared with the other controls and transgenic lines. Although separating the developmental sequence in heterogeneous tissues is not straightforward, overall the observations suggest that SEC11^{149} expression affects primarily cell expansion rather than cell division. Most significant, however, the SEC11^{149} phenotype associated with the presence of the native SYP121 in the parental background and showed a dominance even in the wild-type background, much as we found for secYFP retention. These observations point to a crucial role for the SEC11 and its N-terminal domain in regulating traffic to the plasma membrane, and they underline the specific association of the SM protein with SYP121.

**DISCUSSION**

The Arabidopsis Qa-SNAREs SYP121 (=SYR1/PEN1) and SYP122 exhibit high sequence homology and overlapping functions, and both are present at the plasma membrane of cells throughout the plant (Lipka et al., 2007; Bassham and Blatt, 2008). Along with the Qa-SNARE SYP132, which is constitutively expressed and present at low levels (Enami et al., 2009), these proteins mediate secretory vesicle traffic, contributing to cellular homeostasis, expansion, and growth (Blatt, 2000; Shohe et al., 2003; Campanoni and Blatt, 2007). Functional differences between SYP121 and SYP122 are known (Leyman et al., 1999; Zhang et al., 2007; Rehman et al., 2008; Honsbein et al., 2009, 2011; Grefen et al., 2010a; Eisenach et al., 2012), but as these Qa-SNAREs share cognate partners (Kwon et al., 2008; Karnik et al., 2013b; Yun et al., 2013), studies to date have offered few clues to the mechanistic basis for the differences in their regulation and function. Among other factors, SM proteins are widely recognized to be key components that regulate SNARE-mediated vesicle traffic, both in stabilizing the cognate SNARE complex and in controlling the availability of several Qa-SNAREs for binding (Südhof and Rothman, 2009; Rizo and Südhof, 2012). Arabidopsis harbors six SM proteins, including SYP121, which was identified originally with another Qa-SNARE, SYP111, in membrane traffic during cell division (Waizenegger et al., 2000; Assaad et al., 2001; Park et al., 2012). Only recently was SYP111 shown to bind with SYP121 and affect traffic at the plasma membrane mediated by this Qa-SNARE (Kamik et al., 2013b).

We have examined the specificity of SEC11, taking advantage of the soluble domains of the Qa-SNAREs, SYP121^{149} and SYP122^{149}, that act as dominant-negative (inhibitory) fragments and block secretory traffic (Geelen et al., 2002; Tyrrell et al.,...
SEC11-Regulated Vesicle Traffic

The SEC11 fying the expression of the Qa-SNARE and SEC11 transformed wild-type, stack merged bright-GFP-HDEL rendered as 3D projections, along with the corresponding, mid-secretory marker secYFP and the retained (endoplasmic reticulum) marker.

Figure 9. The SEC11[1-149] Fragment Blocks Secretory Traffic, but Only in the Presence of the Native SYP121.

(A) Representative confocal images (left to right) of fluorescence from the secretory marker secYFP and the retained (endoplasmic reticulum) marker GFP-HDEL rendered as 3D projections, along with the corresponding, mid-stack merged bright-field and GFP-HDEL image. Images are of transiently transformed wild-type, syp121, and syp122 Arabidopsis seedlings (indicated at left) expressing secYFP and GFP-HDEL together with SEC11 alone and together with SYP121C (indicated at right). Immunoblot analysis verifying the expression of the Qa-SNARE and SEC11[1-149] fragments using anti- myc and anti-HA antibodies is shown at right. Bar = 50 μm.

(B) secYFP:GFP-HDEL fluorescence ratios as means ± se of more than eight independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from 3D projections of root images after correcting for the background fluorescence similarly recorded from untransformed seedlings of the same age. Significance is indicated by lettering at P < 0.05.

2007), and of the syp121 and syp122 mutant Arabidopsis. These studies led to a substantial reassessment of roles for the SM protein. Most important, the findings establish a function for SEC11 in selective regulation of SYP121-mediated traffic at the plasma membrane. They offer strong evidence that the SYP121C and SYP122C fragments block traffic by sequestering the cognate SNARE partners, thereby competing for their binding with the full-length Qa-SNAREs at the plasma membrane. They indicate that SEC11 interaction with the SYP121 N terminus is crucial in preparing the Qa-SNARE for vesicle fusion. Finally, they suggest that binding of SEC11 to the SYP121 N terminus has an unexpected and complementary role in “unlocking” the SNARE complex for its recycling following vesicle fusion.

SEC11 Is Selective among Plasma Membrane Qa-SNAREs

Previous studies had indicated a lack of specificity among soluble fragments of the plasma membrane SNAREs to mediate the block of secretion. Tyrrell et al. (2007) noted that the dominant-negative fragments SYP121C and SYP122C were almost equally effective in blocking traffic to the plasma membrane, and specificity was evident only between subsets of SNAREs aligned with their target membranes. It was suggested that block by SYP121C and SYP122C could be related mechanistically either to competition for a common pool of cognate partners (Misura et al., 2001; Pajonk et al., 2008) or to the ability of the Qa-SNARE fragments to bind with the full-length counterparts quasi-indiscriminately in homomultimers, thereby trapping SYP121 and SYP122, possibly in a semi-crystalline, “closed” conformation (Sieber et al., 2007; Grefen et al., 2011). Our data now provide substantive evidence that the SNARE fragments block traffic by competing for the cognate partners. We found that overexpressing SEC11 rescued secretory traffic independent of the dominant-negative SNARE fragment, but only in the background of Arabidopsis expressing the native SYP121 at the plasma membrane (Figure 3). The requirement for the native SYP121, especially, militates against the argument for SEC11 dependent of the dominant-negative SNARE fragment, but only in Figure 4). Because the SYP121C,F9A mutant on its own does not bind with SEC11 (Karnik et al., 2013b), the observations preclude a titration of the soluble Qa-SNARE fragments and lead to the conclusion that these fragments must block traffic by sequestering the cognate SNAREs in complexes that are unable to complete the fusion process.

These experiments also yield insights into the specificity of SEC11 in regulating secretory traffic. Both SYP121 and SYP122 are able to mediate traffic at the plasma membrane, and soluble fragments of both the SNAREs were found to block secretory traffic in wild-type or syp121 and syp122 Arabidopsis (Figure 2).
It is significant, therefore, that SEC11 rescued secretory traffic, but only so long as the native SYP121 was present (i.e., in wild-type and syp122 Arabidopsis) but not in the syp121 mutant, where only native SYP122 was present (Figure 3). Experiments with the N-terminal domain of SEC11 complement these findings, and we will return to this point shortly. Of course, SEC11 is known also to bind with SYP111 (=KNOLLE), a homolog of SYP121 and SYP122 (Assaad et al., 2001). However, SYP111 is expressed only during cytokinesis (Lauber et al., 1997), and it does not overlap functionally with SYP121 (Muller et al., 2003; Reichardt et al., 2011). Indeed, the association with SYP121, and the actions of SEC11 and SEC11\textsuperscript{D149} when stably

**Figure 10.** SEC11\textsuperscript{D149} Expression Affects Growth in Wild-Type and syp122, but Not in syp121, Arabidopsis.

(A) Three-week-old Arabidopsis expressing SEC11\textsuperscript{D149} under the control of the Dex-inducible promoter in the wild-type, syp121, and syp122 backgrounds. Images are of plants treated daily with 10 µM Dex beginning 7 d after germination.

(B) and (C) Mean ± se for shoot fresh and dry weights from the wild-type and mutant backgrounds and three independently transformed lines each, comprising >50 plants per line. Values are normalized to the untransformed wild type, and significance is indicated by letters (P < 0.05).

(D) Developmental progression of leaves from representative plants as in (A). Immunoblots using anti-myc antibodies for expression of the Dex-induced construct in each case are included. Bar = 1 cm.

(E) Mean ± se of leaf numbers from >50 plants as in (A) to (C) with significance indicated by letters (P < 0.05).
overexpressed, also raise questions about whether the effects of the sec11 (=keule) null mutation are solely the result of its impairing cell division (Assaad et al., 2001). Resolving the molecular determinants of SEC11 specificity for SYP111 and SYP121 now presents technical challenges. Like K+ channel specificity for SYP121 (Grefen et al., 2010a), SEC11 binding to the SYP121 N terminus almost certainly depends on residues adjacent to the highly conserved Phe-9 of the Qa-SNARE, but its resolution will require dissecting binding between two distinct sites of the SM protein and two different locations on the SNARE (Karnik et al., 2013b). Regardless, however, SEC11 action is clearly selective for SYP121 at the plasma membrane.

**SYP121 and SEC11 N Termini Mediate in Traffic Control at the Plasma Membrane**

Binding of SEC11 to SYP121 exhibits two distinct modes that roughly parallel those described for SM proteins in mammals and yeast (Dulubova et al., 2007; Südhof and Rothman, 2009) and associate with two binding sites on the SM protein (Karnik...
et al., 2013b). In contrast with its mammalian and yeast counterparts, however, SEC11 binding to the N terminus of SYP121 is competitive with that of VAMP721 and SNAP33, and it has been associated with a stable, quasi-open conformation of the Qa-SNARE that is unable to assemble with these cognate partners (Karnik et al., 2013b). Our findings here complement these observations with a similar divergence in the effects of the SM protein in vivo. Whereas in mammals, overexpressing Munc18-1 promotes traffic block by the cytosolic domain of the Qa-SNARE Syn1A (Khvotchev et al., 2007), we observed SEC11 overexpression to rescue traffic blocked by the SYP121DC fragment, and in a manner dependent on the native SYP121 (Figure 3). Not only do these findings underscore the specificity of SEC11 binding, but they point to a mass-action effect to promote traffic through binding with the native Qa-SNARE.

Bimolecular interactions of SEC11 are suppressed with SYP121F9A (Karnik et al., 2013b). Thus, the syp121SYP121F9A complementation provided an important test of the requirement for binding with the SYP121 N terminus. Since SEC11 was expected to bind SYP121F9A only in complex, we reasoned that the complementation might counter traffic block by SYP121AC by enhancing the stability of SYP121 in the SNARE complex. Instead, we found that secretory traffic was suppressed in plants expressing SYP121F9A and that traffic was not recovered by overexpressing SEC11 (Figure 5). We also tested secretory traffic with the mutant SM protein SEC11L128R. This mutant retains the ability to bind in complex with SYP121, VAMP721, and SNAP33, but its interaction with the Qa-SNARE N terminus is virtually lost in binary complex (Figure 6) (Karnik et al., 2013b). Expressing the SEC11L128R mutant in vivo also suppressed secretory traffic independent of SYP121AC, but in this case, the effect was evident only in the syp122 background; in other words, only if SYP121 was the principal Qa-SNARE at the plasma membrane (Figure 7).

How might these several observations be reconciled? We can start from the premise that SEC11 binding is selective for SYP121 (Figures 2 to 4; Supplemental Figures 1 and 2). Keep in mind that the studies were performed against the background of native SEC11 and, for the SYP121F9A-complemented plants, of SYP122 expression. Therefore, SEC11L128R will have competed with the native SEC11 to block traffic, but only when SYP121 dominated secretion, whereas the exchange of SYP121F9A for SYP121 eliminated all binding of the Qa-SNARE N terminus with

Figure 12. SEC11AC Expression Affects Epidermal Cell Size and Inflorescence Development.

(A) Epidermis of fully expanded leaves from 3-week-old Arabidopsis expressing SEC11AC under the control of the Dex-inducible promoter in the wild-type, syp121, and syp122 backgrounds. Images are from plants treated as in Figure 10 and were acquired at 40× objective magnification. Bar = 20 μm. (B) Mean ± se for epidermal cell size from the wild-type and mutant backgrounds and three independently transformed lines each, comprising >5 plants per line and >40 images taken randomly from leaf numbers 6 to 8 of each plant. Values are normalized to the untransformed wild type, and significance is indicated by letters (P < 0.05). (C) Closeup images showing aberrant and necrotic leaves (arrows) formed in lines expressing SEC11AC in the syp122 mutant background. (D) Images and mean ± se for inflorescence length of the lines in (A) after 8 weeks of growth. Statistical differences are indicated by letters (P < 0.01). Bar = 5 cm.
SEC11. Note, too, that SEC11 binding is associated both early in the SNARE cycle with the closed and quasi-open conformations of SYP121 and also late in the SNARE cycle with SYP121 in the SNARE complex (Karnik et al., 2013b). One simple explanation aligns with these two binding modes: it postulates that SEC11 binding to the N terminus of SYP121 is essential for “levering” the SM protein from SYP121 in the quasi-open conformation early in the SNARE cycle and is equally important for discharging SEC11 from SYP121 and the SNARE complex late in the cycle. This explanation is compatible with the binding characteristics of SEC11, SEC11-L128R, and especially with the weaker effect of the SYP121-F9A mutant in bimolecular interaction with SEC11 (Karnik et al., 2013b). It predicts that the SYP121-F9A complementation, like SYP121-AC,F9A expression (Supplemental Figure 2), suppresses SYP121- and SYP122-mediated traffic, because it sequesters the cognate SNAREs that are shared between these pathways. In other words, by preventing SEC11 binding to the N terminus at a late stage in the cycle, the cognate SNAREs become trapped in complex with SYP121 and SEC11. Of course, exit from the quasi-open conformation with SEC11 may also be suppressed on complementing the syp121 mutant with SYP121-F9A, but this action cannot explain a block of traffic via SYP122.

By contrast, because SEC11-L128R must compete with the native SEC11, its efficacy in block will be diluted, especially when traffic can also pass through the SYP122 pathway (Figure 7). This explanation accords with present models for SM interactions with several Qa-SNAREs (Rathore et al., 2010; Rizo and Südhof, 2012; Archbold et al., 2014). It is consistent, too, with recent studies of the yeast and mammalian SM proteins Sly1p (Demircioglu et al., 2014) and Munc18-1 (Christie et al., 2012), which appear to bind their cognate Qa-SNAREs concurrently via the major cleft and the N-terminal site on the SM protein. In the case of Sly1p, binding to the N-terminal site has been suggested to be important for forming a quasi-open or “loosened” conformation of the Qa-SNARE. In effect, binding between the N termini of Sly1p and its cognate Qa-SNARE Sed5p may create a molecular fulcrum that helps in prising open the Qa-SNARE conformation of the Qa-SNARE. In effect, binding between the N termini of Sly1p and its cognate Qa-SNARE Sed5p may create a molecular fulcrum that helps in prising open the Qa-SNARE conformation of the Qa-SNARE fragment (Figure 13). This pattern of block is a close counterpoint to SEC11 rescue from secretory block by the SYP121-AC and SYP122-AC fragments (Figure 3). SEC11-L128R incorporates determinants of interaction with the N terminus of SYP121, and it retains the ability to bind with SYP121 in vitro and in vivo (Figure 8). However, it lacks the C-terminal half of SEC11 that is essential to form the major SM binding cleft (Rizo and Südhof, 2012; Karnik et al., 2013b; Archbold et al., 2014). So a straightforward interpretation of its functional activity is that SEC11-L128R must bind with SYP121 at the plasma membrane, competing with the native SEC11 for the Qa-SNARE N terminus. Furthermore, this binding also must occur late in the SNARE cycle in order to suppress disassembly of the SNARE complex and sequester the cognate SNARE proteins. As with the syp121:syp121-F9A results, this prediction is the simplest that explains the dominance of SEC11-L128R block when expressed on its own in wild-type Arabidopsis that maintains the SYP122-dependent pathway: SEC11-L128R may also bind in binary complex with SYP121, but such binding cannot explain its action in blocking traffic in the wild-type plants. The prediction also provides a plausible explanation for traffic rescue in the syp121 mutant when coexpressed with SYP121-AC (Figure 9). In the latter case, the Qa-SNARE fragment will have been the only ligand available for SEC11-L128R, and its binding can be understood to sequester SYP121-AC from interaction with the cognate SNAREs, especially if the latter were enhanced in the absence of the native SYP121. Indeed, this interpretation finds support in our experiments showing that SEC11-L128R failed to rescue traffic in the syp121 mutant background when coexpressed with SYP122-AC and SYP121-AC,F9A (Supplemental Figures 3 and 4). Since the SM protein does not bind either (Supplemental Figure 1) (Karnik et al., 2013b), its fragment will not have sequestered the Qa-SNARE fragment and therefore failed to rescue traffic in the syp121 mutant background.

Finally, the characteristics of traffic block by SEC11-L128R were mirrored in the growth patterns of stable lines expressing this construct. Following Dex treatment to induce SEC11-L128R expression, we observed reduced rosette size, leaf number, and fresh and dry weights in the wild-type and syp122 backgrounds, but not in the syp121 mutant background, when compared with the corresponding controls (Figures 10 and 12). Complementary results were observed on stably overexpressing the wild-type SEC11 (Figure 11). In this case, vegetative growth was consistently accelerated in the wild-type and syp122 backgrounds, and again, no effect on growth was recovered when overexpressing SEC11 in the syp121 mutant. Translating effects on secretory traffic from the molecular and cellular levels to vegetative growth is inevitably fraught with difficulty. Nonetheless, our analysis indicated that the consequences, notably of traffic block by SEC11-L128R, were allied to differences in mean cell size, implying an action mediated through the expansion of the tissue rather than through an interference in cell division, such as might have entailed its interaction with SYP111 (Assaad et al., 2001; Sollner et al., 2002). These observations also call into question the earlier premise that the keule (sec11) mutant phenotype is solely the result of impaired cell division (Assaad et al., 2001).

A Unifying Model for SEC11 Action at the Plasma Membrane

Figure 13 presents a simple model that incorporates all these findings and the background of our knowledge relating to SEC11 and SYP121 in vesicle traffic. SYP121 assembles with the Qbc-SNARE SNAP33 and the R-SNARE VAMP721 (also the nearly identical VAMP722) to form a functional SNARE complex. The
Figure 13. Model of SEC11 Interaction with SYP121 and Its Impact on Secretory Traffic.

Panels 1 to 4 encapsulate the sequence of SNARE assembly, vesicle fusion, and their block by SYP121^{DC}, SYP122^{DC}, and SEC11^{L128R}. Panels 5 to 8 summarize steps in SNARE complex disassembly and its block by SEC11^{D149} and SYP121^{F9A}. Additional SNARE components, including SNAP33, are omitted for clarity.

Panel 1. SYP121-mediated traffic is greatly accelerated by binding between Phe-9 of SYP121 and Leu-128 of SEC11, which facilitates opening of the Qa-SNARE (a) and its assembly in complex with cognate SNARE proteins (b). SEC11 then stabilizes the SNARE complex (c) during vesicle fusion.

Panel 2. Traffic proceeds via SYP122 and, to a much lesser extent, via SYP121, independent of SEC11 through opening of the Qa-SNARE (d), which enables SNARE complex assembly (e). Note that SYP121 and SYP122 share a common pool of vesicles as well as the R-SNAREs VAMP721 and VAMP722. Note, too, that SEC11 may bind with SYP121^{F9A} in the SNARE complex, effectively crossing from (e) to (c).

Panel 3. The Qa-SNARE fragments SYP121^{DC} and SYP122^{DC} are able to compete for cognate SNAREs (f), including VAMP721 and VAMP722, thereby preventing the assembly of functional SNARE complexes through both pathways illustrated in panels 1 and 2 and suppressing vesicle fusion at the membrane (here shown only in relation to panel 2).

Panel 4. SEC11^{L128R} is unable to bind and promote SYP121 opening (g). It competes with traffic dependent on SEC11 (panel 1), but not with traffic independent of SEC11 (panel 2), and therefore affects vesicle fusion only when SYP122-dependent traffic is eliminated in the syp122 mutant.

Panel 5. After vesicle fusion, disassembly of the SYP121 SNARE complex is greatly accelerated through binding between Phe-9 of SYP121 and Leu-128 of SEC11, which facilitates opening of the complex (h) to release SEC11 (i) and, following the action of the NSF ATPase and associated binding factors (omitted for clarity) (Lipka et al., 2007; Bassham and Blatt, 2008), the cognate SNAREs.

Panel 6. Disassembly of SNARE complexes without SEC11 also proceeds through binding with the NSF ATPase and associated binding factors (omitted for clarity) (Lipka et al., 2007; Bassham and Blatt, 2008).

Panel 7. The SNARE complex with SYP121^{F9A} is unable to bind Leu-128 of SEC11, thereby preventing its release and trapping the Qa-SNARE and its cognate partners in complex.

Panel 8. SEC11^{L149} competes for binding with Phe-9 of SYP121 and thereby prevents the conformational changes required for full-length SEC11 to release the complex. As a consequence, SEC11^{L149} traps SYP121 and its cognate SNARE partners in complex.
major cleft of SEC11 binds and stabilizes this complex; although not essential for the final stages of vesicle fusion, it greatly accelerates the process. The major cleft of SEC11 also binds SYP121 in the closed conformation. This binary interaction is stabilized by binding between the SYP121 N terminus, which includes Phe-9, and the minor cleft in SEC11 incorporating Leu-128, and it requires the two binding sites of SEC11 to reside on a single polypeptide to lever SYP121 into a quasi-open but fusion-incompetent conformation (Karnik et al., 2013b).

Four conclusions can now be drawn from our observations as outlined above. First, overexpressing SEC11 with SYP121-ΔC rescues traffic, unlike the mammalian model (Khvotchev et al., 2007), and it relies on the native SYP121 (Figure 3). These results imply a mass-action effect through binding with the SNARE complex to promote traffic. Second, we understand the action of SEC11ΔC (Figure 7) to arise primarily through competition with the wild-type SEC11 for binary association with the major cleft of the SM protein while still permitting the wild-type SEC11 to bind to the SYP121 N terminus. Thus, by binding with two SM polypeptides, it effectively "locks" the closed mode of SYP121 and suppresses traffic when SYP121 is the dominant QA-SNARE. Third, and by contrast, the SYP121-ΔPA mutant does not compete for SEC11 and must sequester the common pool of cognate SNAREs in order to affect both SYP121- and SYP122-dependent traffic (Figure 5). It follows that SEC11 binding to the SNARE complex, and more importantly its release following assembly and, presumably, vesicle fusion, must be affected by the loss of SEC11 interaction with the SYP121 N terminus. Finally, we interpret traffic block by SEC11Δ149 to arise similarly through interaction with SYP121 in the SNARE complex. As illustrated, SEC11Δ149 competes with the bound SEC11 for the SYP121 N terminus and thereby prevents its levering from, and opening of, the SNARE complex. However, it is equally plausible, for example, that SEC11Δ149 binding to the SNARE complex precludes SEC11 binding as well as locking up the SNAREs in complex. Resolving the detailed mechanics of SEC11 binding that drives SYP121 conformations between the closed, quasi-open, fusion-competent, and SNARE complex forms will now benefit from kinetic and binding affinity analysis. Regardless of the details, our studies lead us to the inevitable conclusion that binding of SEC11 is selective for SYP121 and that its association with the SYP121 N terminus is important not only for the conformational transition of SYP121 to a fusion-competent form but also later for the recycling of SYP121 and its cognate SNAREs from the SNARE complex.

METHODS

Plasmids and Recombinant Proteins

Clones for the expression of tagged recombinant proteins were constructed as described previously (Honsbein et al., 2009; Karnik et al., 2013b). Open reading frames for SYP121, SYP122, and SEC11 were amplified with gene-specific primers to include Gateway attachment sites, and primers for mutagenesis were designed using SDM-Assist software (Karnik et al., 2013a) to introduce the desired mutations along with silent mutations that could be used to identify the mutants in restriction digests. Supplemental Table 1 lists the primers used in cloning and site-directed mutagenesis.

Secretion analysis made use of the tricistronic vector pTeqG-2in1-CC (Karnik et al., 2013b), which incorporates four 35S-driven expression cassettes, two carrying the coding sequences for secYFP and for GFP-

HDEL. The remaining two cassettes provide Gateway recombination sites (attR3-lacz-Z-attR2 and attR1-ChloramphenicolR, ccdB-attR4) and a C-terminal tag, either 3xHA or myc (Grefen and Blatt, 2012a). Assays were performed with SEC11 constructs incorporating the C-terminal 3xHA tag and the QA-SNARE constructs incorporating the C-terminal myc tag (Karnik et al., 2013b). Protein-protein interaction studies in vivo were performed by rBIFC using the tricistronic vector pBIFC-2in1-NC (Grefen and Blatt, 2012a) with SEC11 constructs nYFP-tagged N terminally and cYFP-tagged SYP121ΔC tagged C terminally.

SYP121ΔC, SYP122ΔC, and SYP121ΔC-PA for pull-down assays were PCR amplified using overlap extension to add 3′ NcoI sites, 5′ Flag (DYKDDDDK) tag-StrepII (WSHPQFEK) tag, and HindIII sites. The genes were introduced into a modified pET28-Duet vector (Invitrogen) for transformation and expression in Escherichia coli. Constructs for SUS assays were prepared by cloning the coding sequences for SEC11 and SEC11Δ149 in the Gateway-compatible pMetOYC-Dest bait vector below; sequences for SYP121 and SYP122 were cloned in the pNXS5-Dest prey vector with a 2xHA tag for verifying expression (Grefen and Blatt, 2012b).

Constructs for stable transformation of Arabidopsis thaliana were generated using the Gateway-compatible vectors pDXI-cmyc-Dest and pUBQ10-GFP-Dest (Grefen et al., 2010b) following LR reactions.

pDXIsV-Dest Vector

The RFP expression cassette of the binary vector pBIFC-BB (Grefen and Blatt, 2012a) was replaced by a codon-optimized (Arabidopsis codon usage), gene-synthesized phosphinothricin acetyltransferase (Barstar resistance) cassette (flanked by the nos promoter and terminator), yielding vector pBBv. This vector also contains 5′ of the resistance cassette a couple of unique blunt-end restriction sites (AfeI, PmlI, Eco35I, and SnaBI) to facilitate the insertion of other expression cassettes. pDXIsV-Y-Dest was assembled using different gene-synthesized expression cassettes. First, a 35S:GVG-T35S construct was gene synthesized (GVG = GAL4 DNA binding domain, VP16 transactivation domain) and cloned into the Gateway-compatible pMetOYC-Dest bait vector (below); sequences for SYP121 and SYP122 were cloned in the pNXS5-Dest prey vector with a 2xHA tag for verifying expression (Grefen and Blatt, 2012b).

SUS and CytoSUS Assays

The CytoSUS vector pMetOYC-Dest was created using a combination of gene synthesis and classical cloning. A fragment of the met25 promoter, starting at the internal PmlI site, followed by the 108 bp of OSt44 without stop codon and the first 946 bp of the Gateway recombination cassette were gene-synthesized (Genscript). This construct as well as pMetYC-Dest (Grefen et al., 2009) were cleaved using restriction endonucleases PmlI and BssHII (New England Biolabs), and the gene-synthesized expression cassette 6xUAS-35S:GateWaymyc-T35S was inserted to create pDXI-Cmyc. This "side product" can be used as a binary Dex-inducible, Gateway-compatible vector. Cutting a 35S:secYFP-T35S construct from pBIFC-BB-secYFP (Karnik et al. 2013b) via SmaI/SnaBI and inserting it into pDXI-Cmyc via SnaBI yielded the final vector pDXIsV-Y-Dest.

Pull-Down Assays and Immunoblotting

Protein expression was induced in E. coli BL21 DE3 cells (Life Technologies) with 1 mM isopropyl β-D-1-thiogalactoside for 4 h, and the proteins were purified by glutathione- and streptactin-coupled Sepharose affinity chromatography and the SYP121ΔC was immobilized as bait. Pull-downs included GST alone as a background control, and the ratios of proteins
bound to bait were normalized against common standards for comparison as before (Kamik et al., 2013b).

For immunoblot analysis of plant tissues, leaves were excised and flash-frozen in liquid N$_2$. Frozen tissue was ground in equal volumes (w/v) of homogenization buffer containing 500 mM sucrose, 10% glycerol, 20 mM EDTA, 20 mM EGTA, Protease Inhibitor (Roche), 10 mM ascorbic acid, 5 mM DTT, and 50 mM Tris-HCl, pH 7.4, and centrifuged at 13,000 g and 4°C for 30 min to pellet debris. Supernatant was diluted 1:1 in 2× Laemmli buffer containing 2.5% 2-mercaptoethanol, heated to 95°C for 10 min, and separated by SDS-PAGE. Proteins from yeast were prepared as described previously (Grefen et al., 2010a). All samples were diluted in loading buffer to 5 μg protein/mL per lane. Porcine S-stained Rubisco bands were used as loading standards for plant samples. ImmunobLOTS (Kamik et al., 2013b) used primary αSYP121 (1:40,000 dilution), αmyc (1:5000 dilution; Abcam), αHA (1:10,000 dilution; Roche), αSEC11 (1:2000 dilution; Assaad et al., 2001), αGFP (1:10,000 dilution; Abcam), and αVP16 (Roche) antibodies and goat anti-rabbit secondary antibody. Cross-reacting bands were visualized using WestFemto SuperSignal chemiluminescence detection (Thermo Pierce) and imaged by scanning at 1200-dpi resolution. In some experiments, membranes were reprobed after stripping in 100 mM b-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, at 70°C for 45 min.

Plant Growth, Transformation, and Analysis

Seedlings of Arabidopsis Columbia-0 (wild type) or syp122-1 (syp121) mutants (Honsbein et al., 2009) were transformed transiently as described previously (Grefen et al., 2010b). Stable Arabidopsis lines were selected by growth on soil with phosphinothricin (Basta; Bayer CropScience). Homozygous T4 lines were verified as described previously (Grefen et al., 2010b). All samples were diluted in loading buffer to 5 μg protein/mL per lane. Porcine S-stained Rubisco bands were used as loading standards for plant samples. ImmunobLOTS (Kamik et al., 2013b) used primary αSYP121 (1:40,000 dilution), αmyc (1:5000 dilution; Abcam), αHA (1:10,000 dilution; Roche), αSEC11 (1:2000 dilution; Assaad et al., 2001), αGFP (1:10,000 dilution; Abcam), and αVP16 (Roche) antibodies and goat anti-rabbit secondary antibody. Cross-reacting bands were visualized using WestFemto SuperSignal chemiluminescence detection (Thermo Pierce) and imaged by scanning at 1200-dpi resolution. In some experiments, membranes were reprobed after stripping in 100 mM b-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7, at 70°C for 45 min.

Confocal Microscopy

Roots of Arabidopsis seeds were imaged after 3 to 4 d of cocultivation with Agrobacterium tumefaciens using a Zeiss LSM510 META confocal microscope. Images were routinely collected as Z-stacks using Planapo 20x/0.75NA objective (Zeiss).

Statistical Analysis

All data are reported as means ± se of n independent measurements. Statistical analysis was performed using Sigmmaplot 11.2 (Systat, SPSS). Significance was determined using the Holm-Sidak test and ANOVA.

Supplemental Data

Supplemental Figure 1. SEC11 Interacts Selectively with SYP121 over SYP122.

Supplemental Figure 2. Phe$_9$ of SYP121AC Is Not Essential for Secretory Block.

Supplemental Figure 3. The SEC11:D149 Fragment Fails to Rescue Secretory Traffic Block by SYP122AC in the syp121 Mutant.

Supplemental Figure 5. Time-Course and Dexamethasone Concentration Dependence of SEC11:D149 Expression.

Supplemental Figure 6. Normal Growth of Lines Carrying the Dexamethasone-Inducible SEC11:D149 Construct.

Supplemental Table 1. Primer Sequences Used for Cloning SYP121AC, SYP122AC, SEC11, SEC11:D149, VAMP721, and SNAP33, for Site-Directed Mutagenesis of SEC11 and SYP121, and PCR for StrepII-Tagged SYP121AC.

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Author Contributions

R.K. designed the experimental strategies with M.R.B. R.K. and B.Z. carried out the SUS assays. C.G. and R.K. designed constructs and vectors. R.K. and M.R.B. analyzed the data and wrote the article with support from C.A. B.Z., C.G., S.W., and pull-downs with S.W. and immunoblot assays with C.A. B.Z., C.G., S.W., and C.R. designed the experimental strategies with M.R.B. R.K. and B.Z. carried out the SUS assays. C.G. and R.K. established the mOST4-anchored SEC11F9A chimeric construct. M.R.B. provided and pull-downs with S.W. and immunoblot assays with C.A. B.Z., C.G., S.W., and M.R.B. analyzed the data and wrote the article with support from C.G.

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Binding of SEC11 Indicates Its Role in SNARE Recycling after Vesicle Fusion and Identifies Two Pathways for Vesicular Traffic to the Plasma Membrane
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