SNAREs SYP121 and SYP122 Mediate the Secretion of Distinct Cargo Subsets[1][OPEN]

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SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins drive vesicle fusion and contribute to homoeostasis, pathogen defense, cell expansion, and growth in plants. In Arabidopsis (*Arabidopsis thaliana*), two homologous Qa-SNAREs, SYNTAXIN OF PLANTS121 (SYP121) and SYP122, facilitate the majority of secretory traffic to the plasma membrane, and the single mutants are indistinguishable from wild-type plants in the absence of stress, implying a redundancy in their functions. Nonetheless, several studies suggest differences among the secretory cargo of these SNAREs. To address this issue, we conducted an analysis of the proteins secreted by cultured wild-type, *syp121*, and *syp122* mutant Arabidopsis seedlings. Here, we report that a number of cargo proteins were associated differentially with traffic mediated by SYP121 and SYP122. The data also indicated important overlaps between the SNAREs. Therefore, we conclude that the two Qa-SNAREs mediate distinct but complementary secretory pathways during vegetative plant growth.

SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins drive vesicle fusion and are critical for the movement of membrane and cargo proteins within the cell. SNAREs contribute to cell growth, homoeostasis, pathogen defense, and development in plants (Lipka et al., 2007; Bassham and Blatt, 2008). At the plant cell plasma membrane, vesicle fusion serves to deliver cell wall material and other components for secretion, enabling wall remodeling for cell expansion. Cargo proteins destined for secretion across the plasma membrane generally are first transported from the endoplasmic reticulum (ER) to the Golgi, where they are actively sorted and packaged for export to the plasma membrane. Fusion events with each transit between membranes depend on vesicle fusion driven by SNARE proteins.

SNAREs are classified mainly into either R- or Q- (Qa, Qb, and Qc) SNAREs based upon the presence of a conserved Arg or Gln residue at the center of the SNARE domain. R-SNAREs typically are localized to the vesicle membrane and the Q-SNAREs to the target membrane for fusion (Fasshauer et al., 1998; Bock et al., 2001; Bassham and Blatt, 2008). These proteins, providing one domain of each of these four subgroups, assemble in complex to bring vesicle and target membrane surfaces together for fusion. In Arabidopsis (*Arabidopsis thaliana*), several Qa-SNAREs, which also are known as syntaxins of plants (SYPs), reside at the plasma membrane and mediate in the final stages of secretion (Bassham and Blatt, 2008). Of these Qa-SNAREs, substantial evidence from genetic and physiological studies have shown that three, SYP121 (also known as SYNTAXIN RELATED PROTEIN1/PENETRATION1 [PEN1]), SYP122, and SYP132, are expressed widely throughout the vegetative plant and are responsible for much of the secretory activity (Uemura et al., 2004; Sanderfoot, 2007). The single mutants *syp121* and *syp122* show phenotypes only under abiotic and biotic stress, whereas the *syp121syp122* double mutant is severely impaired in its growth (Collins et al., 2003; Assaad et al., 2004; Zhang et al., 2007; Karnik et al., 2017). SYP132 typically shows quite low but constitutive expression when compared with SYP121 and SYP122 (Enami et al., 2009; Karnik et al., 2017), but the single *syp132* mutation is lethal. Thus, the functions of SYP121 and SYP122 appear to be

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partially redundant and carry the bulk of traffic to the plasma membrane.

Despite their functional similarities, SYP121 and SYP122 are distinguishable on several accounts. Studies have shown that K^+ channels bind selectively to SYP121 with the effect of promoting channel gating and secretory vesicle fusion (Honsbein et al., 2009; Grefen et al., 2010b; Karnik et al., 2017). This Qa-SNARE has been shown to be important for stomatal movements (Leyman et al., 1999; Eisenach et al., 2012), and it has defined resistance against barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) as the *pen1* mutant (Collins et al., 2003). SYP121 also binds preferentially with SEC11 (Karnik et al., 2013, 2015), a member of the Secretion1/Mammalian uncoordinated-18 protein family that facilitates and regulates SNARE assembly. By contrast, SYP122 has been associated with cell wall deposition during early development (Assaad et al., 2004), bacterial pathogen-related defense (Nühse et al., 2003), and salicylic acid and jasmonic acid signaling (Assaad et al., 2004; Zhang et al., 2008). Rehman et al. (2008) and Ul-Rehman et al. (2011) have indicated potential differences between SYP121 and SYP122 based on heterologous associations with Rab GTPases in tobacco (*Nicotiana tabacum*). These studies show that SYP121 and SYP122 functions are clearly separable physiologically, but they offer few clues to the underlying basis for these differences. Notably, their contributions to the traffic and secretion of different cargo proteins have not been explored in detail.

If SYP121 and SYP122 define two parallel pathways for traffic to the plasma membrane, the different cargo proteins transported by these pathways are likely to underpin many of the phenotypic differences associated with the two Qa-SNAREs and also may help explain their functional redundancies in the absence of stress. To address this question, we explored the cargo proteins carried by the two pathways using mass spectrometry (MS) analysis using the *syp121* and *syp122* mutants. Many of the secreted proteins mediated by the two Qa-SNAREs belonged to common protein families, which may account for aspects of their functional redundancy. Significant subsets of proteins also appeared to be exported via only one or the other of the two pathways identified with the Qa-SNAREs. These findings align with, and thus may help explain, the phenotypic characteristics of the two mutants identified to date.

RESULTS

Arabidopsis Secretome Isolation and Protein Identification

To capture and identify secreted proteins from Arabidopsis, we established the workflow outlined in Figure 1A. We grew seedlings of the mutants, *syp121* and *syp122*, and of wild-type Arabidopsis in Murashige and Skoog liquid medium, collected secreted proteins from the medium, and then separated them by SDS-PAGE. We then excised, washed, trypsin digested, and analyzed protein bands by MS. We used a qualitative

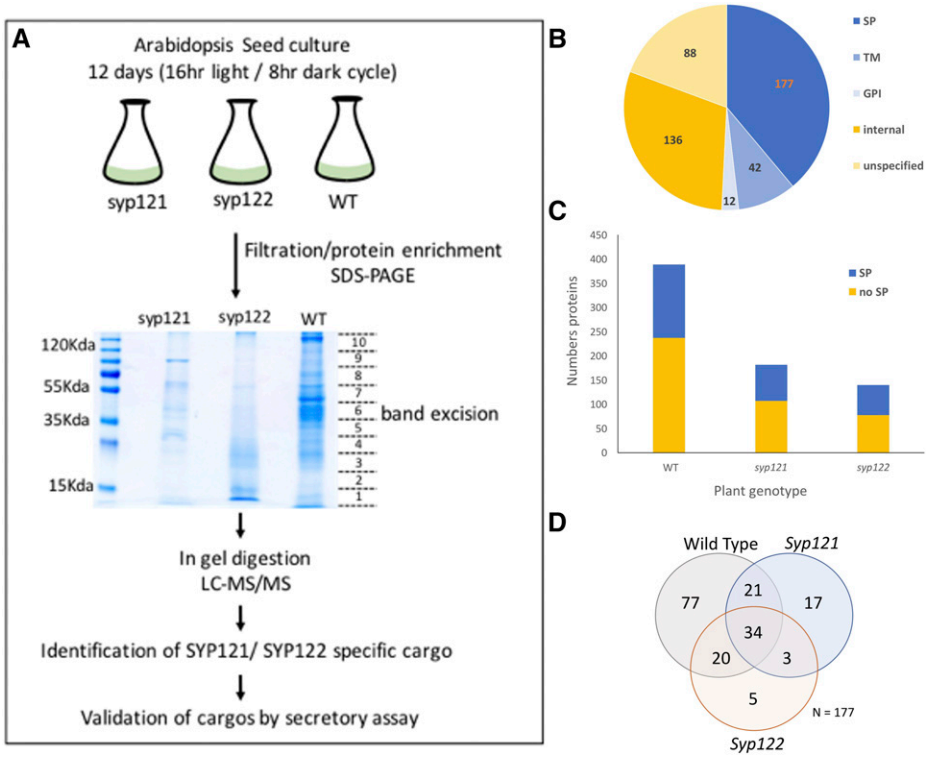

Figure 1. Identification of SYP121- and SYP122-specific cargo. A, Workflow for Arabidopsis secretome isolation. B, Distribution of total proteins with signal peptides (SP), transmembrane domains (TM), glycosylphosphatidylinositol (GPI) anchors (GPI), and those classified as internal proteins (internal) or proteins with unknown locations (unspecified). C, Distribution of proteins with and without signal peptides identified in extracts from *syp121* and *syp122* mutant lines and wild-type (WT) Arabidopsis lines. D, Venn diagram illustrating the overlap of secreted proteins identified from *syp121*, *syp122*, and wild-type Arabidopsis lines.

proteomic analysis based on proteins observed with a minimum of two peptides in at least one of three independent biological replicates. Using these criteria, we identified 455 proteins in total from wild-type and mutant plants (Supplemental Table S1).

We assessed known and predicted subcellular location(s) for all 455 identified proteins based on UNIPROT annotations (UNIPROT consortium; Fig. 1B). Conventionally secreted proteins were defined as containing a signal peptide, lacking membrane anchor, and lacking an ER retention signal. Thus, we classified 177 (38%) proteins as conventionally secreted (Supplemental Table S2). Forty-two proteins that contained at least one transmembrane domain were classified as integral components of membranes, and approximately half of these also contained a signal peptide. Twelve proteins contained a GPI anchor in addition to a signal peptide; five of these were considered to be secreted based on additional UNIPROT annotations. Only 49 proteins were annotated as cytoplasmic, and we combined these with 87 proteins that were annotated by localization to organelles or the endoplasm to classify 136 (30%) proteins as internal. Eighty-eight proteins without a specific UNIPROT assigned location were classified as undefined.

Identification of SYP121- and SYP122-Specific Cargo

Of the total 455 identified proteins, 398 were identified from the wild-type plants, while a partial block of protein secretion was observed in the mutants, from which we identified 182 total proteins from *syp121* and 140 total proteins from *syp122* plants (Fig. 1C; Supplemental Table S2). Of the 177 conventionally secreted proteins, 152 were identified from wild-type plants and 75 and 62 from *syp121* and *syp122*, respectively. We concluded that reduced protein secretion in the mutant plants was a result of absence of the specific SNARE. We cannot rule out that the absence of a protein may simply reflect that its abundance was reduced below the detection threshold of the mass spectrometer. This observed partial secretory block in the mutants challenges past ideas of nearly complete redundancy of the SYP121 and SYP122 SNARE proteins (Lipka et al., 2007; Kwon et al., 2008), suggesting that both SNAREs are functional in the normal growth conditions.

We proposed that conventionally secreted proteins identified in the *syp122* mutant and wild-type samples, but absent in *syp121*, would be secreted preferentially via the SYP121-mediated secretory pathway and vice versa. To identify SYP-specific cargo proteins, we compared the proteins identified from *syp121*, *syp122*, and wild-type plants (Fig. 1D; Supplemental Table S2). The analysis yielded 20 SYP121-specific and 21 SYP122-specific cargo proteins (Table 1). A proportion of secreted proteins, constituting 19% (34 of 177), was shared across all lines, whereas 43% (77 of 177) proteins were absent in both mutants (Fig. 1D). The proteins that were identified in all three lines could be secreted through the SYP132-mediated pathway, or SYP121 and SYP122 could compensate for the loss of function in the single mutant lines, or a combination of both possibilities could occur. However, the 77 putative cargo proteins identified from wild-type plants but absent in both mutants were likely SYP132 independent, which suggests that SYP121 and SYP122 may work together in a single fusion event for the secretion of these cargo proteins. Notably, we identified low numbers of proteins unique to the *syp* mutants (25 in total), suggesting that the assay is sensible, as the presence of many proteins unique to the mutants would imply the induction of secretion caused by the absence of one or the other of the two SNAREs.

Functional Specificities among SYP121- and SYP121-Associated Cargo Proteins

To determine if SYP121- or SYP122-specific cargo proteins play a role in a specific function, we used Gene Ontology classifications and functional annotations from UNIPROT (Table 1; Supplemental Table S3). Of the 152 secreted proteins identified from wild-type plants, we observed that peroxidases, proteases, and glycosyl hydrolases all were well represented, as were several protease inhibitors; these included proteins likely to be trafficked by specific routes: for SYP121 (20 proteins), SYP122 (21 proteins), or SYP132 (34 proteins; Table 2). Major functional categories were carbohydrate metabolism, cell wall modification, and lipid metabolism. SYP121-specific proteins were involved predominantly in lipid metabolism, most notable being GDSL lipases (GDSL refers to the consensus amino acid sequence of Gly, Asp, Ser, and Leu around the active site Ser), and cargos associated with oxidative stress responses and protein folding. Several protease inhibitors were identified mainly as SYP122-specific cargo, which also had more cell wall-associated proteins, protease inhibitors, and seed storage proteins.

Validation of SNARE-Specific Cargo Proteins

To test the findings of our proteomic analysis, we analyzed a subset of cargo proteins to determine if their secretion was mediated by SYP121 and SYP122. We performed secretion assays in Arabidopsis roots after transient transformation to express the fluorescently tagged cargo proteins (Honsbein et al., 2009; Grefen et al., 2010b; Zhang et al., 2017). We tested Protein Disulfide Isomerase-Like1 (PDIL1; AT1G21750), GDSL (AT1G28660), and Pectin Methylesterase Inhibitor Protein (PMEI; AT2G26440) as potential candidates associated with traffic mediated by SYP121 and BETA-GLUCOSIDASE34 (BGLU34; AT1G47600) as mediated by SYP122. We also tested the traffic of MERISTEM-5 (MERI-5; AT4G30270) as a cargo identified in wild-type lines only. The coding sequences of these proteins were used to generate mCherry fusions downstream of the 35S promoter. We incorporated each construct in a

Table 1. *Proteins identified as SYP121- or SYP122-specific cargo proteins*

Locus	Protein Name	Identified in	Secretion Mediated by	Function
AT1G76160	SKU5, similar5	Wild type, *syp121*	SYP122	Cell wall
AT5G26280	TRAF-like family protein	Wild type, *syp121*	SYP122	Other
AT1G30870	Peroxidase superfamily protein	Wild type, *syp121*	SYP122	Oxidative stress
AT5G10560	Glycosyl hydrolase family protein	Wild type, *syp121*	SYP122	Carbohydrate metabolism
AT1G54000	GDSL-like lipase	Wild type, *syp121*	SYP122	Lipid metabolism
AT4G16500	Cystatin/monellin superfamily protein	Wild type, *syp121*	SYP122	Protease inhibitor
AT5G20950	Glycosyl hydrolase family protein	Wild type, *syp121*	SYP122	Carbohydrate metabolism
AT4G22470	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	Wild type, *syp121*	SYP122	Protease inhibitor
AT1G29090	Cys proteinase superfamily protein	Wild type, *syp121*	SYP122	Protease
AT4G04460	Saposin-like aspartyl protease family protein	Wild type, *syp121*	SYP122	Protease
AT1G28290	AGP31, arabinogalactan protein31	Wild type, *syp121*	SYP122	Cell wall
AT4G28520	CRU3, cruciferin3	Wild type, *syp121*	SYP122	Seed storage
AT5G44120	CRU1, RmlC-like cupin superfamily protein	Wild type, *syp121*	SYP122	Seed storage
AT1G47600	BGLU34, β-glucosidase34	Wild type, *syp121*	SYP122	Carbohydrate metabolism
AT5G34940	GUS3, glucuronidase3	Wild type, syp121	SYP122	Other
AT1G09750	Eukaryotic aspartyl protease family protein	Wild type, *syp121*	SYP122	Protease
AT3G54400	Eukaryotic aspartyl protease family protein	Wild type, *syp121*	SYP122	Protease
AT1G30700	FAD-binding berberine family protein	Wild type, *syp121*	SYP122	Cell wall
AT1G30720	FAD-binding berberine family protein	Wild type, *syp121*	SYP122	Cell wall
AT4G20860	FAD-binding berberine family protein	Wild type, *syp121*	SYP122	Cell wall
AT1G17860	Kunitz family trypsin and protease inhibitor protein	Wild type, *syp121*	SYP122	Protease inhibitor
AT2G10940	Seed storage 2S albumin superfamily protein	Wild type, *syp122*	SYP121	Seed storage
AT4G22485	Seed storage 2S albumin superfamily protein c	Wild type, *syp122*	SYP121	Seed storage
AT4G22513	Seed storage 2S albumin superfamily protein c	Wild type, *syp122*	SYP121	Seed storage
AT5G07360	Amidase family protein	Wild type, *syp122*	SYP121	Protease
AT1G14540	Peroxidase superfamily protein	Wild type, *syp122*	SYP121	Oxidative stress
AT5G22410	RHS18, root hair specific18	Wild type, *syp122*	SYP121	Oxidative stress
AT4G23560	GH9B15, glycosyl hydrolase9B15	Wild type, *syp122*	SYP121	Carbohydrate metabolism
AT1G77510	PDI6, PDIL1-2	Wild type, *syp122*	SYP121	Protein folding
AT3G07320	*O*-Glycosyl hydrolases family17 protein	Wild type, *syp122*	SYP121	Carbohydrate metabolism

(*Table continues on following page.*)

Table 1. (*Continued from previous page.*)

Locus	Protein Name	Identified in	Secretion Mediated by	Function
AT1G52050	Man-binding lectin superfamily protein	Wild type, *syp122*	SYP121	Cell wall
AT5G19890	Peroxidase superfamily protein	Wild type, *syp122*	SYP121	Oxidative stress
AT1G21750	PDI5, PDIL1-1	Wild type, *syp122*	SYP121	Protein folding
AT3G45310	Cys proteinase superfamily protein	Wild type, *syp122*	SYP121	Protease
AT1G28660	GDSL-like lipase	Wild type, *syp122*	SYP121	Lipid metabolism
AT4G25810	XTR6, xyloglucan endotransglycosylase6	Wild type, *syp122*	SYP121	Cell wall
AT2G28100	FUC1, α-L-fucosidase1	Wild type, *syp122*	SYP121	Carbohydrate metabolism
AT2G26440	Plant invertase/pectin methylesterase inhibitor superfamily	Wild type, *syp122*	SYP121	Cell wall
AT3G45010	SCPl48, Ser carboxypeptidase-like48	Wild type, *syp122*	SYP121	Protease
AT3G03640	BGLU25, β-glucosidase25	Wild type, *syp122*	SYP121	Carbohydrate metabolism
AT1G28670	ARAB-1, GDSL-like lipase	Wild type, *syp122*	SYP121	Lipid metabolism

tricistronic vector with the ER marker GFP fused to the C-terminal amino acid motif HDEL (GFP-HDEL) as a marker for transformation and for ratiometric analysis.

Figure 2A shows one representative candidate each for the SYP121- and SYP122-specific cargo subsets, and the complete analysis is summarized in Figure 2B. Expressing PDIL1-mCherry in the *syp121* mutant showed increased retention in the ER, as indicated by the elevated mCherry-GFP ratio. By contrast, PDIL1-mCherry traffic was much lower in the *syp122* mutant than in the *syp121* mutant but still significantly higher ($P \leq 0.05$) than observed in wild-type seedlings, as measured by the low mCherry-GFP ratio. These results are consistent with the expectation that the traffic of PDIL1 is specific to SYP121 and not SYP122. Expressing BGLU34-mCherry in *syp122* roots showed increased retention, while mCherry fluorescence in *syp121* and wild-type seedlings was similar, indicating that the secretion of BGLU34 depends on the presence of SYP122. As expected, the traffic of MERI-5 was observed in both mutants comparable with that in wild-type seedlings, indicating that its secretion is not sensitive to the loss of one of either of these SNAREs.

DISCUSSION

The SNAREs SYP121 and SYP122 are closely related and arose from a gene duplication event that facilitated subsequent functional specializations (Dacks and Doolittle, 2002; Sanderfoot, 2007; Pajonk et al., 2008). Previous studies demonstrated functional differences

Table 2. *Functional analysis and comparison of secreted proteins between likely cargo routes*

All 152 secreted proteins identified from wild-type samples were assigned to one of four potential trafficking routes. Broad functional categories were assigned based on Gene Ontology terms.

Category	SYP121	SYP122	Both SYP121 and SYP122	SYP132
Cell wall associated	3 (15%)	5 (24%)	18 (23%)	3 (9%)
Carbohydrate metabolism	4 (20%)	3 (14%)	11(14%)	5 (15%)
Lipid metabolism	5 (25%)	1 (5%)	17 (22%)	0
Defense associated	0	0	1 (1%)	1 (3%)
Oxidative stress response	3 (15%)	1 (5%)	3 (4%)	6 (18%)
Proteases, peptidase	3 (15%)	4(19%)	6 (8%)	10 (29%)
Protease inhibitor	0	3 (14%)	1 (1%)	1 (3%)
Seed storage	0	2 (10%)	0	1 (3%)
Protein folding	2 (10%)	0	0	0
Miscellaneous	0	2 (10%)	20 (26%)	7 (21%)
Total proteins	20	21	77	34

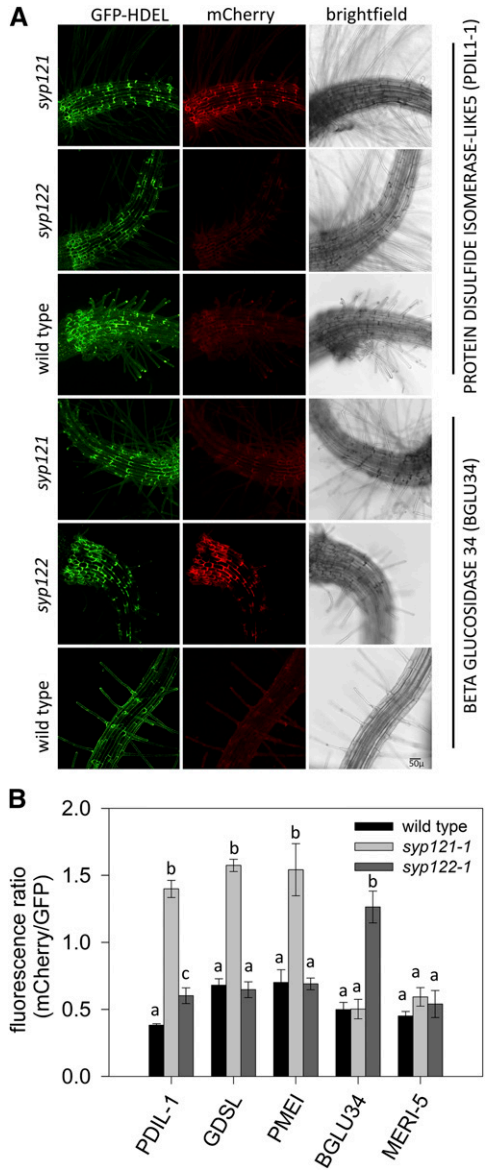

Figure 2. Validation of SNARE-specific cargos by secretory traffic assay. A, Retention and secretion of PDIL1-1-mCherry and BGLU34-mCherry in *syp121*, *syp122*, and wild-type roots. Brighter mCherry fluorescence indicates retention within the cell. GFP-HDEL fluorescence localizes to the ER. Bar = 50 μm. B, mCherry:GFP-HDEL fluorescence ratios as means ± SE of more than three independent experiments for each construct and Arabidopsis line. Fluorescence ratios were calculated as the mean tissue fluorescence determined from three-dimensional projections of root images after correcting for the background fluorescence recorded similarly from untransformed seedlings of the same age. Significant differences across lines within each construct are indicated by different letters at $P \leq 0.05$.

between these SNAREs under stress; otherwise, only the double *syp121syp122* mutant shows impaired growth (Lukowitz et al., 1996; Collins et al., 2003; Assaad et al., 2004; Sutter et al., 2006; Eisenach et al., 2012). Although studies have described the role of SYP121 and SYP122 in the mechanics of vesicle fusion during exocytosis (Fasshauer et al., 1998; Bock et al., 2001; Bassham and Blatt, 2008; Karnik et al., 2015), the molecular mechanism of the selection of specific SNAREs at the plasma membrane and their contribution to the specificity of cargo proteins are not well known. Rehman et al. (2008) and Ul-Rehman et al. (2011) showed that SYP121 and SYP122 differ in their interaction with RABA2c (also known as RAB11A), resulting in differential secreted proteomes. Out of the nine identified tobacco proteins used by Ul-Rehman et al. (2011) in their similarity assessment, our total data set included five possibly homologous proteins; however, these lacked signal peptides and were not included in our detailed analysis. By analyzing the dominant sets of conventionally secreted proteins in the two mutants and in the wild type, we demonstrated that SYP121 and SYP122 differ in their contributions to the secretion of specific cargo proteins across the plasma membrane.

Comparison with Similar Proteomic Studies

The proportions of proteins that we identified with signal peptides, membrane anchors, or transmembrane domains are comparable with similar proteomic studies (Supplemental Table S4). Hervé et al. (2016) recently identified 1,093 proteins from the cell walls of Arabidopsis leaves; of these, they classified 361 (33%) proteins as cell wall localized on the basis of possessing a signal peptide or a transmembrane domain and lacking retention signals. Of the cell wall proteome of Hervé et al. (2016), 72 proteins are in common with our data set. Rutter and Innes (2017) isolated extracellular vesicles from Arabidopsis leaves and identified 598 proteins in total, 204 (34%) of which they classified as extracellular. Compared with the extracellular vesicle proteome of Rutter and Innes (2017), 45 proteins also were identified in our data set. Earlier secretome studies tended to use Arabidopsis suspension cells. For example, Kaffarnik et al. (2009) identified 91 proteins, approximately half of which had signal peptides. We identified 29 proteins in common with the data set of Kaffarnik et al. (2009).

A review based on early secretomes, such as that from Kaffarnik et al. (2009), claimed that approximately 50% of secreted proteins were leaderless, and this concept has remained popular with some authors (Agrawal et al., 2010; Ding et al., 2012). A recent and excellent review of cell wall and secreted proteomes is provided by Ghahremani et al. (2016). However, it is inevitable that some proteins from damaged or dying cells will be identified in proteomic studies, and further direct experimental evidence is required before classifying proteins as leaderless or unconventionally secreted. Predication algorithms have their weaknesses, especially if originally designed for animal systems, as discussed (Lonsdale et al., 2016), and in the absence of experimental evidence, a requirement of a signal peptide is still the best defining feature for a secreted protein and is used by the cell wall proteomic experimental database WallProtDB (San Clemente and Jamet, 2015).

Members of Multigene Families Are Trafficked by Different Routes

We identified four sets of proteins that are likely to be trafficked by different routes, with their secretion dependent on specific Qa-SNAREs; 20 proteins were assigned as SYP121 dependent, 21 proteins as SYP122 dependent, and 77 proteins were sensitive to disruption in either SYP121 or SYP122 and are likely trafficked by a partially redundant route mediated by both. The secretion of 34 proteins was unaffected by disruption to either SYP121 or SYP122. Therefore, the secretion of these proteins is likely mediated by SYP132, the other widely expressed Qa-SNARE in Arabidopsis. The existence of specific and partially redundant routes could explain why the single *syp121* and *syp122* mutants do not show strong phenotypes except under abiotic or biotic stress. In contrast, in the double *syp121syp122* mutant, both the specific cargo proteins and the partially redundant cargo proteins will have defective secretion and could explain the mutant's viable but dwarfed and necrotic phenotype. We did not attempt proteomic identification of the secretome of the *syp121syp122* mutant due to the extreme nature of its small and sensitive phenotype.

While individual proteins were assigned to specific routes, possible functional redundancy among cargo proteins would mean that disruption to either SYP121 or SYP122 alone is less likely to affect growth severely if a closely related cargo protein is secreted via the partially redundant alternative route. Many of the proteins that we assigned to specific routes are members of highly diversified gene families. We identified 13 peroxidases, 13 glycosyl hydrolases, 11 GDSL lipases, and 11 proteases, all of which come from large multigene families. These numbers are too low for a phylogenetic analysis, but there are notable preferences within these protein families for specific routes. For example, the secretion of granulin proteases was unaffected in either *syp121* or *syp122* (and likely depends on SYP132), while aspartyl proteases appeared to depend on SYP122. Among the identified glycosol hydrolases, subfamily 17 appears to be secreted by a SYP121-dependent route. Interestingly, some members of glycosyl hydrolase family 17 are localized to plasmodesmata, while others are secreted to the cell wall (Gaudioso-Pedraza and Benitez-Alfonso, 2014). The secretion of GDSL lipases was sensitive to disruption in either SYP121 or SYP122 (and, therefore, likely independent of SYP132). GDSL lipases have broad substrate and functional specificities and have been associated with plant immunity (Kim et al., 2013; Gao et al., 2017).

Differences between SYP121- or SYP122-Mediated Traffic

Previous studies have shown that the *syp121* mutant is impaired in both abiotic stress (Leyman et al., 1999; Eisenach et al., 2012) and pathogen defense responses (Collins et al., 2003; Zhang et al., 2007). At the core of these findings is the activity of SYP121 in stomatal function, but it is clear that manipulating traffic associated with this SNARE has much broader consequences for cell expansion and plant growth (Geelen et al., 2002; Honsbein et al., 2009; Grefen et al., 2015). A recent study showed that pectin methylesterase is highly expressed in guard cells and that the *pectin methylesterase6* (*pme6-1*) mutant exhibits high levels of methyl-esterified pectin. Of importance, the *pme6-1* mutation leads to elevated CO_2 assimilation, increased conductance, and evaporative cooling (Amsbury et al., 2016; Wu et al., 2017), features that in many ways complement the characteristics of the *syp121* mutation (Eisenach et al., 2012) and our findings here that the traffic of PMEIs and β-1,3-glucanase is dependent on SYP121. While PMEI determines the elasticity of the cell wall by controlling the activity of pectin methylesterase, the β-1,3-glucanase degrades the callose to support stomatal opening and closure. We note that Eisenach et al. (2012) ascribe aspects of the *syp121* phenotype to slowed vesicle recycling to the plasma membrane and reduced K^+ transport on recovery from stress. These characteristics may well be augmented by effects mediated through cell wall modification that affect its plasticity, such as the importance of callose deposition in defense responses (Rehman et al., 2008; De Caroli et al., 2011; Ul-Rehman et al., 2011).

Overexpressing PMEIs also reduces susceptibility to *Botrytis cinerea* (gray mold) and *Pectobacterium carotovorum* (formerly known as *Erwinia carotovora*, the causal agent of soft rot) infection by elevating pectin esterification (Lionetti et al., 2007). There are some indications that PMEIs, glucanases, and xyloglucan endotransglycosylases contribute to plant defense by suppressing microbial pectin-degrading enzymes (Divol et al., 2007). We speculate that the loss of several cell wall-modifying proteins in the *syp121* mutant makes subtle changes to the cell wall architecture and reduces pathogen penetration resistance. In this context, it is interesting that the *syp122* mutant shows more obvious cell wall defects compared with the *syp121* mutant, but its penetration resistance remains unaltered (Reiter et al., 1997). We only identified two clearly defense-associated proteins, Germin3 (AT5G20630) and a lectin family protein (AT3G15356), but these were not assigned as specific to either SYP121 or SYP122.

In contrast to our conclusion that PMEI1 and PMEI2 proteins are secreted through a SYP121-dependent pathway, De Caroli et al. (2011) observed that overexpression of the dominant negative mutant of SYP121 (SP2) had no effect on the secretion of secGFP-PMEI1. Notably, their PMEI-GFP fusion protein did not appear to be secreted but was retained in the Golgi and lacked a GPI modification they identified as necessary for secretion. We suggest that differences in the experimental systems (tobacco protoplasts compared with Arabidopsis plants) and the detection methods could cause the different observations.

In conclusion, we find that the SNAREs SYP121 and SYP122 are associated with different subsets of cargo proteins that are targeted for secretion at the plasma membrane. Our results also show that a substantial proportion of cargo proteins are secreted in both the *syp121* and *syp122* mutants and frequently belong to the same gene families, which may explain why the single mutants do not show a strong phenotype but the *syp121syp122* double mutant growth is severely impaired. We speculate that the *syp121* mutant is susceptible to pathogen invasion in part because of the effects on the traffic of proteins responsible for cell wall modification.

MATERIALS AND METHODS

Arabidopsis Secretome Capture

Arabidopsis (*Arabidopsis thaliana*) wild type (Columbia-0) and *syp121* and *syp122* mutants were sterilized in 70% (v/v) ethanol and 2.5% (v/v) sodium hypochlorite for 20 min, then washed with sterile distilled water and vernalized at 4°C. Seeds were cultured at a density of 4 mg of seeds per 100 mL of filter-sterilized 0.5× Murashige and Skoog medium under an alternating 8-h/16-h dark/light cycle at 18°C and 100 rpm agitation. The medium was recovered after 10 d of growth and filtered through a 0.22-μm filter in a vacuum apparatus to remove seeds and insoluble material. Secreted proteins were precipitated using StrataClean (Stratagene) resin following the manufacturer's instructions, denatured, and separated by SDS-PAGE. Gel lanes were divided into 10 pieces and submitted for protein digestion and analysis by liquid chromatography-MS/MS.

Protein Digestion and MS

All reagents were purchased from Sigma-Aldrich unless specified otherwise. Gel pieces were destained with 50% (v/v) ethanol in 50 mM ammonium bicarbonate (ABC) and 100% ethanol before reduction and alkylation of Cys residues using 10 mM DTT in 50 mM ABC and 55 mM iodoacetamide. Proteins were digested in gel using trypsin (50:1 Progma Gold) and eluted from the gel slices using sequential washes of 50% (v/v), 80% (v/v), and 100% acetonitrile in 50 mM ABC. Peptides were filtered through a 0.22-μm cellulose acetate spin column to remove any residual fragments of gel and concentrated by lyophilization for storage at −20°C.

Lyophilized peptides were resuspended in 2% (v/v)acetonitrile and 0.5% (v/v) trifluoroacetic acid in water for analysis using reverse-phase chromatography prior to MS analysis. Two columns were utilized, an Acclaim PepMap l-precolumn cartridge (300 μm i.d., 3.5 mm long, 5 μm of 100 Å beads) and an Acclaim PepMap RSLC (75 μm i.d., 25 cm long, 2 μm of 100 Å beads; Thermo Scientific), installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Samples were loaded onto the precolumn equilibrated in 2% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid for 8 min at 10 μL min^{-1}, after which peptides were eluted onto the analytical column at 300 nL min^{-1} by increasing the mobile phase B concentration from 4% (v/v) B to 25% (v/v) over 90 min, then to 35% (v/v) B over 10 min and 90% (v/v) B over 5 min, followed by a 15-min reequilibration at 4% (v/v) B. Peptides were injected directly from the liquid chromatograph (300 nL min^{-1}) via a Triversa Nanomate nanospray source (Advion Biosciences) into a Thermo Orbitrap Fusion (Q-OT-qIT; Thermo Scientific) mass spectrometer. Survey scans of peptide precursors from 400 to 1,600 mass-to-charge ratio were performed at 120 K resolution (at 200 mass-to-charge ratio) with automatic gain control. Precursor ions with charge state 2 to 6 were isolated (isolation at 1.2 Thomson in the quadrupole) and subjected to higher energy collisional dissociation fragmentation with a normalized collision energy of 35. MS/MS data were analyzed using the Orbitrap at 30 K resolution. Automatic gain control was set to 5.4 e3, and the maximum injection time was 200 ms. Dynamic exclusion duration was set to 60 s with a 10 ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2-s cycles.

Protein Identification

Raw data were converted to Mascot generic format using MSConvert in the ProteoWizard Toolkit (version 3.0.5759; Kessner et al., 2008). MS spectra were searched with Mascot engine (Matrix Science, version 2.4.1; Nesvizhskii et al., 2003) against the Arabidopsis TAIR10 database (https://www.arabidopsis.org/) and the common Repository of Adventitious Proteins Database (http://www.thegpm.org/cRAP/index.html). Theoretical peptides were generated from a tryptic digestion allowing up to two missed cleavages and variable modifications: carbamidomethyl on Cys and oxidation on Met. Precursor mass tolerance was 10 ppm, and product ions were searched at 0.6 D tolerances. Scaffold (version Scaffold_4.6.1; Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. All raw MS files and associated peptide identifications have been submitted to the public proteomics repository PRIDE, part of the ProteomeXchange consortium (accession no. PXD009640; Vizcaíno et al., 2014, 2016).

Bioinformatic Analysis

Subcellular locations were based on in silico prediction and experimental evidence using UNIPROT (http://www.uniprot.org/). UNIPROT annotations of signal peptides were used to predict secretory proteins in conventional pathways. Proteins containing a signal peptide and lacking membrane anchors, transmembrane domains, vacuolar annotation, or ER retention signals were classified as conventionally secreted proteins. Proteins with the transmembrane domain were annotated as integral components of the membrane. All cytoplasmic and internal organelle proteins were categorized as internal proteins. The remaining proteins that were not assigned to specific locations were designated as undefined.

Cloning of SNARE-Specific Cargo Proteins for Traffic Analysis

For secretory traffic assay, a tricistronic vector was constructed with Gateway-compatible sites containing a 35S promoter and GFP-HDEL on the vector backbone. GFP-HDEL was PCR amplified from pVKH-GFP-HDEL (Batoko et al., 2000) and ligated into pTecGFP-HDEL-2in1-BcFP (Karnik et al., 2013) via the *Hin*dIII/*Kpn*I site. The two 2in1 expression cassettes (Grefen and Blatt, 2012) containing the 35S promoter, Gateway cassette (either attR3-lacZ-attR2 or attR1-ChloramphenicolR, ccdB-attR4), and a C-terminal tag (either 3xHA or myc) were gene synthesized and ligated sequentially via blunt end cloning (*Afe*I and *Sna*BI) into pTecG to create pTecG-2in1-CC.

The genes for selected cargo proteins were PCR amplified using gene-specific primers flanked with Gateway attB1 and attB4 sites and cloned in the pDONR221 vector (Supplemental Table S5). mCherry was cloned in the pDONR221 vector using the B2 and B3 sites. Entry vectors containing mCherry and cargo were transferred to pTecGFP-HDEL-2in1-BcFP to make a destination vector for the secretory assay. The constructs were used for the transformation of *Agrobacterium tumefaciens* (Nottingham Arabidopsis Stock Centre, University of Nottingham).

Transient Transformation and Confocal Microscopy

Roots of Arabidopsis seedlings were grown for 3 d in 0.05× Murashige and Skoog medium before cocultivation with *A. tumefaciens* GV3101 as described previously (Grefen et al., 2010b). Seedlings were imaged 3 to 4 d post-transformation using a Leica SP8-SMD confocal microscope. Images were routinely collected as Z-stacks using a Planapo 20×/0.75NA objective lens. GFP fluorescence was excited with continuous 488-nm light, and fluorescence emission was collected over 500 to 535 nm. mCherry was excited with 552-nm light, and fluorescence was collected over 590 to 645 nm. Control images for background fluorescence were collected in parallel from untransformed seedlings. Fluorescence images were rendered as three-dimensional projections, and fluorescence intensities were quantified after background correction. All experiments were repeated at least three times for each construct.

Statistics

Data are reported as means ± SE where appropriate and are reported with significance ($P < 0.050$) determined by ANOVA.

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. List of all proteins identified from all replicates with unique peptide counts.

Supplemental Table S2. List of all proteins classified as secreted and the UNIPROT field that informed the classification.

Supplemental Table S3. Functional classification of likely SYP121 and SYP122 cargo proteins.

Supplemental Table S4. Comparison of identified proteins with similar proteomic studies by other researchers.

Supplemental Table S5. List of primers used in this study.

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