# The syntaxin SYP132 contributes to plant resistance against bacteria and secretion of pathogenesis-related protein 1

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**In contrast to many mammalian pathogens, potential bacterial pathogens of plants remain outside the host cell. The plant must, therefore, promote an active resistance mechanism to combat the extracellular infection. How this resistance against bacteria is manifested and whether similar processes mediate basal, gene-for-gene, and salicylate-associated defense, however, are poorly understood. Here, we identify a specific plasma membrane syntaxin, *Nb*SYP132, as a component contributing to gene-for-gene resistance in *Nicotiana benthamiana*. Silencing *NbSYP132* but not *NbSYP121*, the apparent orthologue of a syntaxin required for resistance to powdery mildew fungus, compromised AvrPto-Pto resistance. Because syntaxins may play a role in secretion of proteins to the extracellular space, we performed a limited proteomic analysis of the apoplastic fluid. We found that *NbSYP132*-silenced plants were impaired in the accumulation of at least a subset of pathogenesis-related (PR) proteins in the cell wall. These results were confirmed by both immunoblot analysis and imunolocalization of a PR protein, PR1a. These results implicate *Nb*SYP132 as the cognate target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor for exocytosis of vesicles containing antimicrobial PR proteins. *Nb*SYP132 also contributes to basal and salicylate-associated defense, indicating that SYP132-dependent secretion is a component of multiple forms of defense against bacterial pathogens in plants.**

bacterial defense | defense response | *Nicotiana benthamiana*

To combat potential bacterial pathogens, plants have developed multiple levels of defenses. As in mammals, recognition of pathogen-associated molecular patterns (PAMPs) such as bacterial flagellin forms the basis of basal resistance and is thought to provide protection from the majority of potential bacterial invaders (1–6). By contrast, "race-specific" responses may be a second line of defense triggered by the recognition of avirulence (*Avr*) gene products, or effector proteins, injected into the host by the bacterium via a type III secretion system (7–9). These effector proteins normally contribute to the virulence of pathogens by interfering with host defense responses (8, 9). Recognition of the effector protein itself or its activity by the corresponding plant resistance (*R*) gene product, however, limits both the growth of otherwise virulent pathogens and prevents the development of disease symptoms. A third type of resistance is salicylate (SA)-associated defense (10). In response to infection of a limited area of a leaf, SA-associated defense confers broad spectrum bacterial resistance throughout the plant, even against normally virulent pathogens (10). How perception of the bacterial invaders and subsequent signaling events manifest as active resistance against bacteria, however, remains poorly understood. Similarly, although some genetic overlaps have been identified between the different types of defense (7), it is not known whether the ultimate mode of action is related between each form of resistance.

Recent evidence points toward cell wall-based mechanisms as playing an active role in mediating resistance to bacteria. AvrPto, an effector protein from *Pseudomonas syringae*, suppresses cell wall-based defenses arising during infection including callose deposition and accumulation of putatively secreted proteins, and the decrease in extracellular defenses correlated with a loss of suppression of bacterial growth (11). Transcripts encoding many components of the plant's secretion machinery were also found to increase during systemic acquired resistance, and plants with mutations in many of these general components of secretion allowed increased bacterial growth (12). Finally, the HopM1 effector protein from *P. syringae* was recently shown to cause the degredation of the host protein, *At*MIN7, an adenosine diphosphate ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) (13). ARF GEF proteins are key components involved in vesicle trafficking, indicating that bacterial pathogens target the plant secretory pathway as part of their suppression of host defense responses.

Here, we report the characterization of *Nb*SYP132, a specific plasma membrane syntaxin that contributes to gene-for-gene, basal, and SA-associated defense. Syntaxins are target-soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) on the acceptor membrane required for specific docking and fusion of donor vesicles via interactions with corresponding vesicular SNAREs and other proteins (14, 15). Consistent with a potential role for this protein to mediate defense-related secretion, we found that silencing *NbSYP132* inhibited the accumulation of at least a subset of pathogenesis-related (PR) proteins, including PR1a, in the cell wall. These results are consistent with the hypothesis that *Nb*SYP132-mediated secretion is a common mechanism of resistance to bacteria.

## Results

**_Nb_SYP132 Is a Component of Gene-For-Gene Resistance.** To identify new signaling/response components of the plant defense path-

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Abbreviations: BTH, benzothiadiazole *S*-methylester; PAMP, pathogen-associated molecular pattern; PM, plasma membrane; PVX, potato virus X; PR, pathogenesis related; SA, salicylate; TRV, tobacco rattle virus.

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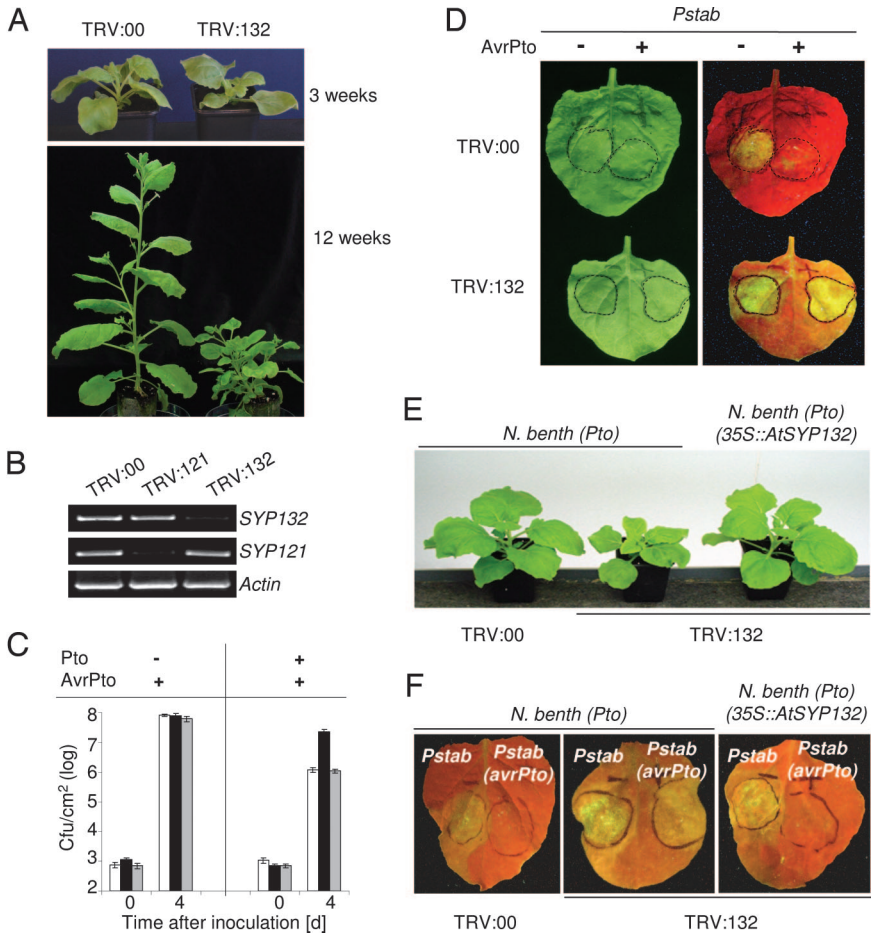


**Fig. 1.** *Nb*SYP132 contributes to gene-for-gene resistance against bacteria in *N. benthamiana*. (*A*) Side view of 3- and 12-week-old plants inoculated with control (TRV:00) or *NbSYP132* (TRV:132) silencing vectors. (*B*) RT-PCR demonstrates that TRV:132 and TRV:121 specifically reduce transcript levels of *NbSYP132* and *NbSYP121*, respectively. Actin was used as a control. (*C*) *Nb*SYP132 contributes to AvrPto-Pto-mediated resistance to the bacterial pathogen, *Pstab*(*avrPto*). Three-week-old nontransgenic (−Pto) or transgenic (+Pto) plants silenced with TRV:00 (white bars), TRV:132 (black bars), or TRV:121 (gray bars) were inoculated with *Pstab*(*avrPto*) (+AvrPto). Bacterial growth results shown are means ± SEM ($n = 6$). Experiments were repeated at least six times with similar results. (*D*) Loss of resistance in TRV:132 plants leads to development of disease symptoms. Leaves of transgenic plants expressing Pto were infiltrated with *Pstab* +/− *avrPto*. Disease symptoms are visualized 3 days after infiltration as loss of chlorophyll fluorescence (appears red under UV light). Experiments were performed three times with similar results. (*E* and *F*) Expression of the orthologous protein, *At*SYP132, complements the growth defect (*E*) and disease resistance phenotype (*F*) caused by TRV:132.

way, we previously performed a phosphoproteomic screen using highly enriched plasma membrane (PM) fractions from *Arabidopsis* suspension cell cultures to identify proteins differentially phosphorylated in response to microbial elicitors of basal defense (16). Expanding on this previous work, we identified another candidate protein, *At*SYP132 (At5g08080; data not shown), a PM syntaxin (17, 18). We sought to investigate whether this syntaxin played a role in defense against bacterial pathogens, but we were not able to isolate an insertional or RNAi knockdown mutant in *Arabidopsis* at the time of these studies. Therefore, we switched to transient virus-induced gene silencing in *Nicotiana benthamiana* to examine the biological function of the putative orthologue. A 200-bp fragment that was predicted to be gene-specific for silencing *NbSYP132* was cloned into a tobacco rattle virus (TRV) silencing vector [TRV:132; see supporting information (SI) Fig. 5*A* for sequence comparisons and SI Fig. 5*B* for phylogenetic comparisons]. Three weeks after silencing, the time used for all biological experiments in this work, TRV:132 plants were slightly smaller than TRV:00 controls (Fig. 1*A Upper*). Morphological differences such as shorter stems and petioles, thicker leaves, and partial loss of apical dominance eventually developed over time (Fig. 1*A Lower*). RT-PCR analysis showed decreased transcript levels for *NbSYP132* (Fig. 1*B*), confirming silencing of the target gene by TRV:132. As a control for specific biological function of *Nb*SYP132 in subsequent assays, we also made a construct (TRV:121) to silence *NbSYP121*. *Nb*SYP121 is the apparent orthologue of other PM-localized syntaxins, *At*SYP121/PEN1 from *Arabidopsis* and ROR2 from barley, which are involved in resistance to powdery mildew fungus (18–20). The TRV:132 construct did not cross-silence *NbSYP121* or vice versa (Fig. 1*B*), indicating specificity for the target gene as would be expected from the divergence in nucleotide sequences (see SI Fig. 5*A*). With this system established, we investigated the biological role of *Nb*SYP132.

In gene-for-gene interactions, resistance to a bacterial pathogen should only be observed when the plant expresses the *R* gene and the bacteria express the corresponding *Avr* gene (7–9). A well studied example is the interaction between Pto from tomato and AvrPto from *P. syringae* pv *tomato* (21). Using *P. syringae* pv *tabaci* (*Pstab*) 11528 expressing AvrPto to infect *N. benthamiana* expressing Pto allows recapitulation of this resistance interaction within a system that allows highly efficient transient silencing of endogenes by using virus-induced gene silencing (22). Indeed, in TRV:00 control plants, growth of *Pstab(avrPto)* was restricted

only in plants expressing Pto (Fig. 1*C*, white bars). This gene-for-gene resistance also prevented the loss of chlorophyll fluorescence (chlorosis) associated with development of disease symptoms (Fig. 1*D Right*, + AvrPto). At the low initial bacterial densities used for growth assays, gene-for-gene resistance did not result in macroscopically visible hypersensitive cell death under white light (Fig. 1*D Left*). Silencing *NbSYP132* did not alter bacterial growth in the compatible interaction (*Pstab*). It did, however, compromise Pto-mediated resistance to *Pstab(avrPto)* as evidenced by bacterial growth more than an order of magnitude higher than on control (TRV:00) plants (Fig. 1*C*, black bars) and development of chlorotic disease symptoms (Fig. 1*D*). Silencing *NbSYP121* had no effect on Pto-AvrPto-mediated resistance (Fig. 1*C*, gray bars), indicating that the effect on resistance was specific for silencing *NbSYP132*.

Finally, to ensure that the phenotypes observed in TRV:132 plants could be attributed to the function of a single gene, we stably transformed *N. benthamiana* to express the *Arabidopsis AtSYP132* (see SI Fig. 6*A*). The nucleotide sequence of *AtSYP132* is sufficiently divergent from the *N. benthamiana* sequence such as not to be targeted for gene silencing by TRV:132. Expression of *At*SYP132 in TRV:132 plants was sufficient to restore normal growth (Fig. 1*E*), full Pto-AvrPto resistance (Fig. 1*F*), and all other TRV:132-associated phenotypes described in this work (data not shown). These complementation experiments confirm that all observed effects are a result of silencing the target gene, *NbSYP132*, and not secondary targets.

**Silencing *Nb*SYP132 Does Not Eliminate Gene-for-Gene or PAMP Responses.** In an attempt to elucidate the role of *Nb*SYP132 in restricting bacterial growth, we examined a number of responses typically associated with resistance. The majority of mutants impaired in gene-for-gene resistance display a loss of either the initial recognition between *R* and *avr* gene products or the responses subsequently leading to hypersensitive cell death (7). However, using *Agrobacterium tumefaciens* transiently expressing avrPto, we found that TRV:132 plants are still capable of dose-dependent Pto-avrPto-dependent cell death (see SI Fig. 7*A*). TRV:132 plants also accumulate transcripts used as molecular markers for SA-dependent resistance responses (23) within the first 24 h, timing similar to that observed in a WT response (see SI Fig. 7*B*). As recently reported, PAMP perception of bacterial flagellin via *Nb*FLS2 contributes significantly to restricting bacterial growth in *N. benthamiana* (24). Therefore, we investigated whether silencing *NbSYP132* resulted in a loss of *Nb*FLS2-mediated responses. However, treatment of TRV:132 plants with 100 nM flg22 resulted in the production of reactive oxygen species (SI Fig. 7*C*) and activation of a mitogen-activated protein kinase (SI Fig. 7*D*) to a similar extent as observed in control plants. These results do not eliminate the possibility that either gene-for-gene or PAMP responses are attenuated in *NbSYP132*-silenced plants, but they do demonstrate that the pathway components are present and functional in these plants.

***Nb*SYP132 Is Required for the Extracellular Accumulation of PR1a.** Because its orthologue, *At*SYP132, is a PM-localized syntaxin (17, 18), it was possible that *Nb*SYP132 was involved in the secretion of antimicrobial proteins or compounds across the PM to the apoplast (extracellular space). To compare proteins secreted in TRV:00 and TRV:132 transgenic plants expressing *Pto*, proteins from the apoplastic fluid were collected 1 day postinoculation with *Pstab(avrPto)* and separated by SDS/PAGE (Fig. 2*A* and SI Fig. 8*A*). A few differentially accumulating proteins were identified by liquid chromatography-MS/MS, and all were PR proteins (see SI Table 1). Many PR proteins are small secreted proteins with antimicrobial activities (23). Immunoblot analysis confirmed that the level of one of these proteins,

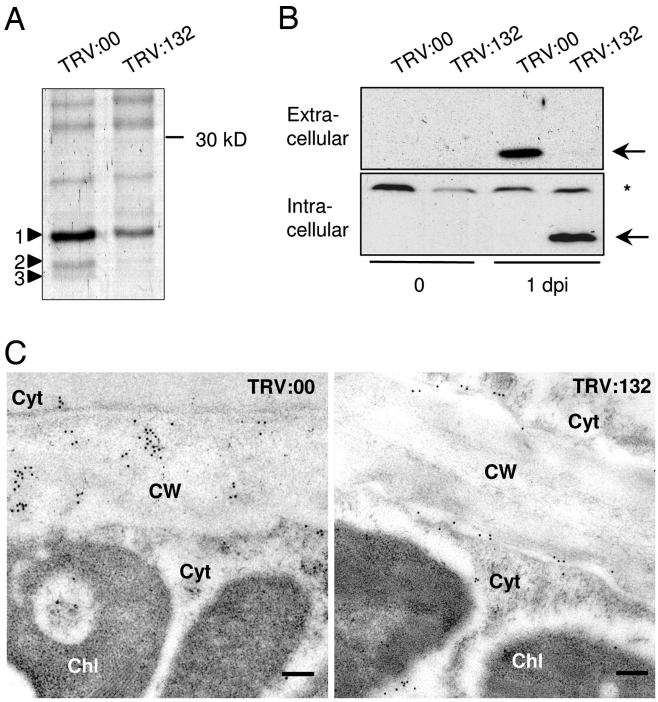


**Fig. 2.** *Nb*SYP132 is required for accumulation of PR1a in the cell wall. (*A*) TRV:132 plants show a reduced accumulation of several PR proteins in the apoplastic fluid. Apoplastic proteins were isolated from TRV:00 and TRV:132 1 day after infection with *Pstab*(*avrPto*) and separated by SDS/PAGE. Differentially accumulating proteins from the bands numbered 1–3 were identified by liquid chromatography-MS/MS (see SI Table 1). Experiments were performed four times with similar results. (*B*) PR1a is produced by TRV:132 plants but does not accumulate in the apoplastic fluid. Apoplastic proteins isolated as in *A* and intracellular proteins from 0.5-cm$^2$ discs of the remaining leaf tissue were separated by SDS/PAGE and subjected to immunoblot analysis using an anti-PR1 antibody (31). Arrows indicate PR1a. The asterisk indicates a cross-contaminating band found only in the intracellular samples. Experiments were performed at least six times with similar results. (*C*) Immunogold labeling of PR1a shows that the protein accumulation in the cell wall in TRV:00 but not TRV:132 plants 24 h after infiltration with *Pstab*(*avrPto*). cw, cell wall; cyt, cytoplasm; chl, chloroplast. See *SI Text* for detailed procedure for transmission electron microscopy. (Scale bars: 200 nm.)

PR1a, was reduced in the apoplastic fluid of TRV:132 plants compared with TRV:00 plants (Fig. 2*B Upper*) and that the antibody only cross-reacts with a single band corresponding to PR1a in the extracellular fluid (SI Fig. 8*B*). The fact that PR1a was easily detected in the remaining tissue after isolation of extractable apoplastic proteins indicated that the protein was produced but not secreted in TRV:132 plants (Fig. 2*B Lower*). These results are further supported by immunolocalization of PR1a using leaves from *Pstab(avrPto)*-infected plants. PR1a consistently accumulated in the cell wall of TRV:00 plants (Fig. 2*C Left* and SI Fig. 9 *A* and *C*). Conversely, relatively little immunolabeling of PR1a in the cell wall was observed in TRV:132 plants (Fig. 2*C Right* and SI Fig. 9 *B* and *D*). As was the case with the resistance responses, silencing *NbSYP121* had no effect on PR1a accumulation in the apoplastic fluid (data not shown), again demonstrating nonoverlapping functions for these PM syntaxins. These results confirm that *Nb*SYP132 is required for the proper secretion of, and is possibly the cognate target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor for, vesicles containing PR1a.

***Nb*SYP132-Dependent Secretion of PR1 Correlates with Resistance to Bacteria.** Because the defect in protein secretion was the only molecular phenotype we could identify that was associated with

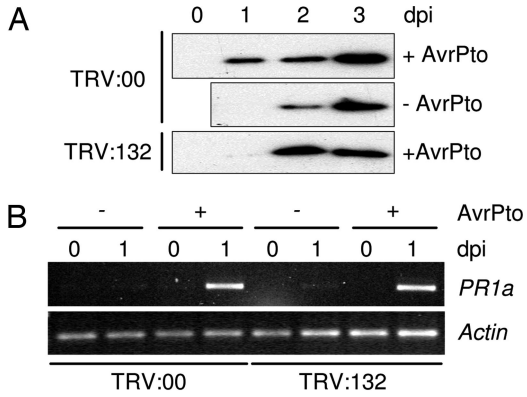


**Fig. 3.** A delay in accumulation of extracellular PR1a correlates with the loss of resistance. (*A*) Secretion of PR1a in TRV:132 plants is delayed after infection with avirulent bacteria (+AvrPto) as compared with control (TRV:00) plants and phenocopies the delay in secretion after infection by virulent bacteria (−AvrPto). Experiments were performed three times with similar results. (*B*) Transcriptional induction of PR1a is delayed in the virulent compared with the avirulent interaction but is not affected by silencing *NbSYP132*. Experiments were performed at least three times with similar results.

enhanced bacterial growth and formation of disease symptoms in TRV:132 plants, we directly compared the correlation between secretion of PR proteins and resistance. In both cases in which disease symptoms formed (i.e., TRV:00 − AvrPto, TRV:132 + AvrPto), a 1-day delay in the extracellular accumulation of PR1a was observed as compared with that found during a resistance response (TRV:00 + AvrPto) (Fig. 3*A*). The eventual accumulation of PR1a in the apoplastic fluid of TRV:132 plants most likely occurs because of residual low levels of *Nb*SYP132 as a result of incomplete gene silencing (Fig. 1*B*). Mechanistically, we found that the delayed accumulation of PR1a in the cell wall was accomplished differently. In response to treatment with virulent pathogens, production of the transcript for PR1a was delayed (Fig. 3*B*), whereas in TRV:132 plants treated with avirulent bacteria (Fig. 3*B*), transcript accumulation was similar to WT (Fig. 3*B*) but protein secretion did not occur (Fig. 3*A*).

***Nb*SYP132 Contributes to Basal and SA-Associated Defense Against Bacteria But Is Not Required for *R*-Gene-Mediated Resistance Against a Virus.** Although little is known about whether components of gene-for-gene-based resistance also are used during basal resistance, a correlation has been found between extracellular defense mechanisms and basal resistance (11). Using the hrpA$^-$ mutant of *P. syringae* pv *tomato* DC3000 (*Pst* DC3000) deficient in the type III secretion system to assay basal defense, we found that TRV:132 plants (Fig. 4*A*, black bars) allowed greater bacterial growth of *hrpA*$^-$ mutants than TRV:00 plants (Fig. 4*A*, white bars). Similar trends in bacterial growth were observed with the nonpathogenic, plant-colonizing bacterium, *A. tumefaciens* (data not shown). Thus, *Nb*SYP132 also contributes to basal defenses that restrict bacterial numbers in the apoplast in the absence of *R* gene-mediated recognition.

A recent report provided evidence that general components of the secretion machinery such as the endoplasmic reticulum-resident chaperone BiP2 were required for another form of bacterial resistance, namely SA-associated defense responses in *Arabidopsis* (12). Interestingly, a correlation also was observed between enhanced susceptibility and loss of secretion of PR proteins as measured by a decrease in PR1 (12). This similarity to our secretion phenotype indicated a possible role for *Nb*SYP132 in SA-associated defense. Pretreatment of TRV:00 plants with benzothiadiazole *S*-methylester (BTH), a chemical

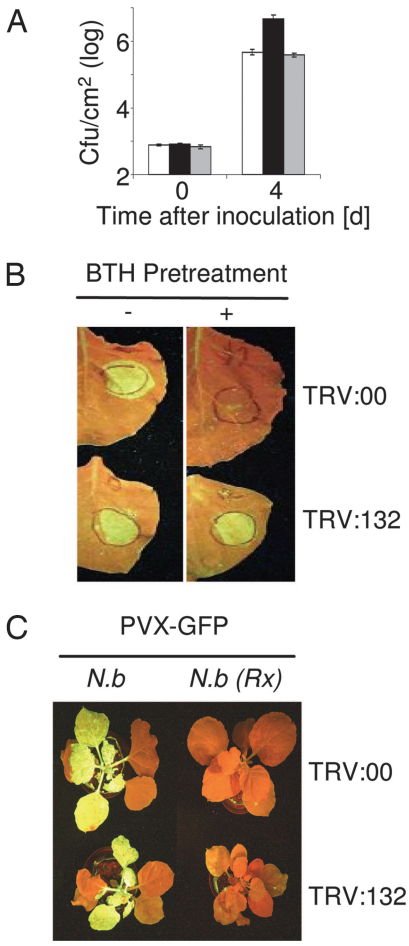


**Fig. 4.** SYP132 contributes to multiple forms of plant defense against bacteria but not against a virus. (*A*) *Nb*SYP132 contributes to basal resistance. Bacterial growth of the *hrpA*$^-$ mutant of *P. syringae* pv. *tomato* [*P.s tomato* (*hprA*$^-$)] in leaf tissue of TRV:00 (white bars), TRV:132 (black bars), or TRV:121 (gray bars) *N. benthamiana*. Experiments were performed four times with similar results. (*B*) Pretreatment with BTH for 14 h does not induce SA-associated defense against the virulent pathogen, *Pstab*, in TRV:132 plants. Experiments were performed three times with similar results. (*C*) *Nb*SYP132 is not required for gene-for-gene resistance against PVX. Two leaves of non-transgenic plants (*N.b.*) or transgenic plants expressing *Rx*, the gene conferring specific resistance against PVX [*N.b.*(*Rx*)], were infiltrated with PVX virus expressing GFP. After 1 week, photographs were taken under UV light. Chlorophyll in the leaves fluoresces red. GFP from the virus fluoresces green. Experiments were performed three times with similar results.

inducer of defense, conferred resistance against the normally virulent pathogen, *Pstab*, as evidenced by prevention of chlorotic disease symptoms (Fig. 4*B*). TRV:132 plants pretreated with BTH, however, developed full disease symptoms if the bacterial infection followed the BTH treatment within 14 h (as in Fig. 4*B*), but full SA-associated defense was restored if infection followed after longer periods of pretreatment (data not shown). These results are consistent with the fact that even though the timing was delayed, TRV:132 plants eventually secreted PR proteins after infection (Fig. 3*A*). Given sufficient time after pretreatment with BTH, TRV:132 plants are likely to accumulate a critical level of PR proteins (or other molecules) to confer resistance.

Finally, we investigated whether *Nb*SYP132 was required for resistance against unrelated pathogens. Potato virus X (PVX) will spread through solanaceous plants in the absence of the corresponding *R* gene, *Rx* (25). Using PVX-GFP to visualize

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viral movement from the site of inoculation, we found that PVX spread to a similar extent through both TRV:00 and TRV:132 plants not expressing *Rx* (Fig. 4*C Left*). Transgenic plants expressing *Rx* stopped the virus at the site of inoculation, and this resistance was unaffected by silencing *NbSYP132* as demonstrated by the lack of viral accumulation (Fig. 4*C Right*). These results further demonstrate that *NbSYP132*-silenced plants are not generally deficient in *R* gene-mediated responses.

## Discussion

We identified a previously uncharacterized PM syntaxin, *At*SYP132, in a phosphoproteomic screen targeting proteins differentially phosphorylated in response to the bacterial elicitor molecule, flg22 (data not shown). Silencing *NbSYP132* in *N. benthamiana* expressing the Pto resistance gene compromised gene-for-gene resistance against *Pstab(*avrPto), allowing bacteria to grow nearly to levels found in the compatible interaction. Interestingly, silencing *NbSYP132* did not alter virulence in the compatible interaction. This result may be explained if virulent bacteria already interfere with the production of proteins (or other molecules) dependent on *Nb*SYP132 for extracellular accumulation, so loss of the syntaxin itself caused no further effect. Alternatively, *Nb*SYP132-mediated events may be a primary mode of resistance; but a secondary mode of resistance may still be present to limit further bacterial growth.

An important consideration arising from the present work together with other recent studies (11–13) is that protein secretion appears to be an important, and possibly terminal, step mediating active resistance to bacterial infection. Further support for the role of *Nb*SYP132-dependent secretion in defense came from our comparison of two forms of susceptibility, the compatible interaction in WT plants with the incompatible interaction in *NbSYP132*-silenced plants. Similar levels of bacterial growth and disease symptoms were observed in both interactions, but the molecular phenotypes were different. In the compatible interaction, the transcription of PR1a was delayed by 1 day. In the incompatible interaction, the timing of transcript accumulation was unchanged but secretion was delayed to a similar extent. Thus, the correlation between these two susceptible interactions was the 1-day delay in extracellular accumulation of PR1a. These results do not, however, imply that PR1a itself mediates resistance. More likely is the hypothesis that there are numerous proteins or compounds requiring *Nb*SYP132 for proper secretion that evoke resistance against the invading pathogen. Functional redundancy of these components may have masked their genetic identification, and only by blocking the secretion of the whole subset of proteins (e.g., by silencing *NbSYP132*) were we able to observe the effect on resistance.

Our results strongly indicate that *Nb*SYP132-dependent secretion of antimicrobial proteins and/or other antimicrobial compounds plays a role in active resistance against bacteria during gene-for-gene, basal, and SA-mediated defense responses. Whether the secreted cargo in all three responses is truly the same remains to be determined, but the PR proteins are likely candidates for being conserved components because all three responses result in the transcriptional up-regulation of PR genes (23, 26). The differences in the genetic components associated with these responses, however, indicate that multiple signaling pathways may converge on the regulation of these genes.

Intriguingly, two syntaxins have been implicated in mediating defense-related secretion in plants, SYP132 for resistance to bacteria as shown in this work and SYP121/ROR2 for resistance against powdery mildew fungus (18–20). In this work, we demonstrated that SYP121 is dispensable for resistance against bacteria, whereas SYP132 contributed significantly, indicating a potential specificity for particular syntaxins in the secretion of defense-related cargo. Understanding the mechanisms by which these syntaxins mediate secretion of specific vesicles not only will contribute to our understanding of plant defense, but also should provide unique insights into the regulation of protein secretion in plants.

## Materials and Methods

**Virus-Induced Gene Silencing in *N. benthamiana*.** Gene fragments of *NbSYP132* or *NbSYP121* (see *SI Text*) were cloned into the binary pTV00 vector (27) with a modified pGreen backbone (pGIITV00). Silencing was performed as described (22) except that cultures were adjusted to $OD_{600}$ = 0.5 and 2-week-old *N. benthamiana* plants were infiltrated with the 10:1 mixture of RNA2 and RNA1. Plant genotypes used in this study included nontransgenic *N. benthamiana* plants and transgenic plants crossed to express three resistance genes *Pto*, *N*, and *Rx* (individual lines described in ref. 22). Plants were grown in environment-controlled chambers at 22°C under 16-h light/8-h dark cycle. Experiments with silenced plants were performed 3–4 weeks after induction of silencing.

**Expression Analyses by RT-PCR.** Leaf tissue was ground in liquid nitrogen, and RNA was isolated by using TRI Reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. For reverse transcription, 2 $\mu$g of total RNA, SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), and Oligo-$dT_{17}$ primers were used. Subsequent PCRs were performed with gene-specific primers and 50–100 ng of cDNA per reaction. Primers were designed to be specific for: actin (NbAct1, 5′-ATGGCAGACGGTGAGGATATTCA-3′; NbAct2, 5′-GCCTTTGCAATCCACATCTGTTG-3′); SYP132 (NbSYP132_1, 5′-GGAGCTAGGCCTCGACGATTTC-3′; Nb132_2, 5′-CTCGCCTTTCAACAACCTCACGG-3′); SYP121 (NbSYP121_1, 5′-GTGGTGGTATCAATCTTGACAAG-3′; NbSYP121_2, 5′-CCATTCTTTGCCGGAGTTCATTG-3′), PR1a (PR1a_1, 5′-AATATCCCACTCTTGCCG-3′; and PR1a_2, 5′CCTGGAGGATCATAGTTG-3′).

**Pathogen Growth Assays.** Cultures of *P. syringae* pv. *tabaci (Pstab)* 11528, containing an empty vector or expressing AvrPto, were grown overnight at 28°C on L medium (10 g·liter$^{-1}$ bacto tryptone, 5 g·liter$^{-1}$ yeast extract, 5 g·liter$^{-1}$ sodium chloride, and 1 g·liter$^{-1}$ D-glucose) containing 100 mg·liter$^{-1}$ rifampicin and 50 mg·liter$^{-1}$ kanamycin for selection. Syringe inoculations and measurements of bacterial growth *in planta* were performed as described for *Arabidopsis* (28) except that $10^4$ cfu·ml$^{-1}$ was used for initial inoculations. Each experiment was repeated at least three times. To assay viral movement, *Agrobacterium* expressing the PVX:GFP construct (29) was inoculated at a 1,000-fold dilution from an $OD_{600}$ = 1 into two expanded leaves of 4-week-old *N. benthamiana* plants. Photographs were taken under UV light 1 week after inoculation.

**Isolation of Apoplastic Fluid.** One day after inoculation with *Pstab(avrPto)*, leaves were vacuum-infiltrated for 5 min with apoplastic fluid (AF) buffer (50 mM phosphate buffer, pH 7/150 mM sodium chloride) following published methods (30). AF was collected by centrifugation (5 min, 1,500 × *g*), mixed with 2× SDS sample buffer, and subjected to SDS/PAGE.

**Immunoblot Analysis.** Proteins separated by SDS/PAGE were transferred in the presence of 0.01% SDS for 2 h at 250 V onto nitrocellulose (Hybond-ECL; Amersham, Piscataway, NJ). The membrane was blocked by using 5% skimmed milk powder in TBS containing 0.05% Tween-20 and probed with anti-PR1 polyclonal antiserum (31). Peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody, and the reaction was visualized by using the Supersignal Pico detection kit (Pierce, Rockford, IL).

**Transmission Electron Microscopy.** Leaf samples were fixed in 2.5% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate, pH 7.3 overnight, then placed in tissue-handling devices and processed at low temperature to improve antigenicity as described (32) with the following modifications: infiltration steps were performed at −20°C with LR White resin plus 0.5% (wt/vol) benzoin methyl ether and polymerization was in Beem capsules with indirect UV irradiation for 24 h at −20°C followed by 16 h at room temperature. Ultrathin sections of ≈90 nm were taken using an ultramicrotome (Leica, Milton Keynes, U.K.) and picked up on pyroxylin- and carbon-coated gold grids. For the immunogold labeling procedure, grids were incubated on drops of 50 mM glycine/PBS for 15 min followed by drops of preprepared Aurion blocking buffer (5% BSA/0.1% cold water fish skin gelatin/5–10% normal goat serum/15 mM sodium azide/PBS, pH 7.4) (Aurion, Wageningen, The Netherlands) for 30 min then equilibrated in 0.1% BSA-C/PBS (Aurion). Grids were incubated with anti-PR1 antibody diluted 1:300 in equilibration buffer overnight at 4°C, washed five times in equilibration buffer, and incubated for 3 h with goat anti-rabbit antibody conjugated to 10 nm gold (BioCell; Agar Scientific Ltd., Essex, U.K.) diluted 1:50 in equilibration buffer. After four washes in equilibration buffer, three 20-min washes in PBS and two 30-min washes in water, the grids were contrast-stained with uranyl acetate and lead citrate before observation in an 1200 EX transmission electron microscope (JEOL, Tokyo), at 80 kV. Photographs were taken on electron image film (Kodak, Rochester, NY).

**Treatment of Plants with BTH.** Leaves were sprayed using a solution with and without 0.3 mM BTH. Solutions were sprayed onto leaves of 4-week-old *N. benthamiana* plants 12 h before syringe inoculation with *Pstab* ($10^3$ cfu/ml) bacteria resuspended in 10 mM magnesium chloride. Disease symptoms were visualized under UV light 3 days after inoculation.

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