S-Glycoprotein-Like Protein Regulates Defense Responses in *Nicotiana* Plants against *Ralstonia solanacearum*[1][C][W]

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RsRGA4 (for *Ralstonia solanacearum*-responsive gene A4) encodes a polypeptide similar to S-locus glycoprotein (SGP) from *Brassica rapa* and SGP-like proteins from *Ipomoea trifida* and *Medicago truncatula*. Therefore, we designated RsRGA4 as *NtSGLP* (for *Nicotiana tabacum* SGP-like protein) and *NbSGLP* (its *Nicotiana benthamiana* ortholog). *NbSGLP* is expressed in root, leaf, petal, gynoecium, and stamen. Expression of *NbSGLP* was strongly induced by inoculation with an avirulent strain of *R. solanacearum* (Rs8107) and slightly enhanced by inoculation with virulent *R. solanacearum* (RsOE1-1). Expression of *NbSGLP* was induced by inoculation with an *hrpY*-deficient mutant of RsOE1-1 and Rs8107. Expression was also induced by aminocyclopropane carboxylic acid and salicylic acid. Virus-induced gene silencing of *NbSGLP* enhanced the growth of Rs8107. Growth of RsOE1-1 and appearance of wilt symptoms were also accelerated in silenced plants. Expression of *PR-1a* and *EREBP* was reduced, and markers for basal defense, such as callose deposition and reduced vascular flow, were compromised in *NbSGLP*-silenced plants. Moreover, growth of *Pseudomonas cichorii*, *Pseudomonas syringae* pv *tabaci*, and *P. syringae* pv *mellea* was also enhanced in the silenced plants. On the other hand, silencing of *NbSGLP* did not interfere with the appearance of the hypersensitive response. NbSGLP was secreted in a signal peptide-dependent manner. *Agrobacterium tumefaciens*-mediated expression of *NbSGLP* induced *PR-1a* and *EREBP* expression, callose deposition, and reduced vascular flow. NbSGLP-induced callose deposition and reduced vascular flow were not observed in salicylic acid-deficient *N. benthamiana* NahG plants. Taken together, SGLP might have a role in the induction of basal defense in *Nicotiana* plants.

Plants have a variety of active defense mechanisms to protect themselves from microbial pathogen infection. These responses include the hypersensitive response (HR; Levine et al., 1994), production of phytoalexins (Kuc, 1972), activation of pathogenesis-related proteins (Mauch and Staehelin, 1989), induction of an oxidative burst (Baker and Orlandi, 1995), cross-linking of cell wall glycoproteins (Bradly et al., 1992; Brisson et al., 1994), and lignification (Vance et al., 1980). A common feature of plant active defense responses is the transcriptional activation of a large number of genes upon pathogen infection or treatment with pathogen elicitors (Rushton and Somssich, 1998). Some of the pathogen-induced genes encode proteins with direct antimicrobial activities or enzymes involved in biosynthesis of antimicrobial compounds. Other pathogen-induced genes encode proteins with regulatory functions in signal transduction pathways of plant defense responses (Yang et al., 1997).

Ralstonia solanacearum is a devastating, soil-borne pathogen with a global distribution and a wide host range (Hayward, 1991). It causes bacterial wilt on several economically important solanaceous crops. *R. solanacearum* generally invades through wounded roots or natural openings, from which secondary roots subsequently emerge, and then proliferates in the intercellular spaces of the inner cortex and vascular parenchyma, before invasion into xylem vessels (Hayward, 1991; Vasse et al., 1995; Seile et al., 1997). *R. solanacearum* has evolved a highly specialized protein secretion system, known as the type III secretion system, whose main function is to deliver proteins from the bacterial cytoplasm into the host cell cytosol. The *hrp* genes encode the type III secretion system proteins and are required for the induction of the HR, a rapid localized programmed death of plant cells at the infection site, in resistant cultivars and in nonhost plants, and for pathogenicity to host plants (Boucher

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et al., 1992; Van Gijsegem et al., 1995; Kanda et al., 2003a).

In the tomato (*Solanum lycopersicum*), resistance to *R. solanacearum* is controlled by several loci (Thoquet et al., 1996a, 1996b). In Arabidopsis (*Arabidopsis thaliana*), resistance is monogenic and is conferred by the *RRS1-R* gene that encodes a novel R protein. This resistance is dependent upon salicylic acid (SA) and the *NDR1* signaling pathway (Deslandes et al., 2002). Recently, PopP2, the cognate Avr protein for *RRS1-R*, was identified and shown to interact with the R protein (Deslandes et al., 2003). Although genetic identification of *R* genes has been extensively employed to analyze *R. solanacearum*-plant interactions, little is known regarding the molecular events in plants during the establishment of resistance or susceptibility to *R. solanacearum*. Previously, we isolated more than 50 gene fragments that were regulated in tobacco (*Nicotiana tabacum*) plants by inoculation with *R. solanacearum* (*R. solanacearum*-responsive genes [RsRGs]; Kiba et al., 2007). Many RsRGs showed no similarity with any other known genes and thus might represent novel genes related to plant defense responses. To identify RsRGs that are essential for defense responses, potato virus X (PVX) vector-mediated virus-induced gene silencing was performed in *Nicotiana benthamiana*. We previously reported that RsRGA6, encoding a small heat shock protein, was found to be essential for basic defense against *R. solanacearum* (Maimbo et al., 2007). In this study, we focused on clone RsRGA4, which encoded a protein with similarity to the S-locus glycoprotein (SGP). SGPs have been reported to be involved in the determination of self-incompatibility of *Brassica* plants (Takayama and Isogai, 2003). However, the role of proteins with similarity to SGP (SGP-like protein [SGLP]) in plant species other than *Brassica* is unknown. Moreover, little is known about the role of SGLP in plant defenses. Therefore, we carried out expression profiling and functional analysis of SGLP using *N. benthamiana*. We also discuss a possible mechanism by which this SGLP affects disease resistance.

RESULTS

Full-Length Sequence of RsRGA4

As shown in Figure 1, the deduced amino acid sequence of full-length RsRGA4 contains a secretion signal peptide, a bulb lectin domain, and a PAN apple motif. A protein database search showed 99.4%, 30.4%, 25.5%, and 18.1% amino acid identity with its orthologs from *N. benthamiana*, SGLP from *Ipomoea trifida* (AAA97903), *Medicago truncatula* (ABE82226), and SGP from *Brassica rapa* (BAB21000), respectively. Therefore, we designated RsRGA4 as *NtSGLP* (for *N. tabacum SGLP*), and its ortholog from *N. benthamiana* was designated as *NbSGLP*.

Organ-Specific Expression of *NbSGLP*

SGPs are components of the self-incompatibility mechanism and are expressed in the gynoecium (Takayama and Isogai, 2003). To determine the organ-specific expression of *NbSGLP*, total RNAs were isolated from the stamen, gynoecium, petal, leaf, stem, and root of *N. benthamiana*. Expression of *NbSGLP* was observed in all plant organs tested. The relative strength of expression in the tissues was as follows: root >> leaf > petal > gynoecium > stamen (Fig. 2). Therefore, expression of *NbSGLP* was not restricted to the gynoecium, indicating that *NbSGLP* plays a role in *N. benthamiana* other than in pollen-gynoecium interaction.

Expression of *NbSGLP* Is Induced by Inoculation with *R. solanacearum*

RsRGA4 (*NtSGLP*) was isolated as an *R. solanacearum*-responsive gene, which was induced in *N. tabacum* plants inoculated with a virulent strain of *R. solanacearum* (RsOE1-1) and with an avirulent strain of the bacterium (Rs8107), which induces HR on *Nicotiana* plants (Supplemental Fig. S1). To determine the expression profile of *NbSGLP* in response to *R. solanacearum*, RNA samples were isolated from leaves taken from *N. benthamiana* plants that had been inoculated with virulent strain RsOE1-1 of *R. solanacearum* and with an avirulent strain of the bacterium (Rs8107). The total RNAs were isolated from *N. benthamiana* leaves at 5, 15, and 30 min and at 1, 6, 9, 12, 24, 36, 48, and 72 h after inoculation (HAI) with RsOE1-1 and Rs8107. Expression analysis of *NbSGLP* by quantitative reverse transcription (qRT)-PCR showed that strong induction of *NbSGLP* was observed in Rs8107-inoculated *N. benthamiana*, and the strong expression was observed at 15 to 30 min after inoculation and 12 and 48 HAI. Expression of *NbSGLP* was slightly induced by inoculation with RsOE1-1 at 1 and 24 HAI (Fig. 3A).

Relationship between Expression of *NbSGLP* and Induction of Cell Death

Expression of *NbSGLP* was significantly induced in tobacco leaves inoculated with the HR-causing bacterial strain (Rs8107). To examine the relationship between cell death and induction of *NbSGLP* expression, the effect of a cell death-triggering agent, INF1-expressing *Agrobacterium tumefaciens* (Katou et al., 2003), was determined. We also examined the expression pattern of *NbSGLP* induced by inoculation with an *hrpY* (encoding the Hrp pilus) mutant of Rs8107 (Rs8107ΔY) that is not able to induce an HR and an *hrpY* mutant of RsOE1-1 (RsOE1-1ΔY). Expression of *NbSGLP* was induced in *N. benthamiana* plants inoculated with GUS-expressing control *A. tumefaciens*. INF1-expressing *A. tumefaciens* induced NbSGLP expression similarly to the GUS-expressing control at 12 and 24 HAI (Fig. 3B). *NbSGLP* was also up-regulated in *N. benthamiana* leaves inoculated with Rs8107ΔY, with

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NtSGLP   1: M-------EAINIHFF-L-FFILILY--G-AADTIPVDQPLTD--GN-TFISSGGKFELG 45
NbSGLP   1: .-------........-.-.......--.-.............--..-.I.......... 45
ItSGLP   1: .------SSSSHCSL.S.-..F..FHKISW.....TATLSVK.GE.KT.IV..N.T.... 53
MtSGLP   1: .DSI-KVL--VY--C.L.--.---HFIPTFN.LETIVSGQS--IKD.E.L..KD.T..A. 48
BrSGP    1: .KGVRKPYDNSYTFS.L.V..V...FRPTF-SINTLSSTESLTVSI.R.LV...NV..L. 59

NtSGLP  46: FFSPGTSRKRYIGIWFNKVSVQTVVWVANGDSPLNDRDGMLNFTRQGILTLFNGSGHVIW 105
NbSGLP  46: .........W......S...I................N.............L........ 105
ItSGLP  54: .....K.EN..V....KNI..T.......RET...TTS.I.KIIKP...V.L.EDNAT.. 113
MtSGLP  49: ..NFGN.NNQ.F.V.YKNI.PK.L..I..R.V..GNSS.V..L.DK.T.VIVDSKEVT.. 108
BrSGP   60: ..RTNS.SRW.LGI.YKKM.ER.Y.....R.N..SNSI.T.KISGN-N.V.LGYSNKPV. 118

NtSGLP 106: SSNATRRVKN--SKAQLLDSGNLVVRDA-TV----NYLWQSFDYPSDTSLPGMKVGIDLK 158
NbSGLP 106: ......YAQ.--.T..............-..----..........T.......E...... 158
ItSGLP 114: .T.TS.S.Q.--PI..........IK..GDGNEEK.F........T..Y.....I.WNFV 171
MtSGLP 109: ...-.STTTSKP.L-...E....IVK.---EIDPDKI......L.G..L....SIRTN.V 163
BrSGP  119: .T.R..GNESSLVV.E...N..F.M..-SNNNEASQF........T..L..E..L.YD.K 177

NtSGLP 159: TGFHRSLWSWKSTNDPSRGEF-TWTFDPRGFPQPFIMNGSTERHRFGPWNGRGFASAPSR 217
NbSGLP 159: ...R......E..........-......H...........I..........L........ 217
ItSGLP 172: ..HETF.S....SE..AT...-KYSINRN.Y....LKR..VV.Y.S.....FQ.SGSLNS 230
MtSGLP 164: N.DYKG.V..RD.Q..AT.LYSYHIDTN-.Y..VV.TK.D.LFF.I.S....ILSGI..E 222
BrSGP  178: K.LN.F.T..RNSD...S..ISYKLDIQ..M.EFYLLKS.LRAH.S.P...DR.SGI.-. 236

NtSGLP 218: LPSPGY-KYIYVSDPEKISIVYQLTDSSIFARVVMQLDGVLQLSIWNNQTQNWDNYFGSA 276
NbSGLP 218: ......-..T...N......M.E.....................L............... 276
ItSGLP 231: RQ..F.-EIGF.FN.REAYFTNH.LQ-PVITKATLSWN.L.ERTT.VDR..R.VL.L-NV 287
MtSGLP 223: TLYKA.-NFSF.ITEKE..YG.E.LNK.VVS.YLVSST.QIARYMLSD..NS.QLF.VGP 281
BrSGP  237: DQYLS.MV.NFIKNSEEVVYTFRLNDN.IYSRLKISSE.F.ERLT.TPTSIA.NLFWSAP 296

NtSGLP 277: PADNCDIYSRCHAYSLCNNGNSSICSCLDQFEPKNPTEWARENWTSGCVRKATLN QKEV 336
NbSGLP 277: ...D........G...............................S......T...  ..K. 336
ItSGLP 288: .T.T....KL.G..GK..IQT.PV.G...K.V...EAD.LKAD.S..CE.RTA.S NEGE 347
MtSGLP 282: AD-S..N.AI.G.N.N.DIDK.PV.E..EG.V..SQAN.SLQ..SD.....VK.D DNND 340
BrSGP  297: VDLK..VYKA.GV..Y.DENT.PV.N.IQG.M.L.EQR.DLRD.....T.RTR.S -SGD 355

NtSGLP 337: KFLKYPGIKLPDTRFSWYDQGVNLSACEELCLRNCSCVAYANPDITGTNEGCLLWFDELI 396
NbSGLP 337: ..................N......T...........A...................... 396
ItSGLP 348: G....S.......QSV.FN.TMT.EE.KTR.......M..SNIE.RNGGT..FM...... 407
MtSGLP 341: G...HMRM.....SK..FNKSM..EE.ERF.I.....T....L.VRDGGS......NN.L 400
BrSGP  356: D.TMMKNM.....TMATVDRSIDVKE..KR..SD.N.T.F..T..RDGGT..VI.TG..D 415

NtSGLP 397: DIRDLGAS-GQDIYIKLDSSQ-S-G-TS----LLLY----------LCM            427
NbSGLP 397: ...RSWVLV.K.........G-.EN-S.VEKVKK.RIS.PLAASIL.LA            443
ItSGLP 408: ...LVPNE......RVAA.ELGKSFAPIH..VIST..FRTLCKVMIT--            454
MtSGLP 401: .V.K.PSG...L..RVA..ASASELG.NT.FSSVR--------------            435
BrSGP  416: .M.TYF.N...L.VR.APADLV---------------------------            437
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Figure 1. Deduced amino acid sequences of *NtSGLP* and *NbSGLP*. Alignment of the deduced amino acid sequences of S-locus glycoprotein-like proteins from *N. tabacum* (NtSGLP), *N. benthamiana* (NbSGLP), *Ipomoea trifida* (ItSGLP), and *Medicago truncatula* (MtSGLP) and S-locus glycoprotein from *Brassica rapa* (BrSGP). Dots indicate identical amino acids. Dashes show amino acids that are not present in the sequences. The white box shows the signal peptide for extracellular secretion. The gray box indicates the bulb lectin domain. The black box indicates the PAN apple domain.

kinetics similar to that in *N. benthamiana* leaves inoculated with the wild-type Rs8107. In addition, RsOE1-1ΔY also induced *NbSGLP* expression in *N. benthamiana* leaves (Fig. 3C). Therefore, expression of *NbSGLP* was induced in *N. benthamiana* by *A. tumefaciens* and by an *hrp* mutant of *R. solanacearum*. These results suggested that expression of the *NbSGLP* gene could be induced without cell death.

Effect of Intracellular Signaling Molecules on *NbSGLP* Expression

To elucidate the signaling pathways related to *NbSGLP* expression, well-known intracellular signaling molecules were infiltrated into *N. benthamiana* leaves. The signaling molecules used in this study were SA, methyl jasmonate (JA), the ethylene (ET) precursor aminocyclopropane carboxylic acid (ACC), and hydrogen peroxide (H_2O_2). Total RNA was isolated at 12, 24, and 48 h after treatment. Expression of *NbSGLP* was barely affected by the treatment with JA and H_2O_2. *NbSGLP* was induced at 24 h after treatment with ACC. Among these chemicals, SA was the most effective at inducing the expression of *NbSGLP*. Expression of *NbSGLP* increased at 12 to 48 h after SA treatment, and the expression level increased by a factor of 40 in comparison with nontreated tobacco plants at 48 h after treatment (Fig. 3C). These results suggested that induction of *NbSGLP* was mediated by both the ET and SA pathways.

Virus-Induced Gene Silencing of *NbSGLP* in *N. benthamiana*

Expression of *NbSGLP* was observed in *N. benthamiana* plants inoculated with *R. solanacearum* and in those

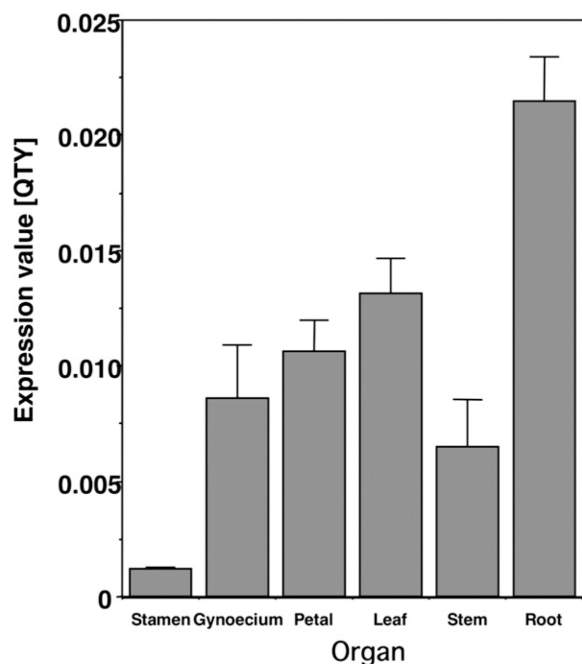

Figure 2. Organ-specific expression of *NbSGLP*. Total RNA was isolated from root, leaf, stem, petal, gynoecium, and stamen of *N. benthamiana*. Quantitative real-time PCR was carried out as described in "Materials and Methods." Expression values for *NbSGLP* are expressed as Qty values after normalization with the actin value. Values represent means and SD from triplicate experiments.

treated with well-known signaling molecules, such as ACC and SA (Fig. 4). This information prompted us to test the function of *NbSGLP* in plant defense. We carried out a virus-induced gene silencing approach in *N. benthamiana* using the PVX vector. Three weeks after inoculation, there were no phenotypic differences between plants infected with *A. tumefaciens* carrying an empty pPVX201 vector and those with bacteria carrying pPVX-SGLP (data not shown). Analysis by qRT-PCR confirmed that the *NbSGLP* gene was silenced (Fig. 4).

Silencing of *NbSGLP* Compromised the Expression of Defense-Related Genes

To test the influence of the silencing of *NbSGLP* on expression of defense-related genes, total RNA was extracted from *NbSGLP*-silenced and control leaves at 6, 9, 12, and 24 HAI with Rs8107. As shown in Figure 4, expression of *PR-1a* (a marker gene for the SA signal pathway) was induced in control plants at 24 HAI with Rs8107, whereas *PR-1a* expression was compromised in *NbSGLP*-silenced plants. Expression of *EREBP* (a marker gene for the ET signal pathway) was observed in control plants at 6 to 24 HAI with Rs8107. However, the expression of *EREBP* was greatly reduced in *NbSGLP*-silenced leaves throughout the experiment. In contrast, the expression level of *PR-4* (a marker gene for the JA signal pathway) was enhanced in *NbSGLP*-silenced leaves. The reductions in *PR-1a* and *EREBP* expression levels were consistent with the *NbSGLP* responses to SA and ACC (Fig. 3C). These results suggested that *NbSGLP* might have an important role (s) in the expression of defense-related genes through the SA and ET pathways.

Silencing of *NbSGLP* Accelerates Growth of *R. solanacearum* and Disease Development of Bacterial Wilt

A reduction of defense-related gene expression was observed in *NbSGLP*-silenced plants, which raised the possibility that disease resistance to nonpathogenic bacteria is compromised and disease susceptibility to pathogenic bacteria might increase in the silenced plants. To address whether silencing of *NbSGLP* affected the growth of Rs8107, the bacterial suspension was inoculated into *NbSGLP*-silenced leaves and control leaves. The bacterial population was determined at 18 and 24 HAI. As shown in Figure 5A, growth of Rs8107 was significantly enhanced in *NbSGLP*-silenced plants at 24 HAI, showing an approximately 10-fold increase in comparison with control plants. We then confirmed the effect of *NbSGLP* silencing on the growth of RsOE1-1. Enhancement of the growth of RsOE1-1 was also observed in *NbSGLP*-silenced plants at 1 and 2 d after inoculation (Fig. 5B). However, acceleration of the RsOE1-1 growth (approximately 5-fold) was not remarkable in comparison with the growth of Rs8107 (approximately 10-fold). These results might reflect the expression level of *NbSGLP* in response to the two bacterial strains. We also observed the phenotypes of *NbSGLP*-silenced and control plants challenged with RsOE1-1. In control plants, bacterial wilt was first observed at 13 d, and the plants were completely wilted at 15 d after inoculation with RsOE1-1. When challenged with RsOE1-1, *NbSGLP*-silenced plants started to wilt at 9 d and were completely wilted at 12 d (Fig. 5, C and D).

Silencing of *NbSGLP* Accelerates Growth of Phytopathogenic Bacteria

The growth of *R. solanacearum* and appearance of bacterial wilt were accelerated in *NbSGLP*-silenced plants. We then tested the role of *NbSGLP* in defense responses of *N. benthamiana* against bacterial pathogens other than *R. solanacearum*. As shown in Figure 6, growth of *Pseudomonas cichorii*, which is nonpathogenic and induces HR to *N. benthamiana*, was significantly enhanced in *NbSGLP*-silenced plants at 12 and 24 HAI, and an approximately 10-fold increase was observed in comparison with control plants at 24 HAI. Intriguingly, enhancement of the growth of *Pseudomonas syringae* pv *tabaci* and *P. syringae* pv *mellea*, both of which are pathogenic to *N. benthamiana*, was also observed in the silenced plants. How-

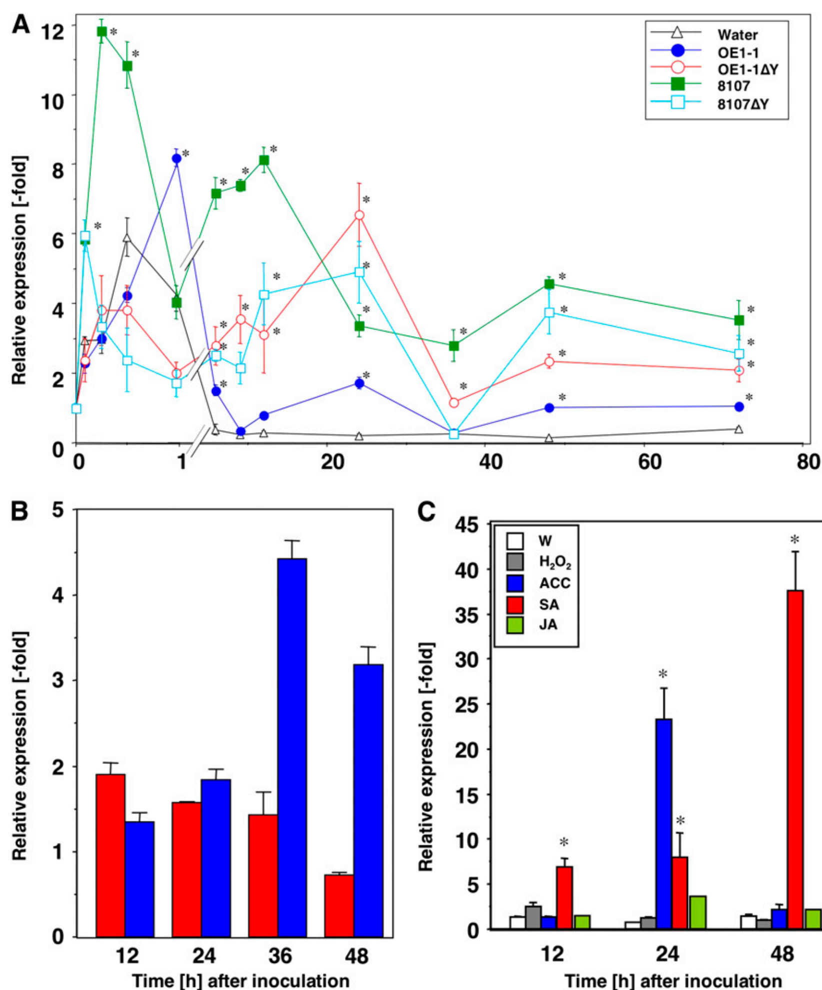

Figure 3. Induction pattern of *NbSGLP* expression. Expression values of *NbSGLP* are relative to the absolute nontreated control level and are normalized against actin values. Values represent means and SD from triplicate experiments. A, Total RNA was isolated from *N. benthamiana* leaves infiltrated with water, RsOE1-1 (OE1-1), *hrpY* mutant of RsOE1-1 (OE1-1ΔY), Rs8107 (8107), or *hrpY* mutant of Rs8107 (8107ΔY) after incubation at 25°C for the indicated times. B, Total RNA was isolated from *N. benthamiana* leaves infiltrated with INF1-expressing (red bars) or GUS-expressing (blue bars; control) *A. tumefaciens* at the indicated time points. C, Total RNA was isolated from *N. benthamiana* leaves at 12, 24, and 48 h after infiltration with water (W), SA, JA, H_2O_2, or ACC. Quantitative real-time PCR was carried out as described in "Materials and Methods." Asterisks denote values significantly different from nontreated control leaves ($P < 0.05$).

ever, acceleration of the growth of *P. syringae* pv *tabaci* and *P. syringae* pv *mellea* was only observed in later stages of infection (24 HAI) and was not remarkable (about 5-fold increase) in comparison with *P. cichorii* (10-fold increase).

HR Cell Death Caused by *R. solanacearum*, *P. cichorii*, and INF1 Are Not Affected by Silencing of *NbSGLP*

The HR is one of the best-characterized plant defenses against pathogens. To examine the response of *NbSGLP*-silenced plants to HR triggering, nonpathogenic bacteria Rs8107 and *P. cichorii* and HR-like cell death-inducing INF1-expressing *A. tumefaciens* were inoculated into *NbSGLP*-silenced plants and control plants. An HR lesion developed in both the control and *NbSGLP*-silenced plants at 48 HAI with Rs8107, *P. cichorii*, and INF1-expressing *A. tumefaciens* (Fig. 7A). Cell death timing was similar in control and *NbSGLP*-silencing plants by all HR-triggering infiltrations, as detected by Evans blue staining (Fig. 7B). These results suggested that NbSGLP might not act upstream of cell death induction.

Silencing of *NbSGLP*-Compromised Basal Defenses

Expression of *NbSGLP* was induced by the inoculation of an *hrp* mutant of *R. solanacearum*, and growth of several phytopathogenic bacteria was also accelerated in *NbSGLP*-silenced plants. These results suggested a role for NbSGLP in basal defenses. To check whether silencing of *NbSGLP* affected the induction of basal defenses, *hrp* mutants of *R. solanacearum* (Rs8107ΔY and RsOE1-1ΔY) were inoculated into *NbSGLP*-silenced leaves and control leaves. Callose deposition, a well-known marker of basal defense, was induced in control plants inoculated with both Rs8107ΔY and RsOE1-1ΔY. In contrast, callose deposition was reduced in *NbSGLP*-silenced plants (Fig. 8A). As another marker of basal defense, we also analyzed vascular flow in *NbSGLP*-silenced leaves and control leaves. Reduction of vascular flow was observed in control plants inoculated with both Rs8107ΔY and RsOE1-1ΔY. On the other hand, the reduction of vascular flow was compromised in *NbSGLP*-silenced plants (Fig. 8B). These results suggested that NbSGLP might have a role in the induction of basal defenses.

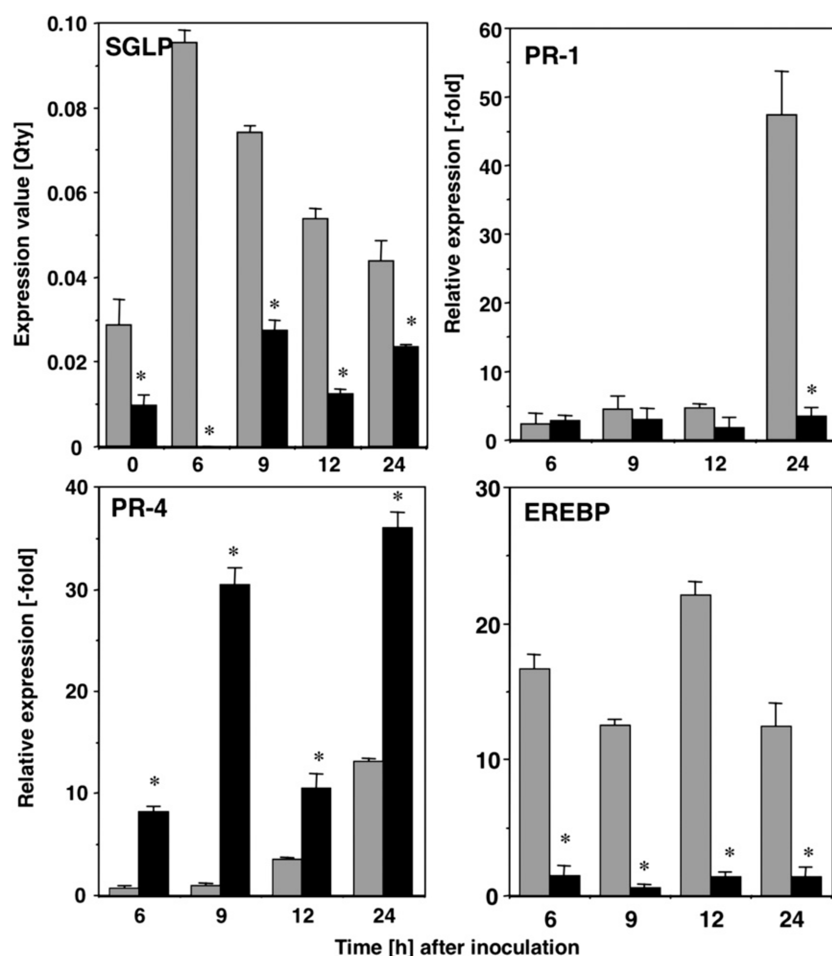

Figure 4. Virus-induced gene silencing of *NbSGLP* and effect of silencing of *NbSGLP* on the expression of defense-related genes. *N. benthamiana* plants were infected with *A. tumefaciens* carrying either PVX (gray bars) or PVX:SGLP (black bars). Three weeks later, the fourth leaves above the primary *A. tumefaciens*-infected leaves were infiltrated with Rs8107, and total RNA was isolated at the indicated time points. The relative abundances of *NbSGLP*, *PR-1a*, *PR-4*, and *EREBP* transcripts were analyzed using quantitative real-time PCR with primer combinations described in Supplemental Table S1. Quantitative real-time PCR was carried out as described in "Materials and Methods." Expression values of *NbSGLP* are expressed as Qty values after normalization with the actin value. Expression values of *PR-1a*, *PR-4*, and *EREBP* are relative to the absolute nontreated control level and are normalized against the actin values. Values represent means and SD of results from triplicate experiments. Asterisks denote values significantly different from empty PVX controls ($P < 0.05$).

NbSGLP Is an Extracellular Secreted Protein

The deduced amino acid sequence of *NbSGLP* contained a signal peptide for extracellular secretion. To determine whether NbSGLP protein is secreted outside the cell or not, the full-length open reading frame (ORF) of *NbSGLP* and an ORF encoding a signal peptide-deleted *NbSGLP* (*NbSGLPΔS*) were transiently expressed in *N. benthamiana* leaves under the control of the 35S promoter. Immunoblot analysis was carried out with intercellular washing fluids (IWFs) and intracellular fractions (ICFs) from *NbSGLP*- or *NbSGLPΔS*-expressing *N. benthamiana*. As shown in Figure 9A, immunoreactive protein was detected in ICFs from both *NbSGLP*- and *NbSGLPΔS*-expressing *N. benthamiana* by immunoblot analysis with anti-Flag antibody, confirming the synthesis of NbSGLP and NbSGLPΔS proteins in *N. benthamiana* leaves. Immunoblot analysis of IWF from *NbSGLP*-expressing leaves showed the presence of an immunoreactive protein band. On the other hand, there was no immunoreactive protein detected in the IWF from *NbSGLPΔS*-expressing *N. benthamiana* leaves. An antibody against cytochrome oxidase (an intracellular marker protein) recognized a protein in the ICFs but not in the IWFs. These results suggested that NbSGLP protein was secreted outside plant cells mediated by the function of the N-terminal signal peptide.

Induction Pattern of Defense-Related Genes by Transient Expression of *NbSGLP*

To analyze the effect of transient expression of *NbSGLP* on defense-related gene expression, full-length *NbSGLP* and signal peptide-deleted *NbSGLPΔS* were transiently expressed in *N. benthamiana* leaves under the control of the 35S promoter (Fig. 9B). qRT-PCR confirmed high-level expression of *NbSGLP* and *NbSGLPΔS* in *N. benthamiana* leaves in comparison with GUS-expressing control leaves, and similar levels of expression were achieved for both constructs. Expression of *PR-1a* and *EREBP* were significantly up-regulated in *N. benthamiana* leaves that transiently expressed the full-length *NbSGLP*. These results suggested that NbSGLP has a role in the regulation of SA- and ET-dependent signaling pathways. The expression level of both PR genes was drastically reduced in *NbSGLPΔS*-expressing leaves in comparison with the full-length *NbSGLP*-expressed leaves. In addition to these experiments, we analyzed the induction of these defense-related genes by treatment with

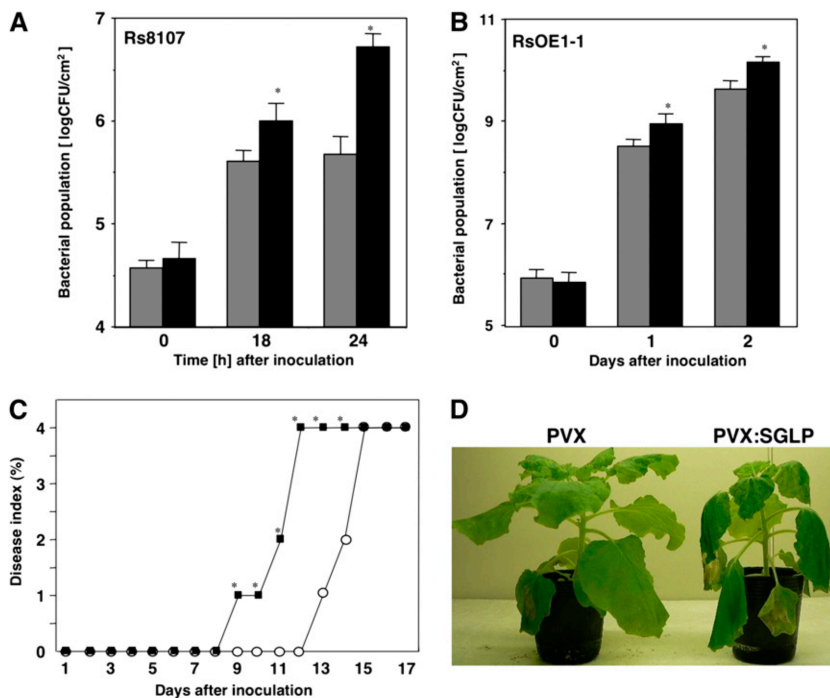

Figure 5. Effects of *NbSGLP* silencing on growth of *R. solanacearum* and development of bacterial wilt. *N. benthamiana* leaves were preinoculated with *A. tumefaciens* either carrying empty PVX (gray bars) or PVX:SGLP (black bars). A, Control and *NbSGLP*-silenced leaves were infiltrated with a bacterial suspension of Rs8107. B, Three weeks later, the fourth leaves above the primary *A. tumefaciens*-infected leaves were infiltrated with a bacterial suspension of RsOE1-1. The bacterial population was determined by plating at specified time points. Values are means of four replicate experiments with SD. Asterisks denote values significantly different from empty PVX controls ($P < 0.05$). C, Disease development of bacterial wilt was rated daily on a 0 to 4 disease index in empty PVX (white circles) or PVX:SGLP (black boxes). Each point represents the mean disease index of 10 plants combined from three separate experiments. Asterisks denote values significantly different from empty PVX controls ($P < 0.05$). D, Characteristic symptoms of bacterial wilt in control (PVX) and *NbSGLP*-silenced (PVX:SGLP) *N. benthamiana*. The photograph was taken 12 d after inoculation with RsOE1-1. [See online article for color version of this figure.]

the IWF fraction from the full-length *NbSGLP*- and *NbSGLPΔS*-expressing leaves. As shown Figure 9C, both *PR-1a* and *EREBP* expression was induced by the treatment with IWF fraction from the full-length *NbSGLP*-expressed leaves. In contrast, no significant induction was observed in *N. benthamiana* leaves treated with IWF fraction from the *NbSGLPΔS*-expressed leaves. These results suggested that induction of these defense-related genes requires an extracellularly secreted NbSGLP.

Induction of Basal Defenses by Transient Expression of *NbSGLP* and Its Dependency on SA

The callose deposition and reduction of vascular flow were compromised in *NbSGLP*-silenced plants (Fig. 8), indicating the role of NbSGLP in the induction of basal defenses. Transient expression of *NbSGLP* induced callose deposition and reduction of vascular flow, whereas these basal defenses were not induced in *N. benthamiana* by transient expression of *NbSGLPΔS* (Fig. 10). Intriguingly, *NbSGLP*-induced callose deposition and reduction of vascular flow were not observed in SA-deficient *N. benthamiana* NahG plants (Fig. 11). These results suggested that NbSGLP has an ability to induce basal defenses, and SA is required for induction of the basal defenses.

DISCUSSION

In this study, we isolated genes from *N. tabacum* and *N. benthamiana* that were similar to *SGP* from *Brassica rapa* (*NtSGLP* and *NbSGLP*). SGPs were originally reported to be encoded by the S-locus, which is well known to be involved in the determination of self-

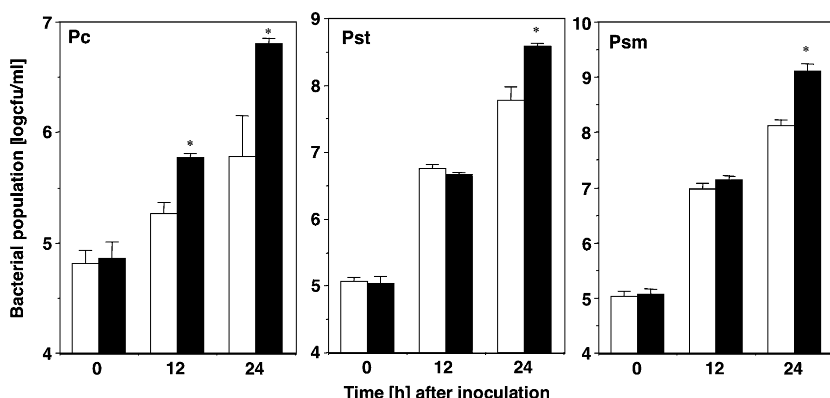

Figure 6. Effects of *NbSGLP* silencing on growth of phytopathogenic bacteria. *N. benthamiana* leaves were preinoculated with *A. tumefaciens* either carrying empty PVX (white bars) or PVX:SGLP (black bars). Control and *NbSGLP*-silenced leaves were infiltrated with a bacterial suspension of *P. cichorii* (Pc), *P. syringae* pv *tabaci* (Pst), or *P. syringae* pv *mellea* (Psm). The bacterial population was determined by plating at specified time points. Values are means of four replicate experiments with SD. Asterisks denote values significantly different from empty PVX controls ($P < 0.05$).

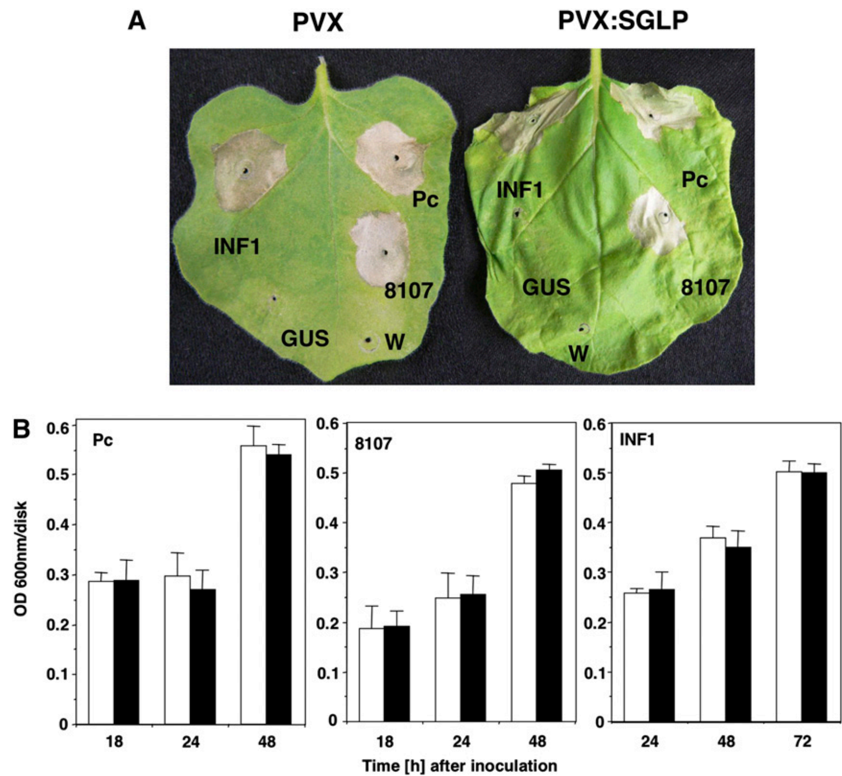

Figure 7. Effects of *NbSGLP* silencing on induction of HR. *N. benthamiana* plants were infected with *A. tumefaciens* carrying either PVX or PVX:SGLP. Control and silenced leaves were infiltrated with Rs8107 (8107), *P. cichorii* (Pc), or *A. tumefaciens* harboring GUS (control; GUS) or INF1 (INF1). A, Photographs were taken 4 d after infiltration of each bacterium. W, Water. B, Cell death was estimated in control (white bars) and *NbSGLP*-silenced (black bars) plants inoculated with *P. cichorii*, Rs8107, or *A. tumefaciens* harboring INF1 by Evans blue staining, as described in "Materials and Methods." [See online article for color version of this figure.]

incompatibility of *Brassica* plants (Takayama and Isogai, 2003). Database searching showed that SGLPs exist not only in *Nicotiana* plants but also in *Medicago* and *Ipomoea* plants. Therefore, SGLPs are evolutionally conserved in plants. However, little is known about the role of SGLP in plant species other than *Brassica*. The organ-specific expression patterns of *NbSGLP* indicated a different role of SGLP than in pollen-stigma interaction (Fig. 2). Expression analysis of *NtSGLP* and *NbSGLP* showed greatly increased expression in *Nicotiana* plants in response to an avirulent strain of *R. solanacearum* (Rs8107) in comparison with the virulent strain of *R. solanacearum* (RsOE1-1; Fig. 3A; Supplemental Fig. S1). These results suggested that SGLP might have a role in plant defense responses in *Nicotiana* plants against *R. solanacearum*.

Plant defense responses are initiated by the recognition of pathogen-derived molecules (Nurnberger and Brunner, 2002; Schulze-Lefert, 2004) and are divided into two categories: effectors-triggered immunity (ETI) and pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI; Jones and Dangl, 2006). In the case of pathogenic bacteria, the type III secretion apparatus encoded by the *hrp* genes enables effector proteins to be injected into plant cells. Effector proteins are recognized by plant cells, after which ETI develops. *hrp*-deficient bacterial pathogens lose the ability to induce ETI but still induce PTI. PTI is triggered by the recognition of generally conserved elicitors (PAMPs). PTI effectively restricts the growth of a majority of potential pathogens (Ma and Guttman, 2008). Rapid induction of *NbSGLP* expression was

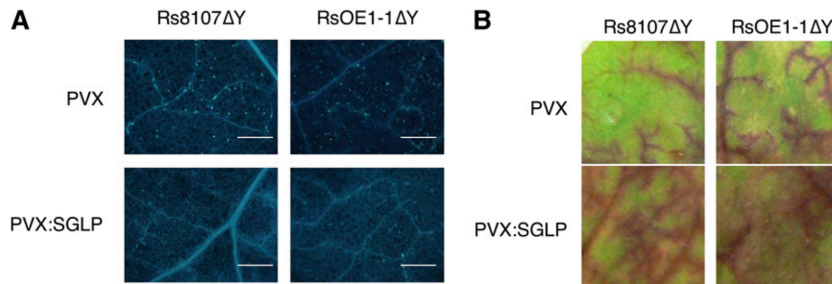

Figure 8. Effects of *NbSGLP* silencing on induction of basal defenses. *N. benthamiana* plants were infected with *A. tumefaciens* carrying either PVX or PVX:SGLP. Control and *NbSGLP*-silenced leaves were infiltrated with the *hrp* mutant of Rs8107 (Rs8107ΔY) and RsOE1-1 (RsOE1-1ΔY) and incubated for 24 h. A, Callose deposition was detected by aniline blue staining. Bars = 100 μm. B, Vascular flow was visualized by staining with Safranin O. [See online article for color version of this figure.]

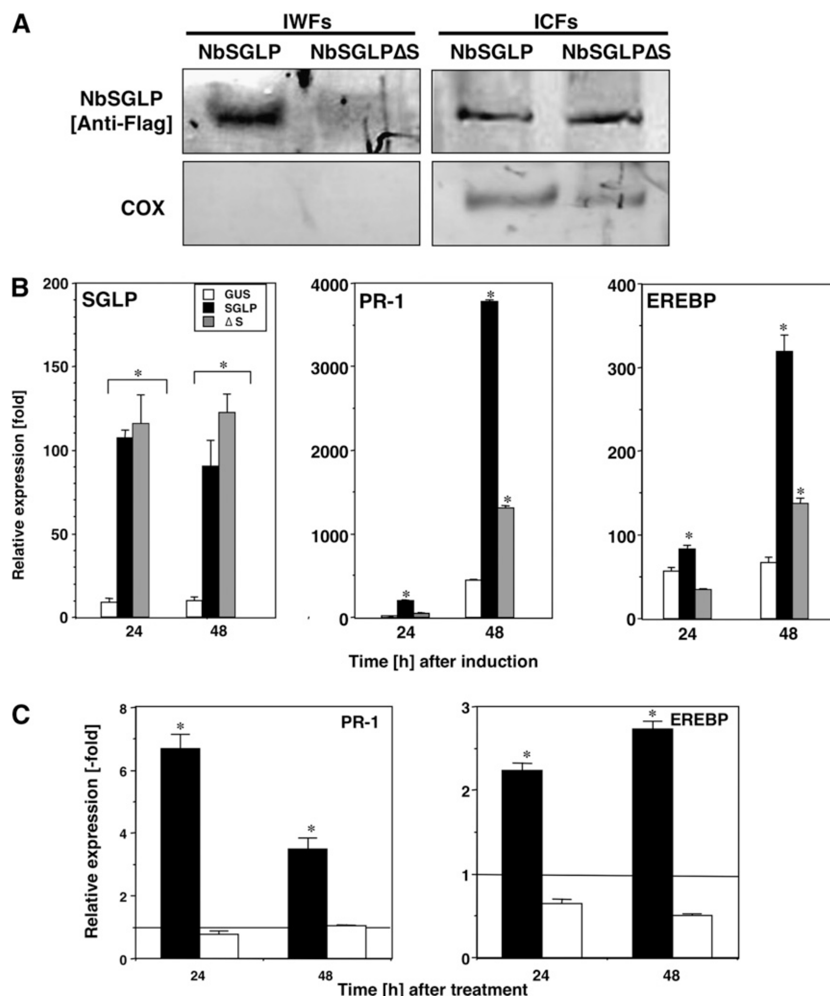

Figure 9. Transient expression of *NbSGLP* and induction of defense-related genes in *N. benthamiana*. A, The IWFs and ICFs were prepared from *N. benthamiana* leaves inoculated with full-length *NbSGLP*- and signal peptide-deleted *NbSGLPΔS*- expressing *A. tumefaciens*. Both IWFs and ICFs were separated by 15% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes. The blots were subjected to western-blot analyses with monoclonal antibodies raised against the Flag tag sequence (NbSGLP [Anti-Flag]) and cytochrome oxidase (COX). B, Total RNA was isolated from *N. benthamiana* leaves inoculated with GUS, full-length *NbSGLP*-, and signal peptide-deleted *NbSGLPΔS*-expressing *A. tumefaciens* at the indicated time points. C, Total RNA was isolated from *N. benthamiana* leaves treated with the IWF fraction from *NbSGLP*-expressing (black bars) or *NbSGLPΔS*-expressing (white bars) leaves. The relative abundances of *PR-1a* and *EREBP* transcripts were analyzed using quantitative real-time PCR with primer combinations described in Supplemental Table S1. Expression values of *NbSGLP*, *PR-1a*, and *EREBP* are relative to the absolute nontreated control level and are normalized against the actin values. Lines showed expression levels of respective genes in nontreated control plants. Values represent means and SD from triplicate experiments. Asterisks denote values significantly different from nontreated controls ($P < 0.05$).

induced in *N. benthamiana* leaves within 5 min after inoculation of Rs8107. The expression of *NbSGLP* was also induced in *N. benthamiana* leaves inoculated with *hrp* mutants of *R. solanacearum* (RsOE1-1ΔY and Rs8107ΔY). Moreover, GUS-expressing *A. tumefaciens* also enhanced *NbSGLP* expression (Fig. 3). Therefore, *NbSGLP* might be induced by PAMPs recognition and be related to induction of PTI.

The growth of Rs8107 was accelerated in *N. benthamiana* plants in which *NbSGLP* was silenced (Fig. 5A), whereas Rs8107 did not systemically spread, was restricted to the infiltrated area, and did not cause bacterial wilt in the silenced plants (data not shown). Reduction in expression of defense-related genes, including *PR-1a* and *EREBP*, was observed in *NbSGLP*-silenced plants challenged with Rs8107 (Fig. 4). Generally, plant resistance responses to avirulent bacterial pathogens are extremely complex and are likely to involve myriad cellular processes, including expression of PR proteins (Maleck et al., 2000; Mysore et al., 2002; Tao et al., 2003). Therefore, reduction of disease resistance seems to be caused by a reduction in the expression of defense-related genes. In contrast, induction of HR by Rs8107 and INF1 was scarcely

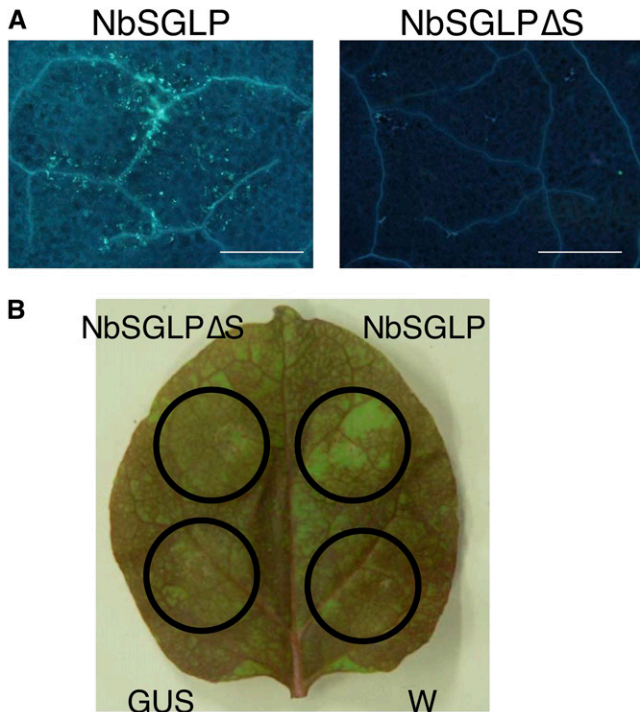

Figure 10. Transient expression of *NbSGLP* and induction of basal defenses in *N. benthamiana*. A, *N. benthamiana* plants were inoculated with full-length *NbSGLP*- and signal peptide-deleted *NbSGLPΔS*-expressing *A. tumefaciens*. Callose deposition was detected by aniline blue staining. Bar = 100 μm. B, *N. benthamiana* plants were infiltrated with water (W), GUS, and full-length *NbSGLP*- and signal peptide-deleted *NbSGLPΔS*-expressing *A. tumefaciens*. Vascular flow was visualized by staining with Safranin O. [See online article for color version of this figure.]

affected by *NbSGLP* silencing (Fig. 7). Therefore, disease resistance is at least partially mediated by NbSGLP, but HR-mediated defense seems not to be related to NbSGLP.

In addition to HR-mediated defense, plants show a symptomatic defense. The defense is defined as basal defense and is reportedly induced by *hrp*-deficient bacterial pathogens (Klement et al., 1999; Szatmari et al., 2005). Basal defense is also activated by virulent pathogens on susceptible hosts (Jones and Dangl, 2006). Our study showed that expression of *NbSGLP* was induced in an *hrp* gene-independent manner (Fig. 3B). Expression of *NbSGLP* was induced not only by avirulent Rs8107 but also by virulent RsOE1-1 (Fig. 3A). Growth of RsOE1-1, and development of bacterial wilt, were accelerated in *NbSGLP*-silenced plants (Fig. 5, C and D). In addition to changing the expression pattern of *NbSGLP*, *hrp* mutants of *R. solanacearum*, such as RsOE1-1ΔY and Rs8107ΔY, induced callose deposition and reduced vascular flow, both of which are markers for basal defense in *N. benthamiana*, whereas these basal defenses were reduced in *NbSGLP*-silenced plants (Fig. 8). The same phenomenon was observed by transient expression of *NbSGLP* (Fig. 10). Taken together, these results suggest that NbSGLP (and NtSGLP) might have a role in the regulation of basal defense responses.

During the initiation of defense responses, signal transduction cascades are triggered and endogenous signaling compounds are produced. In tobacco, SA is a critical signaling molecule leading to the expression of acidic types of PR genes (Ohashi and Ohshima, 1992; Pontier et al., 1994; Guo et al., 2000). JA also mediates the expression of basic types of PR genes. It has also been shown that SA and JA antagonistically inhibit SA and JA production and SA- and JA-dependent PR gene expression, respectively (Niki et al., 1998). In addition to SA and JA, ET also has a crucial role in plant defense (Block et al., 2005). An ET-responsive factor, TSRF1, is reportedly up-regulated by ET, SA, or *R. solanacearum* infection. Overexpression of TSRF1 in tobacco and tomato constitutively activates the expression of PR genes and subsequently enhances transgenic plant resistance to the bacterial wilt caused by *R. solanacearum* (Zhang et al., 2004). Our previous study demonstrated that expression of *PR-1a* was induced by the infiltration of Rs8107, and Rs8107-induced acquired resistance was mediated by the SA-dependent signaling pathway (Kiba et al., 2003). Therefore, SA- and ET-dependent defense responses might have crucial roles in plant defense against *R. solanacearum*. In this study, *NbSGLP* was induced by treatment with SA and ACC (Fig. 3). Transient expression of *NbSGLP* showed significant induction of *PR-1a* and *EREBP* but not PR-4 (Fig. 9B; Supplemental Fig. S2). In contrast, a reduction in expression of *PR-1a* and *EREBP* was observed in

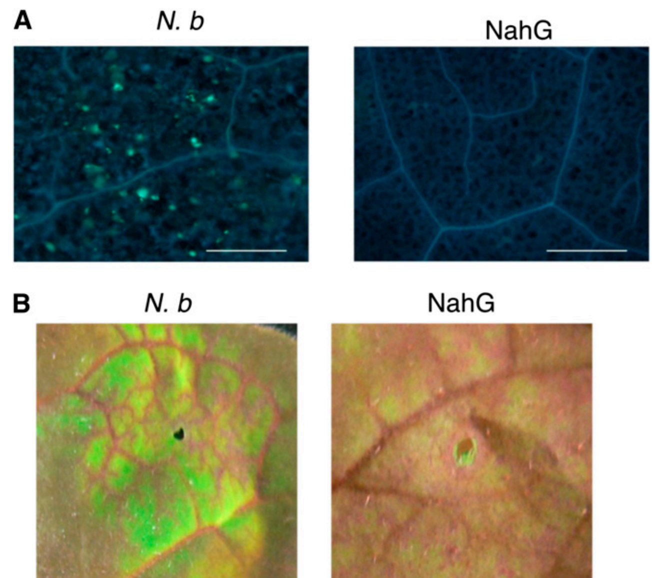

Figure 11. Role of SA in *NbSGLP*-induced basal defenses in *N. benthamiana*. The full-length *NbSGLP*-expressing *A. tumefaciens* was inoculated into *N. benthamiana* (N. b) and *N. benthamiana* NahG. A, Callose deposition was detected by aniline blue staining. Bars = 100 μm. B, Vascular flow was visualized by staining with Safranin O. [See online article for color version of this figure.]

NbSGLP-silenced plants, whereas expression of *PR-4* was enhanced in the silenced plants (Fig. 4). Therefore, NbSGLP might have a role in the regulation of SA- and ET-dependent defense and might antagonistically suppress the JA-dependent signaling pathway. Intriguingly, *NbSGLP*-induced basal defenses were compromised in SA-deficient NahG plants. Recent reports indicate that SA has a critical role in the induction of basal defense in potato against *Phytophthora infestans* (Halim et al., 2007). SA-dependent basal immunity was also reported to be required for plant defense against bacterial pathogens, such as *Erwinia amylovora*, *P. syringae*, and *Pantoea stewartii* in Arabidopsis plants (DebRoy et al., 2004). Taken together, these results suggest that NbSGLP (and NtSGLP) might have a role in the regulation of basal defense responses through the SA-dependent pathway.

Structural analysis of the deduced amino acid sequence of *NbSGLP* showed two conserved motifs: a bulb lectin domain (B-type lectin) and a PAN apple domain. The most characterized proteins containing B lectin and the PAN domain are S receptor kinases and the SGP encoded by the S-locus of *Brassica* plants, which is well known to be involved in the determination of self-incompatibility of *Brassica* plants (Takayama and Isogai, 2003). Recently, the dominant resistance gene, *Pi-d2*, which confers resistance to rice (*Oryza sativa*) blast, has been shown to encode a receptor-like kinase protein with a predicted extracellular domain comprising B-lectin and PAN domains (Chen et al., 2006). Taken together, there are at least two hypothetical models for the function of SGLP in *Nicotiana* plants. A possible hypothetical model is that SGLP might act as a component of a receptor complex and recognize pathogen-derived molecules (PAMPs/elicitors). Another possible model is that SGLP might be involved in intercellular (infected cell to peripheral cells) signal transduction. Further analysis will be required to determine the cognate receptor or elicitor molecule(s) to clarify the SGLP-mediated signaling cascade leading to plant defense.

MATERIALS AND METHODS

Plant and Bacterial Material

Nicotiana tabacum 'Samsun NN', *Nicotiana benthamiana*, and *N. benthamiana* (NahG) were grown in a growth room with a 16-h/8-h photoperiod at a light intensity of 10,000 lux at 25°C (Kiba et al., 2003).

Bacterial strains used in this study are listed in Supplemental Table S2. *Ralstonia solanacearum* strains OE1-1 (RsOE1-1) and 8107 (Rs8107) were grown for 16 h at 30°C in peptone-yeast extract (PY) medium. *Pseudomonas cichorii* SPC9018, *Pseudomonas syringae* pv *tabaci* 6605, and *P. syringae* pv *mellea* MAFF302303 were cultured in PY medium containing 20 μg mL^{-1} rifampicin (Marutani et al., 2005; Kiba et al., 2006b). *hrpY* mutants of *R. solanacearum* 8107 (Rs8107ΔY) and OE1-1 (RsOE1-1ΔY) were cultured in PY medium containing 50 μg mL^{-1} spectinomycin or 50 μg mL^{-1} kanamycin, respectively (Kanda et al., 2003a; Maimbo et al., 2007). The bacterial population was measured spectrophotometrically at an optical density at 600 nm, and the suspension was adjusted to 10^8 colony-forming units (cfu) mL^{-1} for inoculation. Inoculation of bacteria was carried out by leaf infiltration with the bacterial suspension using a syringe. The leaf-infiltration method produces the same phenotype in tobacco plants against *R. solanacearum* strains when compared with the root-inoculation method (Kanda et al., 2003a, 2003b; Shinohara et al., 2005). Reproducible expression of defense-related genes was also observed in tobacco leaves inoculated with *R. solanacearum* isolates RsOE1-1, Rs8107, and a mutant strain of the bacteria (Kanda et al., 2003a, 2003b; Kiba et al., 2003; Maimbo et al., 2007).

Treatment with Intracellular Signaling Molecules

N. benthamiana plant leaves were treated by leaf infiltration using a syringe by the method described previously (Maimbo et al., 2007). Concentrations of chemicals used in the experiment were as follows: 0.3 mM H_2O_2 (Nacalai Tesque), 50 μM SA (sodium salicylate; Sigma), 50 μM JA (Nacalai Tesque), and 100 μM ACC (Sigma).

Isolation of RNA and cDNA Synthesis

Total RNA was isolated from *N. tabacum* 'Samsun NN' and *N. benthamiana* leaves by a previously described method (Kiba et al., 2003). RNA samples were treated with DNase I (RNase free; Takara) to degrade contaminating genomic DNA, according to the manufacturer's instructions. RT was carried out with 1 μg of total RNA and the oligo(dT) primer (Supplemental Table S1) using Moloney murine leukemia virus reverse transcriptase (Takara) according to the manufacturer's instructions.

Isolation of Full-Length cDNA

For isolation of the complete cDNA of RsRGA4, a modified RACE method was performed (Frohman et al., 1988). PCR amplification was performed with a primer combination of A4-S and oligo(dT)-AD, as listed in Supplemental Table S1. cDNAs from *N. tabacum* and *N. benthamiana* were used as templates. Cycling parameters were as follows: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Amplified cDNA fragments were cloned into the vector pGEM T-Easy (Promega) creating pGEMNtA4 (full-length RsRGA4 from *N. tabacum*) and pGEMNbA4 (its ortholog from *N. benthamiana*; Supplemental Table S3).

Sequencing

Sequence analysis was performed using M4 and RV primers (Supplemental Table S1) with the reagents for the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 3100 Avant Automated Sequencer according to the manufacturer's instructions. The sequence analysis was carried out using DNASIS (version 3.6; Hitachi) and the BLAST network service from the National Center for Biotechnology Information (Altschul et al., 1990).

Quantitative Real-Time PCR

Quantitative real-time PCR was carried out by the method of Maimbo et al. (2007). RT was carried out with 1 μg of total RNA and the oligo(dT) primer (Supplemental Table S1) using Moloney murine leukemia virus reverse transcriptase (Takara) according to the manufacturer's instructions. Real-time PCR was carried out with a 20-μL reaction mixture containing 1 μL of the cDNA stock and 10 pM of the respective primers (Supplemental Table S1) using the SYBR premix ExTaq (Takara) with an Applied Biosystems 7300 real-time PCR system. Cycling parameters were the same for all primers: an initial 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min. Melting curve runs were also performed at the end of each PCR to verify the specificity of the primers by the presence of a single product. Relative quantification of gene expression was carried out according to the instructions for the Applied Biosystems 7300 real-time PCR system, using the comparative cycle threshold method for the calculation of Qty value. All values were normalized to the expression values of the actin gene as an internal standard in each cDNA stock. Expression analyses were carried out with at least two biological replications to ensure that expression patterns were reproducible. We have shown characteristic data in the figures. SD values and differences between expression ratios of controls and other samples were tested for statistical significance using the *t* test.

Plasmid Construction for *Agrobacterium tumefaciens*-Mediated Transient Expression

For detection of NbSGLP protein by immunoblot analysis, a Flag tag sequence was added to the C-terminal region of both constructs and expressed

under the control of the cauliflower mosaic virus 35S promoter. A full-length ORF of *NbSGLP* was amplified with primers A4ORF-S and A4Flag-A (Supplemental Table S1) using pGEMNbA4 as a template. A cDNA fragment containing a signal peptide-deleted ORF of NbSGLP was amplified with primers A4dS-ATG and A4Flag-A (Supplemental Table S1) using pGEMNbA4 as a template. These cDNA fragments were subcloned into the TA cloning site of pGEM T-Easy, creating pGEMSGLP and pGEMSGLPdelS. The pGEMSGLP and pGEMSGLPdelS plasmids were digested with *Bam*HI and *Sac*I (Takara) and ligated into the pBI121 vector (Clontech) digested with the same enzymes. The constructs containing these inserts were designated pBI-SGLP and pBI-SGPLdelS, respectively. We also used the binary vector p35S-INF1 containing a fusion between the signal peptide of *N. tabacum PR-1a* and the *Phytophthora inf1* gene driven by the 35S promoter of *Cauliflower mosaic virus* (Huitema et al., 2005). The binary vector p35S-GUS containing the GUS gene driven by the 35S promoter of the *Cauliflower mosaic virus* (Katou et al., 2003) was used as a control. These binary plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, which harbors the transformation helper plasmid pSoup (Hellens et al., 2000), and inoculated into *N. benthamiana* leaves as described previously (Katou et al., 2003; Supplemental Table S3).

Preparation of IWFs and ICFs, and Immunoblot Analysis

The IWFs from *N. benthamiana* leaves from full-length *NbSGLP*- and signal peptide-deleted *NbSGLPΔS*-expressing *A. tumefaciens* were extruded by the method of De Wit and Spikeman (1982). After isolation of IWFs, leaf samples were homogenized with 10 mM Tris-HCl (pH 7.6) containing a protease inhibitor cocktail (Nacalai Tesque). The homogenates were centrifuged at 800*g* for 10 min. The supernatants were used as the ICFs. The IWF (10 μg) and ICF (20 μg) were separated by 10% SDS-PAGE and then electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). The blots were subjected to western-blot analyses with a monoclonal antibody raised against the Flag-tag sequence (Sigma) or with a cytochrome oxidase monoclonal antibody (Abnova). Cross-reacting proteins were visualized with an alkaline phosphatase-conjugated secondary antibody raised in goat against mouse or rabbit IgG (Bio-Rad) with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Nacalai Tesque).

DNA Constructs and Seedling Infection for Virus-Induced Gene Silencing

A 189-bp cDNA fragment representing the 3′ end sequence of *NbSGLP* was amplified with primers PVX:A4U and PVX:A4L (Supplemental Table S1) using pGEMNbA4 as a template. This cDNA fragment was subcloned into the TA cloning site of pGEM T-Easy, and pGEMA4PVX was created. The pGEMA4PVX plasmid was digested with *Pst*I and *Sal*I (Takara) and ligated into pPVX201 (Maimbo et al. 2007) digested with *Sse*8387I and *Sal*I (Takara). The construct containing this insert in the antisense orientation was designated pPVX-SGLP. Plasmid pPVX201 that did not contain any insert was used as a control. These binary plasmids were transformed into *A. tumefaciens* strain GV3101 and inoculated into *N. benthamiana* leaves as described previously (Katou et al., 2003). Three weeks after the initial *A. tumefaciens* inoculation, *R. solanacearum*, *P. cichoriim*, *P. syringae* pv *mellea*, *P. syringae* pv *tabaci*, and *A. tumefaciens* were inoculated into a *N. benthamiana* leaf three to four leaves above the *A. tumefaciens*-inoculated leaf as a challenge inoculation.

Evaluation of Cell Viability

For evaluation of viability, leaf discs were stained with Evans blue (Wako) by the method of Kiba et al. (2006a). Bacterial suspensions were adjusted to give an initial optical density at 600 nm of 0.1 (1.0×10^8 cfu mL^{-1}). Fifty microliters of water or bacterial suspension was infiltrated into a *N. benthamiana* leaf. Leaf discs (1 cm^2) were punched out and submerged in 1 mL of 0.25% Evans blue and incubated for 20 min. They were then washed several times with water to remove excess and unbound dye. The discs were homogenized, and bound dye was extracted with 1% SDS. The extracted dye was measured spectrophotometrically at 600 nm.

Inoculation of Bacteria and Disease Index

Inoculation of bacteria was carried out by leaf infiltration with the bacterial suspension at 10^8 cfu mL^{-1} using a syringe. The plants were coded and inspected daily for wilting symptoms for 17 d. Each assay was repeated in at least six successive trials, and the disease index was recorded as described previously (Shinohara et al., 2005).

Vascular Dye Accumulation Assay and Detection of Callose

R. solanacearum or *A. tumefaciens* was infiltrated on *N. benthamiana* leaves. Each leaf was detached with a razor at the base of the petiole, and the petiole was directly placed in a 0.5% (w/v) Safranin O solution for 12 h (Oh and Collmer, 2005). Detection of callose deposition was determined by the method of Adam and Somerville (1996). Leaves were cleared in alcoholic lactophenol. Cleared leaves were rinsed with 50% ethanol, rinsed with water, and then stained for 30 min in 150 mM K_2HPO_4 (pH 9.5) containing 0.01% aniline blue. Samples were mounted in 50% glycerol, and callose deposition was detected under epifluorescent illumination (Olympus BX-51).

Sequence data from this article have been submitted to GenBank, EMBL, and the DNA Data Bank of Japan under the accession numbers AB479030 and AB479031 (*NtSGLP* and *NbSGLP*, respectively).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression pattern of *NtSGLP* in response to inoculation with *R. solanacearum*.

Supplemental Figure S2. Transient expression of *NbSGLP* and induction of defense-related genes in *N. benthamiana*.

Supplemental Table S1. List of primers used in this study.

Supplemental Table S2. Bacterial strains used in this study.

Supplemental Table S3. List of plasmids used in this study.

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