Role of a xyloglucan-specific endo- β -1,4-glucanase inhibitor in the interactions of *Nicotiana benthamiana* with *Colletotrichum destructivum*, *C. orbiculare* or *Pseudomonas syringae* pv. *tabaci*

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SUMMARY

A xyloglucan-specific endo- β -1,4-glucanase inhibitor cDNA, *NbXEGIP1*, was amplified from diseased leaves of *Nicotiana benthamiana*. The sequence was similar to the tomato xyloglucan-specific endo- β -1,4-glucanase inhibitor (XEGIP) and tobacco nectarin IV genes that have been described as binding and inactivating fungal Family 12 xyloglucan-specific endo- β -1,4-glucanases. Expression of *NbXEGIP1* was not detected in healthy leaves, but the gene was induced during the later stages of infection by the fungi *Colletotrichum destructivum* and *C. orbiculare*. Induction of *NbXEGIP1* also occurred during disease development by the bacterium *Pseudomonas syringae* pv. *tabaci* and during the hypersensitive response produced by *P. syringae* pv. *tabaci* expressing *avrPto*. A portion of *NbXEGIP1* was cloned into a tobacco rattle virus vector for virus-induced gene silencing in *N. benthamiana*. Silencing *NbXEGIP1* did not affect the interactions with either *Colletotrichum* species but did significantly reduce population levels of *P. syringae* pv. *tabaci* in the compatible interaction and *P. syringae* pv. *tabaci* expressing *avrPto* in the incompatible interaction. In the susceptible response to *P. syringae* pv. *tabaci*, silencing of *NbXEGIP1* also resulted in visibly wilted leaves several hours prior to necrosis, which was not observed in control plants. This was related to a significantly higher level of electrolyte leakage and higher expression of a defensin gene from infected *NbXEGIP1*-silenced leaves compared with control leaves. Silencing appeared to be specific as it did not affect expression of a related gene, *NbXEGIP2*. *NbXEGIP1* may act as an inhibitor of a bacterial enzyme that degrades the xyloglucan–cellulose plant cell-wall network, and degradation of the cell wall results in host membrane disruption and signalling of defence responses.

INTRODUCTION

Xyloglucan-specific endo- β -1,4-glucanases are enzymes that can attack xyloglucan and cellulose but have 10 times more activity against xyloglucan than linear β -glucan (Grishutin *et al.*, 2004). They can greatly damage the plant cell wall as xyloglucan binds with cellulose microfibrils contributing to the structural integrity of the cell walls (Juge, 2006). The cross-binding of xyloglucan with cellulose results in a network that prevents the turgor pressure inside a plant cell from rupturing the cell, while still allowing the cell to grow and develop (York *et al.*, 2004). Xyloglucan-specific endo- β -1,4-glucanases are also important as the degradation of xyloglucan around cellulose microfibrils increases access for further cell-wall degradation (Juge, 2006).

Plants produce xyloglucan-specific endo- β -1,4-glucanase inhibitor proteins (XEGIPs) that bind to the enzyme and inhibit its activity (Naqvi *et al.*, 2005; Qin *et al.*, 2003). XEGIPs are widespread among angiosperms and appear to be specific in inhibiting xyloglucan-specific endo- β -1,4-glucanase and not other cell-wall-degrading enzymes (Juge, 2006; Naqvi *et al.*, 2005; Qin *et al.*, 2003). Both purified tomato XEGIP and tobacco nectarin IV proteins were shown to inhibit a xyloglucan-specific endo- β -1,4-glucanase of *Aspergillus aculeatus* and are believed to have evolved as a defence mechanism against microbial degradation of the host cell wall by counteracting the endoglucanases of plant pathogens (Naqvi *et al.*, 2005; Qin *et al.*, 2003). The tomato XEGIP has 62% amino acid identity with an extracellular dermal glycoprotein (EDGP) from carrot (Satoh *et al.*, 1992). Carrot EDGP is localized to dermal tissues and responds to wounding, and it was hypothesized that EDGP is involved in disease resistance. Direct evidence of an XEGIP in defence comes from silencing a potato homologue of the tomato XEGIP by agro-infiltration that reduced transcript levels to 20–25% that of non-silenced plants and resulted in faster lesion expansion by *Phytophthora infestans* (Jones *et al.*, 2006). Water-soaking was minimal in leaves of non-silenced plants, but there was extensive water-soaking in the silenced plants. It was believed that the potato XEGIP contributed

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to resistance by reducing the ability of *P. infestans* to loosen host cell walls, which limited pathogen penetration and reduced exposure of the host cell-wall components to other *P. infestans* enzymes.

To assess the role of XEGIPs in bacterial and fungal diseases further, an examination was undertaken of an XEGIP homologue during the interactions of *Nicotiana benthamiana* with the fungi *Colletotrichum destructivum* and *C. orbiculare*, and the bacterium *Pseudomonas syringae* pv. *tabaci*. *Colletotrichum destructivum* and *C. orbiculare* are hemibiotrophic pathogens that have an initial symptomless biotrophic phase of infection with large primary hyphae and/or vesicles that invaginate the host plasma membrane followed by a necrotrophic phase several days later with secondary hyphae that results in water-soaked lesions (Shen *et al.*, 2001a,b). *Pseudomonas syringae* pv. *tabaci* is also a hemibiotrophic pathogen that initially grows biotrophically in the apoplast utilizing type III secretion system (TTSS) effectors to interfere with plant defence signal transduction in the living host cells but then becomes necrotrophic, breaking down parenchyma cells by enzymes and toxin, resulting in water-soaked lesions with chlorotic halos (Agrios, 2005; Mudgett, 2005). A resistance response was also examined in *N. benthamiana* transformed with the resistance gene *Pto*, which involves a hypersensitive response (HR) due to the recognition of the TTSS effector AvrPto produced by *P. syringae* pv. *tabaci* transformed with *avrPto* (Rommens *et al.*, 1995).

The role of a *N. benthamiana* XEGIP homologue, *NbXEGIP1*, in these plant–pathogen interactions was assessed by determining its expression pattern following pathogen inoculations and by silencing the gene through virus-induced gene silencing (VIGS) with a tobacco rattle virus (TRV) vector (Liu *et al.*, 2002). VIGS involves the induction of post-transcriptional gene silencing in plants using a plant virus to generate double-stranded (ds)RNA, and TRV is a good VIGS vector because it does not have a strong suppressor of silencing, spreads rapidly in the plant and infects meristematic tissue (Robertson, 2004). *Nicotiana benthamiana* has frequently been used for VIGS because VIGS is generally much more pronounced and persistent in *N. benthamiana* than in other plants (Lu *et al.*, 2003). If the XEGIP homologue directly contributes to resistance, then one might expect that the gene will be induced by infection and silencing it should result in greater pathogen growth and greater symptom expression compared with non-silenced plants.

RESULTS

NbXEGIP1 and *NbXEGIP2* amplification and sequence analyses

Primers NbXEGIP1F1 and NbXEGIP1R1 were designed based on the nucleotide sequence of TC7425, which is annotated in the

N. benthamiana Gene Index of the Computational Biology and Functional Genomics Laboratory database as being similar to a xyloglucan-specific fungal endoglucanase inhibitor protein. A single band of the expected size of 708 bp was amplified with these primers using cDNA from *N. benthamiana* leaves infected with *C. destructivum* at 96 h post inoculation (hpi), and sequencing of the PCR product showed 100% nucleotide identity with the overlapping region of TC7425. BLASTN of the PCR product sequence against the GenBank non-redundant database showed that the closest matches (86 and 84% nucleotide identity, respectively) were to sequences for a *Solanum tuberosum* putative xyloglucanase inhibitor (GenBank accession no. AY321357) and a *Lycopersicon esculentum* xyloglucan-specific fungal endoglucanase inhibitor protein precursor (*LeXEGIP*) (GenBank accession no. AY155579). The gene was designated *NbXEGIP1*. Like *LeXEGIP* and other XEGIP genes, *NbXEGIP1* encoded a protein with a signal peptide that is 23 amino acids long, indicating that the protein is secreted.

Based on the conserved regions of the nucleotide sequences of several XEGIP and EDGP-like genes of *Capsicum annuum*, *L. esculentum* and *S. tuberosum*, primers NbXEGIP2F1 and NbXEGIP2R1 were designed. A single band with the expected size of 275 bp was amplified with these primers using cDNA from *N. benthamiana* leaves infected with *C. destructivum* at 96 hpi. The sequence of this PCR product did not show any close match with nucleotide sequences in the *N. benthamiana* Gene Index and only had 58% nucleotide identity with TC7425. However, BLASTN searches of the Tomato and Potato Gene Indices in the Computational Biology and Functional Genomics Laboratory database revealed 84% identity to a potato putative EDGP gene (TC132377) and 65% identity to the tomato *XEGIP* gene (TC154242). The gene was designated *NbXEGIP2*.

A dendrogram was constructed from 15 amino acid sequences with high identity to the predicted amino acid sequences of *NbXEGIP1* and *NbXEGIP2* from plants in the Solanaceae, as well as the carrot EDGP gene (Table 1, Fig. 1). The sequence of TAXI-IV, which is not an XEGIP but is a *Triticum aestivum* xylanase inhibitor with some sequence similarity to *NbXEGIP1* and *NbXEGIP2*, was used as an outgroup. Two clusters were observed in the dendrogram (Fig. 1). One cluster contained NbXEGIP1, tomato XEGIP, tobacco nectarin IV, carrot EDGP, and other similar proteins from tomato, tobacco and potato. The second cluster contained NbXEGIP2 and similar sequences from tomato, potato and pepper. The two clusters did not correlate with the gene annotations given that XEGIP, EDGP or nectarin IV-like genes were found in both clusters. These results indicate that plants appear to contain multiple genes for xyloglucan-specific fungal endoglucanase inhibitor proteins, which can be placed into at least two different groups for plants in the Solanaceae.

Fig. 1 Comparison of xyloglucanase inhibitor protein sequences from Solanaceae as well as the carrot EDGP protein. Wheat TAXI-IV (BAD72882) was included as an outgroup. Amino acid sequences were aligned using CLUSTALX and a tree generated with the bootstrap neighbour-joining-tree procedure of CLUSTALX. Bootstrap values represent the number of times out of 1000 that the branch was supported by bootstrap analysis. A description of each sequence is provided in Table 1.

Expression of *NbXEGIP1* in *N. benthamiana* following infection by *C. destructivum*, *C. orbiculare*, and *P. syringae* pv. *tabaci*

No expression of *NbXEGIP1* could be detected in water mock-inoculated leaves of wild-type *N. benthamiana* (Fig. 2). In addition, expression levels of *NbXEGIP1* were not detectable prior to infection, during the penetration phase at 24 hpi or during the biotrophic phase at 48 hpi for the interactions with *C. destructivum* or *C. orbiculare*. However, an increase in expression was first detectable at 72 hpi with *C. destructivum*. This corresponded to the development of necrosis and the appearance of water-soaked lesions. There was a further increase at 96 hpi, at which time the lesions had expanded and there was extensive leaf necrosis. A similar expression pattern occurred during the course of infection by *C. orbiculare* (Fig. 2). Because lesions expand more slowly with *C. orbiculare* than *C. destructivum*, samples could be collected at 120 hpi, showing that the increase in *NbXEGIP1* expression continued. Thus, expression levels appeared to correlate directly with the amount of necrosis in the necrotrophic phase of infection.

Fig. 2 Relative RT-PCR of *NbXEGIP1* expression in wild-type *N. benthamiana* leaves inoculated with *C. destructivum* (■) or *C. orbiculare* (●). *N. benthamiana* leaves inoculated with water (▲) were used as control. The quantity of *NbXEGIP1* mRNA was determined relative to the amount of *NbEF-1α* mRNA. Means are shown with standard error bars calculated from three or four replications.

No expression of *NbXEGIP1* could be detected in leaves mock-infiltrated with $MgCl_2$, but increased expression was first detectable by 48 hpi with *P. syringae* pv. *tabaci* (Fig. 3). Expression of *NbXEGIP1* continued to increase as the disease progressed,

Table 1 Xyloglucan-specific endoglucanase inhibitor gene sequences used in this study.

Accession number*	Source	Gene name/annotation
TC7425	<i>Nicotiana benthamiana</i>	<i>NbXEGIP1</i>
EF514216	<i>N. benthamiana</i>	<i>NbXEGIP2</i>
AAN87262	<i>Lycopersicon esculentum</i>	<i>LeXEGIP</i>
TC156214	<i>L. esculentum</i>	Nectarin IV like
TC177196	<i>L. esculentum</i>	XEGIP like
AAP84703	<i>Solanum tuberosum</i>	<i>StXEGIP</i>
TC128826	<i>S. tuberosum</i>	XEGIP like
TC120914	<i>S. tuberosum</i>	EDGP like
TC128827	<i>S. tuberosum</i>	EDGP like
TC132377	<i>S. tuberosum</i>	EDGP like
TC11291	<i>Nicotiana tabacum</i>	Nectarin IV like
AAX81588	<i>Nicotiana langsdorffii</i> × <i>N. sanderae</i>	Nectarin IV
TC3830	<i>Capsicum annuum</i>	XEGIP like
BAA03413	<i>Daucus carota</i>	EDGP precursor
BAD72882	<i>Triticum aestivum</i>	TAXI-IV

*The TC sequences were obtained from TGI databases at The Computational Biology and Functional Genomics Laboratory (<http://compbio.dfc.harvard.edu/tgi/>). The open reading frames by framefinder (+) in TGI databases were used to construct the dendrogram. The amino acid sequence of *NbXEGIP2* was translated using the program GeneRunner (Hastings Software, Hastings, NY). All other sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>).

reaching its highest expression at 96 hpi. Water-soaked lesions and necrosis were first visible at 72 hpi with *P. syringae* pv. *tabaci*, and thus the increased expression of *NbXEGIP1* at 48 hpi occurred prior to the appearance of symptoms.

Expression of *NbXEGIP1* during the hypersensitive response in *N. benthamiana* (Pto) following inoculation with *P. syringae* pv. *tabaci* (avrPto)

There was a transient increase in expression of *NbXEGIP1* at 3 hpi in *N. benthamiana* (Pto) following mock-infiltration with MgCl₂ (Fig. 4). A larger transient increase at 3 hpi was observed following inoculation with *P. syringae* pv. *tabaci* (empty vector), which does not induce an HR because it contains a plasmid cloning vector without the avirulence gene, *avrPto*. This indicates that the infiltration process induces *NbXEGIP1* expression, and this induction is greater when bacterial cells are present. However, expression dropped by 6 hpi with expression levels returning to pre-treatment levels for MgCl₂ and slightly above pre-treatment levels with *P. syringae* pv. *tabaci* (empty vector). Therefore, increased expression of *NbXEGIP1* at 3 hpi with *P. syringae* pv. *tabaci* (avrPto) is mostly due to this non-specific effect. After 6 hpi, increased expression only occurred for leaves undergoing the HR induced by *P. syringae* pv. *tabaci* (avrPto). Expression of *NbXEGIP1* peaked at 18 hpi, which was the time of the appearance

Fig. 3 Relative RT-PCR of *NbXEGIP1* in wild-type *N. benthamiana* plants inoculated with *P. syringae* pv. *tabaci* (■). *N. benthamiana* leaves infiltrated with MgCl₂ were used as control (▲). The quantity of *NbXEGIP1* mRNA was determined relative to the amount of *NbEF-1α* mRNA. Means are shown with standard error bars calculated from three or four replications.

Fig. 4 Relative RT-PCR of *NbXEGIP1* expression in leaves of *N. benthamiana* (Pto) inoculated with *P. syringae* pv. *tabaci* (avrPto) (■). Controls were leaves infiltrated with MgCl₂ (▲) or *P. syringae* pv. *tabaci* containing the vector, pDSK519, without insert (●). The quantity of *NbXEGIP1* mRNA was determined relative to the amount of *NbEF-1α* mRNA. Means are shown with standard error bars calculated from three or four replications.

of HR necrosis. It thus appears that *NbXEGIP1* expression is induced during the HR with increases detectable before the appearance of necrosis.

Silencing *NbXEGIP1* in *N. benthamiana* and the effect on the compatible interactions with *C. destructivum*, *C. orbiculare* and *P. syringae* pv. *tabaci* and incompatible interaction with *P. syringae* pv. *tabaci* (avrPto)

In leaves inoculated with *C. destructivum*, *C. orbiculare* or *P. syringae* pv. *tabaci*, the expression level of *NbXEGIP1* was reduced in the *NbXEGIP1*-silenced wild-type *N. benthamiana* to 8–21% of that observed in the MgCl₂ and TRV-GFP vector controls (Table 2, Fig. 5). In *N. benthamiana* (Pto) inoculated with

Table 2 Relative RT-PCR analysis of *NbXEGIP1* and *NbXEGIP2* expression in wild-type *N. benthamiana* with MgCl_2 control, TRV-GFP control and TRV-*NbXEGIP1* treatments after subsequent inoculation with *C. destructivum*, *C. orbiculare* or *P. syringae* pv. *tabaci* and *N. benthamiana* (*Pto*) with the same treatments after inoculation with *P. syringae* pv. *tabaci* (*avrPto*).

Treatment*	Relative gene expression†							
	<i>C. destructivum</i> (96 hpi)		<i>C. orbiculare</i> (96 hpi)		<i>P. syringae</i> pv. <i>tabaci</i> (72 hpi)		<i>P. syringae</i> pv. <i>tabaci</i> (<i>avrPto</i>) (24 hpi)	
	<i>NbXEGIP1</i>	<i>NbXEGIP2</i>	<i>NbXEGIP1</i>	<i>NbXEGIP2</i>	<i>NbXEGIP1</i>	<i>NbXEGIP2</i>	<i>NbXEGIP1</i>	<i>NbXEGIP2</i>
MgCl_2 control	2.09 a	0.10 a	1.65 a	0.03 a	0.81 a	0	1.06 a	0
TRV-GFP control	2.13 a	0.10 a	1.60 a	0.03 a	0.86 a	0	1.18 a	0
TRV- <i>NbXEGIP1</i>	0.39 b	0.12 a	0.35 b	0.04 a	0.07 b	0	0.15 b	0

*Wild-type *N. benthamiana* and *N. benthamiana* (*Pto*) were infiltrated with *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-*NbXEGIP1*) or with 10 mM MgCl_2 without any *A. tumefaciens* (MgCl_2 control). Plants were grown for 15–18 days after TRV treatment to allow silencing to spread in the plant and then inoculated with *C. destructivum*, *C. orbiculare*, *P. syringae* pv. *tabaci* or *P. syringae* pv. *tabaci* (*avrPto*).

†Expression of *NbXEGIP1* in MgCl_2 control, TRV-GFP control or TRV-*NbXEGIP1* *N. benthamiana* was determined by relative RT-PCR. The expression of *NbXEGIP2* was also examined in TRV-*NbXEGIP1* plants to examine the specificity of *NbXEGIP1* silencing. Each measurement represents the mean relative expression from three experiments. Means in the same column followed by the same letter are not significantly different according to the ANOVA Fisher multiple comparisons test at $P < 0.05$.

Fig. 5 Effect of virus-induced gene silencing on expression of *NbXEGIP1* in wild-type *N. benthamiana* and *N. benthamiana* (*Pto*). Relative RT-PCR of *NbXEGIP1* co-amplified with *NbEF-1α* from cDNA of leaves at 96 h post-inoculation (hpi) by *C. destructivum* (A) and *C. orbiculare* (B), at 72 hpi by *P. syringae* pv. *tabaci* (C) and 24 hpi by *P. syringae* pv. *tabaci* (*avrPto*) (D). Wild-type *N. benthamiana* or *N. benthamiana* (*Pto*) were infiltrated with 10 mM MgCl_2 (MgCl_2), *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP), or *A. tumefaciens* containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-*NbXEGIP1*). After 15–18 days from the TRV treatment to allow for silencing to spread, wild-type *N. benthamiana* plants were inoculated with *C. destructivum*, *C. orbiculare* or *P. syringae* pv. *tabaci*, and *N. benthamiana* (*Pto*) plants were inoculated with *P. syringae* pv. *tabaci* (*avrPto*). Lane M is the 100-bp ladder.

P. syringae pv. *tabaci* (*avrPto*), the expression level of *NbXEGIP1* in *NbXEGIP1*-silenced plants was 13–14% of that observed in the MgCl_2 and TRV-GFP vector controls (Table 2).

Silencing appeared to be specific as there was no significant difference in the expression of *NbXEGIP2* in the *NbXEGIP1*-silenced plants compared with the controls (Table 2). This was not unexpected as *NbXEGIP1* and *NbXEGIP2* have less than 60% nucleotide identity to each other. Detectable *NbXEGIP2* expression levels were very low compared with that of *NbXEGIP1* at 96 hpi with *C. destructivum* or *C. orbiculare*, and no *NbXEGIP2*

expression was detected with *P. syringae* pv. *tabaci* at 72 hpi or *P. syringae* pv. *tabaci* (*avrPto*) at 24 hpi (Table 2). Those values were typical for *NbXEGIP2* expression during the interactions as its expression was only slightly induced by 72 hpi for *C. destructivum*, with a peak relative expression value of 0.14 at 96 hpi and was only slightly induced by 96 hpi for *C. orbiculare*, with a peak relative expression value of 0.04 at 120 hpi. No *NbXEGIP2* expression was detected at any time point during infection by *P. syringae* pv. *tabaci* or the HR by *P. syringae* pv. *tabaci* (*avrPto*) (data not shown).

Table 3 Lesion numbers in *N. benthamiana* leaves from plants with MgCl₂ control, TRV-GFP or TRV-NbXEGIP1 treatments following subsequent inoculation with *C. destructivum* or *C. orbiculare*.

Treatment*	Lesions/cm ² †	
	<i>C. destructivum</i> (72 hpi)	<i>C. orbiculare</i> (96 hpi)
MgCl ₂ control	3.23 a	5.31 a
TRV-GFP control	3.01 a	4.97 a
TRV-NbXEGIP1	2.96 a	5.14 a

**N. benthamiana* plants were infiltrated with *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-NbXEGIP1) or with 10 mM MgCl₂ without any *A. tumefaciens* (MgCl₂ control).

†At 15–18 days after *A. tumefaciens* infiltration to induce silencing, the plants were sprayed with a spore suspension of *C. destructivum* or *C. orbiculare*. Lesion numbers were counted at 72 hpi for *C. destructivum*, and at 96 hpi for *C. orbiculare*. Lesion numbers per square centimetre were means pooled from two separate experiments with a total of 20 replications. Means in the same column followed by the same letter are not significantly different according to the ANOVA Fisher multiple comparisons test at $P < 0.05$.

Table 4 Bacterial populations in wild-type *N. benthamiana* with MgCl₂ control, TRV-GFP control and TRV-NbXEGIP1 treatments after subsequent inoculation with *P. syringae* pv. *tabaci* and *N. benthamiana* (Pto) with the same treatments after inoculation with *P. syringae* pv. *tabaci* (*avrPto*).

Treatment*	CFU/cm ² †	
	<i>P. syringae</i> pv. <i>tabaci</i> (72 hpi)	<i>P. syringae</i> pv. <i>tabaci</i> (<i>avrPto</i>) (20 hpi)
MgCl ₂ control	1.01×10^8 a	7.75×10^5 a
TRV-GFP control	1.10×10^8 a	5.99×10^5 a
TRV-NbXEGIP1	7.32×10^7 b	1.67×10^5 b

*Wild-type *N. benthamiana* and *N. benthamiana* (Pto) were infiltrated with *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-NbXEGIP1) or with 10 mM MgCl₂ without any *A. tumefaciens* (MgCl₂ control).

†At 15–18 days after *A. tumefaciens* infiltration to induce silencing, the two youngest fully developed leaves of wild-type *N. benthamiana* were infiltrated with *P. syringae* pv. *tabaci* and *N. benthamiana* (Pto) were infiltrated with *P. syringae* pv. *tabaci* (*avrPto*). Samples were collected at 72 hpi for *P. syringae* pv. *tabaci* and at 20 hpi for *P. syringae* pv. *tabaci* (*avrPto*). CFU/cm² of leaf were means pooled from four separate experiments with a total of 38 replications. Means in the same column followed by the same letter are not significantly different according to the ANOVA Fisher multiple comparisons test at $P < 0.05$.

No significant difference in numbers of water-soaked lesions was observed on leaves of *NbXEGIP1*-silenced plants after inoculation with *C. destructivum* or *C. orbiculare* compared with MgCl₂ and TRV-GFP vector controls (Table 3). There was also no significant difference among the plants in the timing of the development of necrosis or in the appearance of the lesions among the different treatments.

Table 5 Electrolyte leakage from wild-type *N. benthamiana* leaf discs from plants with MgCl₂ control, TRV-GFP or TRV-NbXEGIP1 treatments at 66 h post-inoculation with *P. syringae* pv. *tabaci*.

Treatment*	Relative conductivity†
MgCl ₂ control	0.41 a
TRV-GFP control	0.44 a
TRV-NbXEGIP1	0.54 b

**N. benthamiana* plants were infiltrated with *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-NbXEGIP1) or with 10 mM MgCl₂ without any *A. tumefaciens* (MgCl₂ control).

†At 15–18 days after *A. tumefaciens* infiltration to induce silencing, the two youngest fully developed leaves of each plant were infiltrated with *P. syringae* pv. *tabaci*. Relative conductivity values were means pooled from three separate experiments with a total of 15 replications. Means in the same column followed by the same letter are not significantly different according to the ANOVA Fisher multiple comparisons test at $P < 0.05$.

Population levels of *P. syringae* pv. *tabaci* in wild-type *N. benthamiana* were significantly lower in *NbXEGIP1*-silenced plants than in MgCl₂ and TRV-GFP vector controls (Table 4). Although the bacterial populations were lower with silencing of *NbXEGIP1*, leaves infected with *P. syringae* pv. *tabaci* showed more severe wilting symptoms approximately 4–6 h prior to water-soaking and necrosis in *NbXEGIP1*-silenced *N. benthamiana* than in the MgCl₂ and TRV-GFP vector controls (Fig. 6). An examination of electrolyte leakage from leaves infected with *P. syringae* pv. *tabaci* at that time after inoculation revealed that the relative conductivity was significantly higher in the *NbXEGIP1*-silenced plants than in the MgCl₂ or TRV-GFP vector controls (Table 5). The higher levels of ion leakage indicated that *P. syringae* pv. *tabaci* infection resulted in greater host membrane disruption when *NbXEGIP1* was silenced.

The numbers of *P. syringae* pv. *tabaci* (*avrPto*) in *N. benthamiana* (Pto) were significantly lower in the *NbXEGIP1*-silenced plants than in the controls (Table 4). However, there was no significant difference in the timing or appearance of HR development for leaves of *NbXEGIP1*-silenced plants compared with controls.

The lower bacterial numbers in the interactions of the *NbXEGIP1*-silenced plants with *P. syringae* pv. *tabaci* and *P. syringae* pv. *tabaci* (*avrPto*) may be related to the activation of other defences. To examine this, the expression of a defensin gene, *NbDef2.2*, which is strongly induced in *N. benthamiana* leaves during bacterial infection (Bahramnejad, 2007) was determined. Expression of *NbDef2.2* in *NbXEGIP1*-silenced wild-type *N. benthamiana* was four times higher than in the MgCl₂ and TRV-GFP control plants at 72 hpi with *P. syringae* pv. *tabaci* (Table 6). However, there was no significant difference in *NbDef2.2* expression between the *NbXEGIP1*-silenced *N. benthamiana* (Pto) and the MgCl₂ and TRV-GFP controls at 24 hpi with *P. syringae* pv. *tabaci* (*avrPto*) (Table 6). No expression of *NbDef2.2* was detected in the

Fig. 6 Appearance of youngest fully matured leaf of *N. benthamiana* at 66 hpi with *P. syringae* pv. *tabaci*. Plants had been infiltrated 15–18 days earlier with 10 mM $MgCl_2$ ($MgCl_2$ control), *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or *A. tumefaciens* containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-*NbXEGIP1*).

Table 6 Relative RT-PCR analysis of *NbDef2.2* expression in wild-type *N. benthamiana* with $MgCl_2$ control, TRV-GFP control and TRV-*NbXEGIP1* treatments after subsequent inoculation with *P. syringae* pv. *tabaci* and *N. benthamiana* (*Pto*) with the same treatments after inoculation with *P. syringae* pv. *tabaci* (*avrPto*).

Treatment*	Relative gene expression†	
	<i>P. syringae</i> pv. <i>tabaci</i> (72 hpi)	<i>P. syringae</i> pv. <i>tabaci</i> (<i>avrPto</i>) (20 hpi)
$MgCl_2$ control	0.16 a	0.40 a
TRV-GFP control	0.15 a	0.53 a
TRV- <i>NbXEGIP1</i>	0.70 b	0.50 a

*Wild-type *N. benthamiana* and *N. benthamiana* (*Pto*) were infiltrated with *A. tumefaciens* containing TRV1 and TRV2 with a GFP insert (TRV-GFP control), or containing TRV1 and TRV2 with a fragment of *NbXEGIP1* (TRV-*NbXEGIP1*) or with 10 mM $MgCl_2$ without any *A. tumefaciens* ($MgCl_2$ control). Plants were grown for 15–18 days after TRV treatment to allow silencing to spread in the plant and then inoculated with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tabaci* (*avrPto*).

†Expression of *NbDef2.2* in $MgCl_2$ control, TRV-GFP control or TRV-*NbXEGIP1* *N. benthamiana* was determined by relative RT-PCR. Each measurement represents the mean relative expression from three experiments. Means in the same column followed by the same letter are not significantly different according to the ANOVA Fisher multiple comparisons test at $P < 0.05$.

$MgCl_2$ control, TRV-GFP control or *NbXEGIP1*-silenced plants inoculated with *C. destructivum* or *C. orbiculare* at 96 hpi (data not shown).

DISCUSSION

NbXEGIP1 showed increased expression late in the infection by *C. destructivum* and *C. orbiculare*, which corresponded to the development of the necrotrophic stage, whereas expression of *NbXEGIP1* increased prior to the appearance of necrosis for the compatible and incompatible interactions with *P. syringae* pv. *tabaci*. The lack of detectable expression of *NbXEGIP1* by RT-PCR

in healthy leaves and leaves during the early stages of infection would be consistent with a role for *NbXEGIP1* in the plant's response during the later stages of infection when the host cells would be considerably disrupted by the activities of the pathogens. The transient increase in expression at 3 h after infiltration with $MgCl_2$ also indicates that the gene can be induced by other stresses, such as wounding.

It has been proposed that XEGIP expression is stimulated by biotic and abiotic stresses, such as wounding, which induced expression of the carrot EDGP gene (Satoh *et al.*, 1992; York *et al.*, 2004). Even though *NbXEGIP1* is similar to the carrot EDGP and nectarin IV genes, carrot EDGP gene expression was not induced due to infection by *Erwinia carotovora*, and an arabidopsis homologue of nectarin IV was not induced during infection by *Alternaria brassicola* (Naqvi *et al.*, 2005; Satoh *et al.*, 1992). However, a homologue of carrot EDGP in *Ageratum conyzoides* showed increased expression following inoculation with *Agrobacterium tumefaciens* (Ditt *et al.*, 2001). It appears that there is considerable variation in the regulation of XEGIP genes by biotic stresses. This is also illustrated during compatible and incompatible interactions of *N. benthamiana* with *P. syringae* pv. *tabaci*, in which *NbXEGIP1* expression was induced but *NbXEGIP2* expression was not.

Other cell-wall-degrading enzyme inhibitor genes of plants can also have a wide range of responses to pathogen attack. For wheat, the endoxylanase inhibitor gene, *Taxi-I*, is not pathogen-inducible, whereas the xylanase inhibitor gene, *Xip-I*, is greatly induced in *Erysiphe graminis*-infected leaves but not in *Fusarium graminearum*-inoculated spikelets (Igawa *et al.*, 2005). Two other endoxylanase inhibitor genes of wheat, *Taxi-III* and *Taxi-IV*, showed increased expression following infection by both *E. graminis* and *F. graminearum* (Igawa *et al.*, 2004). Thus, not only are some cell-wall-degrading enzyme inhibitors induced by disease, but some can show differential induction depending upon the pathogen attacking the plant.

Although *NbXEGIP1* was induced during infection by both *C. destructivum* and *C. orbiculare*, silencing of this gene did

not alter the host reaction to either *Colletotrichum* species. *Colletotrichum* species, such as *C. gloeosporioides* and *C. lindemuthianum*, are known to produce many cell-wall-degrading enzymes, like endoglucanases and xylanases (Acosta-Rodriguez *et al.*, 2005; Ortega, 1994). For other plant pathogenic fungi, such as *Alternaria alternata* and *Macrophomina phaseolina*, it has been established that they express endo- β -1,4-glucanase during invasion of the plant, and production of the enzyme is related to symptom expression, indicating a role in virulence (Eshel *et al.*, 2002; Jones and Wang, 1997). It is possible that if there are xyloglucan-specific endo- β -1,4-glucanases produced by *C. destructivum* or *C. orbiculare*, then the enzymes are not important during infection or are not affected by NbXEGIP1.

Silencing NbXEGIP1 during the susceptible response to *P. syringae* pv. *tabaci* increased the amount of leaf wilting compared with the controls, and this was related to increased electrolyte leakage, indicating greater membrane damage when NbXEGIP1 was silenced. A possible explanation for the effect of silencing NbXEGIP1 is that bacterial xyloglucan-specific endo- β -1,4-glucanases are being secreted during infection that sufficiently affect host cell-wall integrity so that host membrane disruption would occur. With less NbXEGIP1 potentially to bind and inactivate it, xyloglucan-specific endo- β -1,4-glucanases produced by *P. syringae* pv. *tabaci* in the apoplast would be more active, either directly weakening the host cell wall by reducing the cross-binding of xyloglucan with cellulose or indirectly weakening the host cell wall by opening up the wall and permitting greater access to other microbial cell-wall-degrading enzymes. The weakened host wall would be unable to retain the turgor pressure of the cell, resulting in host membrane damage, electrolyte leakage and wilting of the leaves. NbXEGIP1 thus appears to limit the amount of host membrane damage being produced during bacterial infection.

Although NbXEGIP1 contributes to resistance by reducing host cell damage due to *P. syringae* pv. *tabaci* infection, silencing NbXEGIP1 expression reduced rather than increased bacterial cell numbers in the leaf. The lower bacterial numbers in the NbXEGIP1-silenced *N. benthamiana* may be related to the greater induction of expression of defence-related genes, such as NbDef2.2, compared with the controls after inoculation with *P. syringae* pv. *tabaci*. NbDef2.2 encodes a cationic defensin that showed the greatest up-regulation among eight *N. benthamiana* defensin genes following treatment with ethylene, wounding or inoculation with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tabaci* (avrPto) (Bahramnejad, 2007). Although higher levels of this defensin may be partially responsible for the lower population of *P. syringae* pv. *tabaci* in NbXEGIP1-silenced *N. benthamiana* leaves, there may be a number of defence-related compounds that are induced more in the NbXEGIP1-silenced plants. NbDef2.2 expression was chosen as an indicator of the activation of potential defence responses.

One explanation for the effect of NbXEGIP1 silencing on *P. syringae* pv. *tabaci* is that xyloglucan-specific endo- β -1,4-glucanases produced by *P. syringae* pv. *tabaci* are affecting signal molecules, such as hydroxyproline-rich glycopeptides (HypSys) and AtPep1 (Huffaker *et al.*, 2006; Narvaez-Vasquez *et al.*, 2005). Cell-wall damage may cause proteases to process HypSys precursors sequestered in the cell walls into active peptides that interact with receptors to signal defence responses to herbivores and/or pathogens (Pearce *et al.*, 2007). In petunia, a processed HypSys induced expression of an antimicrobial defensin gene, *PhD1* (Lay *et al.*, 2003; Pearce *et al.*, 2007). Wounding can also induce defensin gene expression by other plant peptides involved in defence signalling, such as AtPep1 in Arabidopsis, that are widespread in monocots and dicots (Huffaker *et al.*, 2006). With less NbXEGIP1 in the silenced plants, greater cell-wall damage could occur by xyloglucan-specific endo- β -1,4-glucanases, releasing defence signalling molecules and resulting in more antimicrobial products, such as defensins, that could result in lower populations of *P. syringae* pv. *tabaci* in NbXEGIP1-silenced leaves.

Silencing NbXEGIP1 also resulted in lower population levels of *P. syringae* pv. *tabaci* (avrPto) during the resistance response associated with an HR. Perhaps greater activity of a bacterial xyloglucan-specific endo- β -1,4-glucanase results in a stronger induction of the HR or more amplification of defence signalling during the HR. However, there was no significant difference in NbDef2.2 expression between the NbXEGIP1-silenced plants and the MgCl₂ and TRV-GFP controls at 24 hpi with *P. syringae* pv. *tabaci* (avrPto). This could be due to different defence signalling and activation of defence-related genes other than NbDef2.2 during the HR as compared with the compatible interaction.

Tomato XEGIP and tobacco nectarin IV proteins have only been demonstrated to inhibit fungal xyloglucan-specific endo- β -1,4-glucanase in Family 12 glycoside hydrolases, and it appears that XEGIPs are highly specific in their inhibition (Naqvi *et al.*, 2005; Qin *et al.*, 2003; York *et al.*, 2004). However, silencing a potato XEGIP resulted in greater susceptibility to the stramenopile *P. infestans* (Jones *et al.*, 2006). The Stramenopila are phylogenetically distinct from fungi, and these results indicate that an XEGIP gene can be effective against xyloglucan-specific endo- β -1,4-glucanases of non-fungal plant pathogens, such as *P. infestans*. No Family 12 endoglucanase genes have yet been found in *P. infestans* (Jones *et al.*, 2006). However, a Family 12 endoglucanase gene was obtained from *P. sojae*, suggesting that there may be a similar enzyme in *P. infestans*.

Endoglucanases are also important in the virulence of at least some plant pathogenic bacteria. Endoglucanase mutants of *Ralstonia solanacearum* have a reduced ability to cause wilting of tomato, and tissue maceration is reduced in endoglucanase mutants of *Erwinia carotovora* (Denny *et al.*, 1990; Walker *et al.*, 1994). The genome of *P. syringae* pv. *tabaci* has not been sequenced, but the genome of a related bacterial plant pathogen,

Pseudomonas syringae pv. *tomato* DC3000, contains a Family 8 endoglucanase, *wssD* (locus ID: PSPTO 1029), but no Family 12 endoglucanase (<http://www.pseudomonas.com>). However, there are Family 12 endoglucanases described for a variety of plant pathogenic bacteria, such as cellulase S of *Xanthomonas axonopodis* pv. *citri* (GenBank accession no. NP_643814) and *Xanthomonas oryzae* pv. *oryzae* (GenBank accession no. AAW74331), and *celB* of *Erwinia carotovora* subsp. *carotovora* (Park *et al.*, 1998). It is therefore possible that NbXEGIP1 is inhibiting the action of an as yet undescribed Family 12 endoglucanase of *P. syringae* pv. *tabaci*, or there may be a novel interaction of NbXEGIP1 with an endoglucanase from another glucoside hydrolase family given that silencing *NbXEGIP1* affects the growth of this pathogen in the plant.

Another possible explanation for the reduced bacterial populations, wilting and electrolyte leakage due to silencing *NbXEGIP1* is that it encodes an inhibitor that regulates the activity of endogenous endoglucanases of *N. benthamiana* induced by *P. syringae* pv. *tabaci* infection or during the HR. However, it is considered unlikely that plants have endogenous targets for XEGIPs as plant endoglucanases are not closely related to xyloglucan-specific endo- β -1,4-glucanases (York *et al.*, 2004). For example, the tomato XEGIP did not inhibit a tomato xyloglucan glucosylase that uses xyloglucan as a substrate (Qin *et al.*, 2003). Therefore, it appears more likely that NbXEGIP1 acts to inhibit a bacterial enzyme that can damage host cells rather than a host enzyme.

EXPERIMENTAL PROCEDURES

Biological materials and pathogen inoculations

Nicotiana benthamiana plants were grown at 22 °C until the eight-leaf stage with a photoperiod of 8/16 h dark/light at 150 $\mu\text{mol}/\text{m}^2/\text{s}$. *Colletotrichum destructivum* isolate N150P3 and *C. orbiculare* isolate A20767P1 (Chen *et al.*, 2003) were cultured on sodium chloride–yeast extract–sucrose agar medium (SYAS) (Mandanhari *et al.*, 1986) at 22 °C under continuous fluorescent light, and conidia were washed from 7–12-day-old cultures. For *C. destructivum*, entire plants were sprayed with a 1×10^5 conidia/mL suspension in sterile distilled water, and for *C. orbiculare*, they were inoculated with 2×10^6 conidia/mL. The plants were then incubated at room temperature in containers to maintain high humidity. *Pseudomonas syringae* pv. *tabaci* strain 11528R was grown overnight in Kings medium B (KB) (King *et al.*, 1954) containing 50 mg/L rifampicin, and suspended in 10 mM MgCl_2 to 1×10^6 CFU/mL. *Pseudomonas syringae* pv. *tabaci* strain 11528R transformed with vector pDSK519 or vector pDSK519 containing *avrPto* (Thilmony *et al.*, 1995) was grown overnight in KB containing 50 mg/L rifampicin and 20 mg/L kanamycin, and suspended in 10 mM MgCl_2 to 1×10^8 CFU/mL. For the compatible bacterial interaction, the two youngest fully matured leaves of wild-type

N. benthamiana were infiltrated with a suspension of *P. syringae* pv. *tabaci* strain 11528R using a needle-less syringe (Klement, 1963). For the incompatible bacterial interaction, the two youngest fully matured leaves of *N. benthamiana* transformed with *Pto* (Rommens *et al.*, 1995) were inoculated with *P. syringae* pv. *tabaci* (*avrPto*) in the same manner. The interaction between *N. benthamiana* (*Pto*) and *P. syringae* pv. *tabaci* strain 11528R transformed with the vector pDSK519 without an insert served as the empty vector control. Inoculated plants were kept at 22 °C with a photoperiod of 8/16 h dark/light at 150 $\mu\text{mol}/\text{m}^2/\text{s}$.

The area of the inoculated leaves was measured with a leaf area meter (Model 3100, LI-COR, Lincoln, NE). For quantifying *Colletotrichum* infection, the number of lesions was counted at 72 and 96 hpi for *C. destructivum* and *C. orbiculare*, respectively, and expressed on a per cm^2 leaf basis. For quantifying *Pseudomonas* populations, leaf discs of 0.8 cm diameter collected at 72 hpi for *P. syringae* pv. *tabaci* with wild-type *N. benthamiana* and 20 hpi for *P. syringae* pv. *tabaci* (*avrPto*) with *N. benthamiana* (*Pto*), respectively, were ground in 1 mL 10 mM MgCl_2 containing 0.01% Tween 80 and then dilution-plated onto KB containing the antibiotics described above to determine the number of colony forming units.

RNA extraction

For extraction of RNA for relative RT-PCR, leaf samples were frozen in liquid nitrogen and stored at -80 °C. Total RNA was prepared according to the method of Chen *et al.* (2000) except that phenol/chloroform was added prior to the homogenization buffer, which consisted of 200 mM Tris base, 400 mM KCl, 200 mM sucrose, 35 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 25 mM EDTA, pH 9.0. The RNA was resuspended in 25–50 μL DEPC-treated dH_2O and stored at -80 °C.

Sequence alignments

A dendrogram was created using amino acid sequences homologous to *LeXEGIP* (Qin *et al.*, 2003) from plants in the family Solanaceae. Sequences were obtained from the databases at GenBank at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) and The Computational Biology and Functional Genomics Laboratory (<http://compbio.dfci.harvard.edu/index.html>) (Table 1). In addition, the predicted protein sequences of *NbXEGIP1* and *NbXEGIP2* were included along with the carrot extracellular dermal glycoprotein (EDGP) (Sato *et al.*, 1992) and the *Triticum aestivum* xylanase inhibitor IV (TAXI-IV) (Igawa *et al.*, 2004). The sequences were aligned, and a dendrogram was created with CLUSTAL X (Thompson *et al.*, 1997) using default parameters. The CLUSTAL X alignment was subjected to distance analysis using the PHYLIP program with the neighbour-joining algorithm and 1000 bootstrap replications. Identification

of a putative signal peptide in the predicted protein sequence of *NbXEGIP1* was done using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>).

RT-PCR

Single-stranded cDNA was synthesized using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Burlington, ON, Canada) and oligo (dT) primer with total RNA following the manufacturer's instructions. All PCR reactions were performed using a Mastercycler Personal (Eppendorf, Mississauga, ON, Canada). RT-PCRs were performed in 15- μ L reaction volumes with 1 μ L cDNA, 1.5 units *Tsg* polymerase (Biobasic, Toronto, ON, Canada), 1.5 μ L 10 $\times$ *Tsg* polymerase buffer, 2 mM dNTP, 2.5 mM $MgSO_4$ and 1.0 μ M of each primer. All RNA samples used for reverse transcription were tested for the presence of genomic DNA by using them directly as the PCR template, prior to cDNA synthesis, under the same PCR conditions.

For the amplification of *NbXEGIP1*, primers NbXEGIP1F2 (5'-GGTATGGCTGGCCTTGGTAG) and NbXEGIP1R1 (5'-AGATCAATGGATGGCACAGC) were designed from the nucleotide sequence of the annotated xyloglucan-specific fungal endoglucanase inhibitor gene of *N. benthamiana*, TC7425 (Table 1), to amplify a PCR product of 510 bp. Amplification conditions were 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and one cycle for 10 min at 72 °C. The PCR product was purified using GenElute™ Gel Purification Kit (Sigma, St Louis, MO), cloned into pBluescript (Stratagene, La Jolla, CA), and then transformed into *Escherichia coli* DH5 α . The insert was then sequenced (Laboratory Services Division, University of Guelph).

For the amplification of *NbXEGIP2*, primers NbXEGIP2F1 (5'-AACCTGTCCCTTGTGGT) and NbXEGIP2R1 (5'-CCTTGCTAGTTCTCAAG) were designed based on the conserved regions of the nucleotide sequences of TC3830, TC156214, TC120914, TC132377, TC128826 and TC128827 (Table 1) to amplify a PCR product of 275 bp. The PCR conditions were 94 °C for 3 min followed by 30 cycles of 30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C. The PCR product was directly sequenced (Laboratory Services Division).

Relative RT-PCR

Semi-quantitative relative RT-PCR was done with the translation elongation factor 1 α (*NbEF-1 α*) as an internal constitutive control according to Dean *et al.* (2002). EF-1 α forward primer TobefS (5'-CTCCAAGGCTAGGTATGATG) and reverse primer TobefA (5'-CTTCGTGGTGCATCTCAAC) were designed by Dean *et al.* (2002) to yield a 375-bp PCR product that could be co-amplified with the gene of interest.

Relative RT-PCR was performed in 15- μ L reaction volumes with 1 μ L cDNA, 1.5 units *Tsg* polymerase (Biobasic, Toronto, ON,

Canada), 1.5 μ L 10 $\times$ *Tsg* polymerase buffer, 2 mM dNTP and 2.5 mM $MgCl_2$ with 0.5 μ M of EF-1 α primer pair and 1 μ M of each *NbXEGIP1*, *NbXEGIP2* or *NbDef2.2* primer. For relative RT-PCR of *NbXEGIP1*, forward primer NbXEGIP1F1 (5'-GCCTGCTTCCTGATAACACA), which was 198 bp upstream from NbXEGIP1F2, was paired with primer NbXEGIP1R1 to amplify an RT-PCR product of 708 bp. Relative RT-PCR of *NbXEGIP1* was done at 94 °C for 3 min followed by 26 cycles of 30 s at 94 °C, 1 min at 62 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C. For relative RT-PCR of *NbXEGIP2*, primers NbXEGIP2F1 and NbXEGIP2R1 were used at 94 °C for 3 min followed by 30 cycles of 30 s at 94 °C, 1 min at 45 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C. For relative RT-PCR of *NbDef2.2*, primers NbDef2.2F1 (5'-AAAAGCTTGATCAGTGAG) and NbDef2.2R1 (5'-GGATTGAAGTGCCACAC) were used at 94 °C for 3 min followed by 30 cycles of 30 s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and a final extension for 10 min at 72 °C.

Electrophoresis and quantification of the PCR products were done as per Dean *et al.* (2002). To determine that the number of amplification cycles was not too great for quantification, the cDNA samples were also amplified with three fewer cycles, and the bands again quantified. The results with the different number of cycles were compared to demonstrate that the expression patterns were not significantly different. To confirm the identity of the RT-PCR products, the bands from the gel for both *NbXEGIP1* and *NbXEGIP2* were purified with GenElute™ Gel Purification Kit (Sigma) and directly sequenced (Laboratory Services Division, University of Guelph).

Gene silencing and analysis of silenced plants

The cloned 510-bp RT-PCR fragment of *NbXEGIP1* obtained with primers NbXEGIP1F2 and NbXEGIP1R1 was digested from pBluescript using *EcoRI* and subcloned into *EcoRI*-digested TRV-RNA2 (PYL156 or pTRV2) vector (Liu *et al.*, 2002). The pTRV2 construct was transformed into *E. coli* DH5 α and selected on LB agar containing 50 mg/L kanamycin. The plasmid was extracted from *E. coli* and then transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and selected on LB agar containing 50 mg/L kanamycin and 5 mg/L tetracycline. The antibiotic-resistant GV3101 colonies were checked for the presence of the insert by PCR. Using the same approach, the green fluorescent protein (GFP) gene from pEZS-CL (Yoshimoto *et al.*, 2004) was inserted into pTRV2 and transformed into *A. tumefaciens* as a control.

Agrobacterium tumefaciens inoculations were done following the procedure of Liu *et al.* (2002) with some modifications. Transformed *A. tumefaciens* were grown for 48 h at 28 °C on LB containing 50 mg/L kanamycin and 5 mg/L tetracycline. *Agrobacterium tumefaciens* cultures containing pTRV1 and *A. tumefaciens* containing pTRV2 with *NbXEGIP1* were suspended in 10 mM $MgCl_2$ to an OD₆₀₀ of 0.8 and then mixed in a 1 : 1 ratio.

The bacterial mixture was infiltrated into the lower leaf of four-leaf stage plants using a 1-mL needle-less syringe. As controls, 10 mM MgCl₂ or *A. tumefaciens* containing pTRV1 mixed with *A. tumefaciens* containing pTRV2 with GFP were infiltrated onto *N. benthamiana*. At 15–18 days after *A. tumefaciens* infiltration, the two youngest fully matured leaves of each plant were sprayed either with spore suspensions of *Colletotrichum* spp. or infiltrated with *P. syringae* pv. *tabaci* or *P. syringae* pv. *tabaci* (*avrPto*) suspensions as described above. Lesion numbers were determined at 72 hpi for *C. destructivum* and 96 hpi for *C. orbiculare*. Bacterial populations were checked at 72 hpi for *P. syringae* pv. *tabaci* and 20 hpi for *P. syringae* pv. *tabaci* (*avrPto*). Relative RT-PCR analysis of *NbXEGIP1*, *NbXEGIP2* and *NbDef2.2* expression was performed as described above at 96 hpi for *Colletotrichum* spp., 72 hpi for *P. syringae* pv. *tabaci* and 24 hpi for *P. syringae* pv. *tabaci* (*avrPto*) for plants treated with MgCl₂, *A. tumefaciens* (pTRV1/pTRV2-GFP) or *A. tumefaciens* (pTRV1/pTRV2-*NbXEGIP1*).

For *NbXEGIP1*-silenced and control leaves inoculated with *P. syringae* pv. *tabaci*, five 8-mm-diameter discs were excised per leaf at 66 hpi. The leaf discs were first washed in 15 mL sterile distilled water for 5 min, and then incubated for 2 h in 15 mL of fresh sterile distilled water with shaking at 60 r.p.m. The amount of electrolytes in the water was measured using a conductivity meter (PC 300 series, Oakton, Vernon Hills, IL). After the measurements, the material was autoclaved for 15 min at 121 °C, and then the amount of electrolytes was measured again after the water had returned to room temperature. Relative conductivity was calculated from the electrical conductivity at 2 h incubation divided by the electrical conductivity after autoclaving. Three separate experiments with a total of 15 leaves were examined.

ACKNOWLEDGMENTS

Pseudomonas syringae pv. *tabaci* 11528R and *P. syringae* pv. *tabaci* 11528R containing *avrPto* or vector pDSK519 were kindly provided by Dr G. Martin, Boyce Thompson Institute, Cornell University, Ithaca, NY. Transgenic *N. benthamiana* containing *Pto* was kindly provided by Dr R. Michelmore, Genome Center, University of California, Davis, CA. The plasmid pEVS-CL was kindly provided by Dr M. Raizada, Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada. Funding for this study was provided by the Natural Science and Engineering Research Council of Canada.

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