Induction of a Small Heat Shock Protein and Its Functional Roles in *Nicotiana* Plants in the Defense Response against *Ralstonia solanacearum*[1][W]

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In tobacco (*Nicotiana tabacum*), *Ralstonia solanacearum* OE1-1 (RsOE1-1) is pathogenic, whereas *R. solanacearum* 8107 (Rs8107) is nonpathogenic and induces the hypersensitive response (HR). To elucidate the molecular mechanisms of plant-*R. solanacearum* interactions, we used differential display to isolate a cDNA fragment, A6, regulated in tobacco by inoculation with RsOE1-1. The deduced amino acid sequence predicted from full-length A6-cDNA showed similarity to small heat shock proteins from Arabidopsis (*Arabidopsis thaliana*; hypothetical protein), *Medicago truncatula*, and *Cucumis melo*; we therefore designated A6 to correspond to *Ntshsp17* (for tobacco small heat shock protein 17). Recombinant Ntshsp17 overproduced in *Escherichia coli* exhibited molecular chaperone function. Expression of *Ntshsp17* was increased in tobacco leaves inoculated with both RsOE1-1 and Rs8107. Expression was induced by heat treatment and by treatment with aminocyclopropane carboxylic acid, hydrogen peroxide, methyl jasmonate, and salicylic acid. *Ntshsp17* expression was induced by inoculation with a HR and pathogenicity gene mutant of Rs8107 that does not induce the HR, but not by *Agrobacterium*-mediated transient expression of INF1, an HR elicitor. In *Nbshsp17*-silenced plants (an *Ntshsp17* ortholog in *Nicotiana benthamiana*), expression of *ETHYLENE-RESPONSE ELEMENT-BINDING PROTEIN*, *PATHOGENESIS-RELATED1a* (*PR1a*), and *PR4* genes was compromised, but expression of *ELONGATION FACTOR1α* was scarcely affected. Appearance of the HR was not affected in the silenced plants. In the silenced plants, growth of Rs8107 was accelerated. Bacterial growth and wilt symptoms elicited by RsOE1-1 were also accelerated in the silenced plants. These results indicate that this small heat shock protein might have a role in HR-independent defenses in *Nicotiana* plants.

The ability to recognize invading pathogens and rapidly mount appropriate defense responses is unique to eukaryotic immune systems. In plants, the outcome of many plant-pathogen interactions is determined by the presence or absence of complementary pairs of resistance (*R*) genes in the host and avirulence (*Avr*) genes in the invading pathogens. In the model plant Arabidopsis (*Arabidopsis thaliana*), numerous genetic loci conferring resistance to bacterial pathogens have been identified and cloned, such as *RPM1*, *RPS2*, and *Pto* (for review, see Schulze-Lefert, 2004). Genetic approaches have also been used to define intracellular components required for the action of Arabidopsis *R* genes, such as *EDS1* (Falk et al., 1999), *HSP90* (Hubert et al., 2003), *NPR1* (Cao et al., 1997), *RIN4* (Hubert et al., 2003), *RAR1* (Azevedo et al., 2002), and *SGT1* (Austin et al., 2002). Intracellular components, including *EDS1*, *NPR1*, and a *RAR1* ortholog, reportedly function in *N* gene-mediated resistance to *Tobacco mosaic virus* in the tobacco (*Nicotiana tabacum*) plant (Liu et al., 2002).

Ralstonia solanacearum is a devastating, soilborne pathogen with global distribution and wide host range (Hayward, 1991). It causes bacterial wilt in several economically important solanaceous crops and is well adapted to life in the soil in the absence of host plants. *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 invading into xylem vessels (Hayward, 1991; Vasse et al., 1995; Seile et al., 1997). In the tomato (*Solanum lycopersicum*), resistance to *R. solanacearum* is controlled by several loci (Thoquet et al., 1996a, 1996b), whereas in Arabidopsis, resistance is monogenic and 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 em-

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ployed 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*. An important step in understanding the molecular basis of *R. solanacearum*-plant interactions is isolation and characterization of genes, which are regulated in compatible or incompatible combinations. Godiard et al. (1991) reported the isolation of cDNA clones corresponding to mRNA that accumulated during the early phase of the hypersensitive response (HR) in suspension-cultured cells challenged with an incompatible strain of *R. solanacearum*. However, there have been few other informative reports concerning expression profiling and functional analysis of genes related to *R. solanacearum*-plant interactions.

A

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          10         20         30         40         50         60
GCATAGCCAGTGTTCACAATACAGGAGTTTACATAAACAACCCCAATTACTGAGATGTAC

          70         80         90        100        110        120
CTGAATTGATTAGCTACTGCTCGTAATACATTCTTATATTCCAATTTCAAGGAATGACAC

         130        140        150        160        170        180
ATTTCAGAATCACATATTATGATCAAAGAATTCGACCTCATTGTCTATGCTGAAGCGAAC

         190        200        210        220        230        240
TTGCCTTAGTTCAAATTTCAGCTACGGGATCTCCTTAAGAAAAGAACAGTAAAGCGGGCC

         250        260        270        280        290        300
TGCAGGTAGGAAATTTTTTGACACAACTGTAAGCATGAACTCAGAAGAATGTGCAAAGTC
                                    M  N  S  E  E  C  A  K  S
         310        320        330        340        350        360
TCATCATTCCCAACCAACTGTCACTCTGACTGGGACTGCTGAAAAATGTGCAATTGGACC
 H  H  S  Q  P  T  V  T  L  T  G  T  A  E  K  C  A  I  G  P
         370        380        390        400        410        420
ATCACTTGGTGTGGTTGACATAGGCAATAGTGAAAATGCATATCTTTTTCGTGTTGCGCT
 S  L  G  V  V  D  I  G  N  S  E  N  A  Y  L  F  R  V  A  L
         430        440        450        460        470        480
TCCTGGTGTAAGAAATAAATGTAATATAAAATGTGACATACAGAGAGAAGGGAGAGTCCG
 P  G  V  R  N  K  C  N  I  K  C  D  I  Q  R  E  G  R  V  R
         490        500        510        520        530        540
GATAGAGGGTGTGGTTACAGAGAGTGATGTCTTGAAAAACTCATCCAAGGGTTATGAGAT
 I  E  G  V  V  T  E  S  D  V  L  K  N  S  S  K  G  Y  E  M
         550        560        570        580        590        600
GAAAGTTCAGCAGCTTTCTCCTCCTGGGCCTTTTACCGTATCGTCCAATTTGCCAGGACC
 K  V  Q  Q  L  S  P  P  G  P  F  T  V  S  S  N  L  P  G  P
         610        620        630        640        650        660
GGTTGATCCCAGATTATGTTCCCCACGTTTTAGATCAGATGGCATTCTGGAAGTCATTGT
 V  D  P  R  L  C  S  P  R  F  R  S  D  G  I  L  E  V  I  V
         670        680        690        700        710        720
ATTGAAATACCGGATACCTATTGTGTCAGCCGAAGGTTTGCCTGAGAATTGGTACAATGG
 L  K  Y  R  I  P  I  V  S  A  E  G  L  P  E  N  W  Y  N  G
         730        740        750        760        770        780
CTCTTTCCCAGCTCCTTGAATTCTTGTCGTCCCTGTTTCTTAACATACATGGGATTTATT
 S  F  P  A  P  *
         790        800        810        820        830        840
TGTTTTGTAACATTTCTCGTGAATATAGCAGTCAACTTCTAGGGGGGCTACCTGTACAAC
         850        860        870        880        890        900
AAGGGATTTTGCATTATATGTTTCATTGGATATTTTGGCTATGCTGACGTTGATTTTGAA
         910        920        930        940        950        960
CCCAAGTGTGTACTGTTTCAACAAGAAAAAAATACATGAAGTATGTGACATTATTTACAT
         970        980        990
TCTAAAAAAAAAAAAAAGAAAAAAAAAAA
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B

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Ntshsp17(AB290185)   1:-----MNSEEC--AKSHHSKP-TVIL-TGTAEKCAIGPSLGVVDIGNSENAYLFRVALPG 51
Nbshsp17(AB290186)   1:-----......--...Q.T..-..T.-.............................. 51
AtUNP    (BT000371)  1:-----..A.NNQ-TTTT...VISHVFC....KLGSV..PI.L....V..V......S... 54
Mthsp20  (ABE82163) 301:KSDEPSLMPLISLPDIDSCVQDHSIVL....NRGLL...V......I.KV......S... 360
Cmhsp    (AAO45755) 254:PSEE-DW-A--NL--VAA-T-NSGFAL....AMGHV..II.SM...EC.DS.....S... 305

Ntshsp17(AB290185)  52:V-RNKCNIKCDIQREGRVRIEG-VVTESDVLKNSSKGYEMKVQQLSPPGPFTVSFNLPGP 109
Nbshsp17(AB290186)  52:.-.....................-.I...........D..................... 109
AtUNP    (BT000371)  55:IEK.QDK...E.......C.Q.-.IP.IAIPSDTGCL.R.Q...CP.....SIT.....Q 113
Mthsp20  (ABE82163) 361:.K.EYNQFS...ESD.K.E.R.L-LSGIRTIATQ.RVFQ..T...CS....TIS.S.... 419
Cmhsp    (AAO45755) 306:.K.DP.GFN.EVEKD...V.Q.VTT.GERTV.KH.QVF..VTHN.C...E.SL..Q.... 365

Ntshsp17(AB290185) 110:VDPSLCSPRFRSDGILEVIVLKYRIPIVSAEGLPENWYNGSFPAP                154
Nbshsp17(AB290186) 110:...R...R.............................C.......                154
AtUNP    (BT000371) 114:...R.F..N......F..V.V.LGVR.PTS----------------               143
Mthsp20  (ABE82163) 420:...R.FA.N..D...F.GV.I.-L---------------------               442
Cmhsp    (AAO45755) 366:...QHFLAN.DIA....GV.M.DLQS-------------------                391
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Figure 1. Nucleotide sequence and deduced amino acid sequence of *Ntshsp17* in tobacco. A, Nucleotide sequence and deduced amino acid sequence of *Ntshsp17*. The original A6 sequence is shown in italics. Underlined sequence denotes the HSPS20 motif. B, Alignment of the deduced amino acid sequence of *Ntshsp17*, its ortholog in *N. benthamiana* (*Nbshsp17*), unknown protein from Arabidopsis (AtUNP), HSP20 from *M. truncatula* (Mthsp20), and SHSP from *C. melo* (Cmshsp). Dots indicate identical amino acids. Bars show amino acids that are not present in sequences.

Our objectives are the isolation, characterization, and functional analysis of genes related to *R. solanacearum*-tobacco plant interactions. We employed differential display-PCR to isolate gene fragments from genes regulated in tobacco plants by inoculation with *R. solanacearum* (Kiba et al., 2007). Among the isolated gene fragments, we focused on clone A6, which displayed similarity to small heat shock proteins (SHSPs). A heat shock protein (HSP) family, including HSP70 and HSP90, reportedly has a defensive role against abiotoic stress and some phytopathogens. However, little is known about the role of SHSPs in plant defense. In this study, we carried out expression analysis with quantitative real-time PCR and functional analysis of the SHSP with virus-induced gene silencing (VIGS) using the *Potato virus X* (PVX)-*Nicotiana benthamiana* system. Silencing of this SHSP resulted in compromised expression of defense-related genes, acceleration of growth of virulent and avirulent *R. solanacearum*, and notable acceleration in the development of bacterial wilt. We also discuss a possible mechanism by which this SHSP affects disease resistance.

RESULTS

Ntshsp17 Is Differentially Expressed in RsOE1-1-Infiltrated Leaves

To isolate differentially regulated genes in tobacco in an early stage of infection with *R. solanacearum*, we constructed an equalized cDNA library with mRNA isolated from tobacco 3 h after infiltration (HAI) with water and *R. solanacearum* OE1-1 (RsOE1-1). Products from differential display were then compared to ascertain differentially expressed gene fragments in response to RsOE1-1 inoculation and false-positive clones were eliminated using reverse northern hybridization (Kiba et al., 2007). cDNA fragment A6 showed the strongest signal in reverse northern-blot analysis, using a probe constructed from a labeled cDNA library derived from RsOE1-1-infiltrated tobacco in comparison to signals obtained using a cDNA probe derived from water-infiltrated tobacco (data not shown).We therefore selected clone A6 for further analysis. As shown Figure 1A, the full-length 992-bp cDNA obtained by PCR contained an open reading frame encoding a polypeptide of 154 amino acids, which included the same nucleotide sequence as clone A6. The predicted molecular mass was calculated as approximately 17.6 kD. The deduced amino acid sequence contained a HSP20 motif at amino acid positions 35 to 132 (Fig. 1A). A protein database search showed 58%, 51%, and 48% amino acid identity with putative protein from Arabidopsis (BT000371) and SHSPs from *Medicago truncatula* (ABE82163) and *Cucumis melo* (AA045755; Fig. 1B). Therefore, we designated the gene corresponding to this cDNA as *Ntshsp17* (for *N. tabacum* small heat shock protein 17).

Expression of *Ntshsp17* Is Heat Inducible

To determine the effect of heat treatment on the expression of *Ntshsp17*, RNA samples were isolated

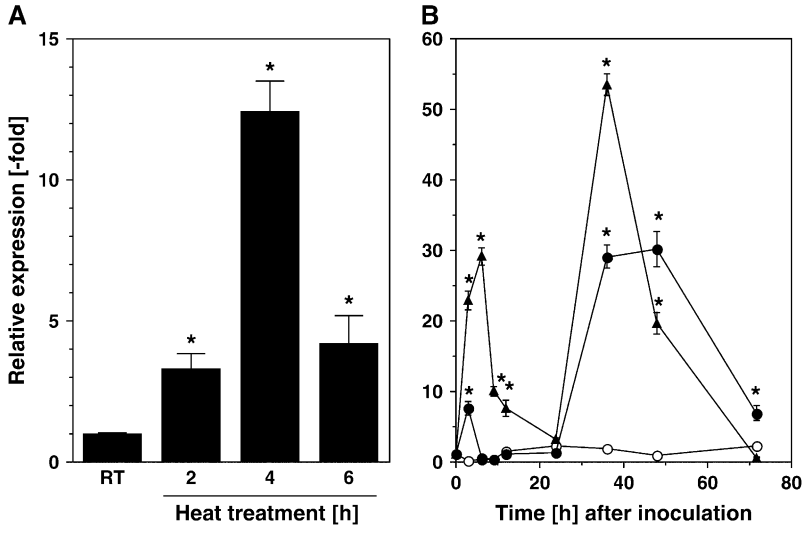

Figure 2. Expression pattern of *Ntshsp17* in response to heat treatment and inoculation with *R. solanacearum*. A, Total RNA was isolated from tobacco 'Samsun NN' leaves after incubation at 42°C for indicated times. Quantitative real-time PCR was carried out as described in "Materials and Methods" with A6rtpF and A6rtpR primers. Expression values are relative to the absolute nontreated control level and are normalized against actin values. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from room temperature (25°C)-incubated controls (RT). *, $P < 0.05$. B, Total RNA was isolated from tobacco 'Samsun NN' leaves infiltrated with water (white circles), RsOE1-1 (black circles), and Rs8107 (black triangles) after incubation at 25°C for indicated times. Quantitative real-time PCR was carried out as described in "Materials and Methods" with A6rtpF and A6rtpR primers. Expression values are relative to the absolute nontreated control level and are normalized against actin values. Values represent the means with SD from triplicate experiments. Asterisks denote values determined to be significantly different from water-infiltrated controls (*, $P < 0.05$).

from leaves taken from tobacco plants that had been incubated at 42°C for 2, 4, and 6 h. Expression analysis of *Ntshsp17* by quantitative reverse transcription (RT)-PCR showed that *Ntshsp17* was induced by heat treatment and that the peak of expression was observed in tobacco 4 h after heat treatment (Fig. 2A). Therefore, *Ntshsp17* is a heat-inducible gene. This result is consistent with the primary sequence of *Ntshsp17*.

Induction of *Ntshsp17* in Response to *R. solanacearum*

In tobacco leaves, the population of RsOE1-1 reached a maximum approximately 48 HAI and necrotic lesions appeared around 48 to 72 HAI (Kanda et al., 2003a). *R. solanacearum* 8107 (Rs8107) is nonpathogenic and induces the HR in tobacco. The bacterial number of Rs8107 increased up to 24 HAI, and the HR can be observed 24 to 36 HAI with Rs8107 (Kiba et al., 2003). The time course of the gene expression analysis was determined by timing bacterial growth and appearance of the HR and necrotic lesions, then isolating total RNA from tobacco leaves at 3, 6, 9, 12, 24 36, 48, and 72 HAI with RsOE1-1 and Rs8107. As shown in Figure 2B, expression of *Ntshsp17* was rapidly and strongly induced in tobacco leaves inoculated with Rs8107. Two-phase expression of *Ntshsp17* was observed and the first peak was detected at 3 to 6 HAI with Rs8107. The second peak was detected during development of the HR (36 HAI with Rs8107). Inoculation with RsOE1-1 also induced two phases of *Ntshsp17* expression at 3 and 36 to 48 HAI. The second peak of expression was also correlated with the appearance of necrotic lesions. However, this increase was not dramatic in comparison to Rs8107 inoculation.

Chaperone Activity of Ntshsp17

Full-length cDNA of A6 showed significant similarity to SHSPs from several plant species. This information prompted us to test the chaperone activity of Ntshsp17 using recombinant Ntshsp17 expressed in *Escherichia coli*. When the total cellular protein in a cell-free extract from control *E. coli* was heated, about 30% of protein was denatured at 40°C and over 50% of proteins decreased their solubility at 90°C. In the protein fraction of Ntshsp17-expressing *E. coli* cells, however, only 5% of proteins disappeared at 40°C, and about 70% of proteins were soluble even at 90°C (Fig. 3A).

The above data indicate that Ntshsp17 is effective in preventing aggregation of bacterial cellular proteins. To test whether chaperone activity is the same using another heat-sensitive substrate, aggregation protection of firefly (*Photinus pyralis*) luciferase (LUC) was examined. SDS-PAGE analysis showed that the purified recombinant protein was a single band with a molecular mass of approximately 17 kD (data not shown). This was consistent with the molecular mass calculated from the deduced amino acid sequence of *Ntshsp17*. LUC incubated with 1 μM Ntshsp17 was recovered almost exclusively in the soluble fraction and was not detected in the pellet fraction (Fig. 3B). Therefore, Ntshsp17 was able to protect LUC from heat-induced insolubilization. These results showed that Ntshsp17 possessed typical chaperone activity.

Effect of Intracellular Signaling Molecules on *Ntshsp17* Expression

To elucidate the signaling pathways related to *Ntshsp17* induction, well-known intracellular signaling molecules were infiltrated into tobacco leaves. The signaling molecules were SA, methyl jasmonate (MeJA), the ethylene (ET) precursor aminocyclopropane car-

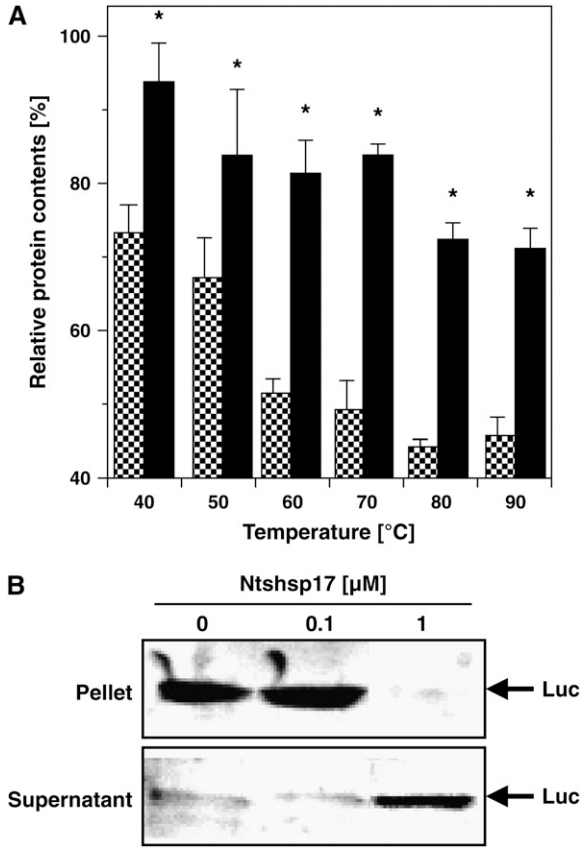

Figure 3. Analysis of chaperone activity of Ntshsp17. A, Thermostability of total protein from *E. coli* with empty pET16b vector or pET16b containing *Ntshsp17*. Total protein extracts were heated at indicated temperatures for 15 min. Heat-denatured proteins were removed by centrifugation and protein content of the supernatant fractions was determined by the method described in the text. Protein content from *E. coli* containing the empty pET16b vector and Ntshsp17-expressing *E. coli* are shown as black and white or black bars, respectively. Values are relative to the levels in unheated controls. Values represent the means and SD from triplicate experiments. Asterisks denote values determined to be significantly different from empty-vector controls (*, $P < 0.05$). B, Aggregation protection of firefly LUC by Ntshsp17. LUC at 1 μM was heated in the absence (0) or presence of an appropriate amount of Ntshsp17 for 15 min at 42°C. After heating, samples were centrifuged, and both supernatant and pellet fractions were subjected to 12% SDS-PAGE and stained with Coomassie Blue.

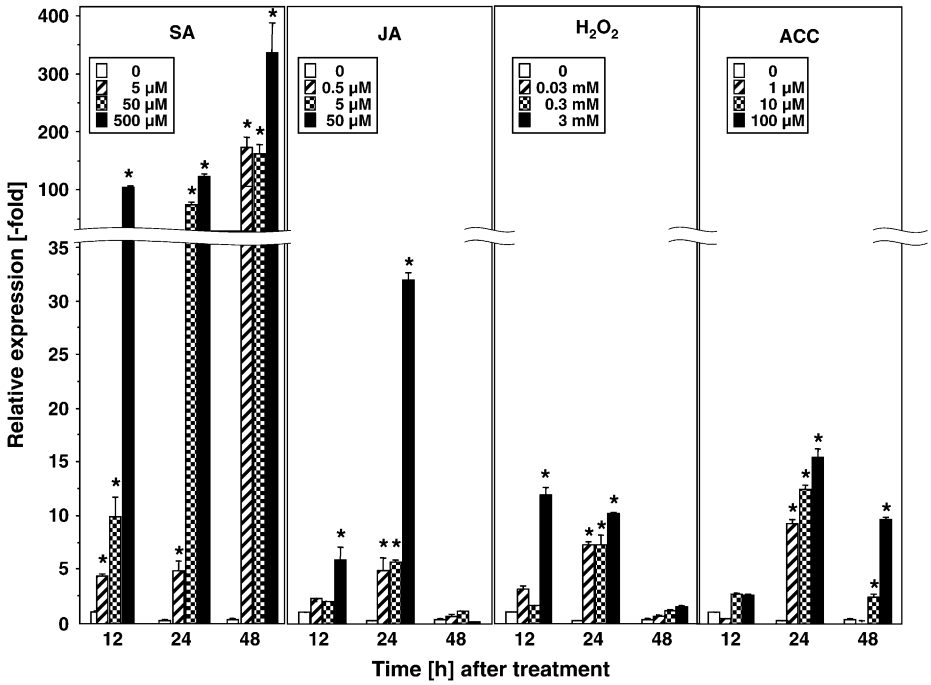

Figure 4. Expression pattern of *Ntshsp17* in response to intracellular signaling molecules. Total RNA was isolated from tobacco 'Samsun NN' leaves at 12, 24, and 48 HAI with water, SA (5, 50, 500 μM), MeJA (0.5, 5, 50 μM), H_2O_2 (0.03, 0.3, 3 mM), and ACC (0, 1, 10, 100 μM). Quantitative real-time PCR was carried out as described in "Materials and Methods" with A6rtpF and A6rtpR primers. Expression values are relative to the absolute nontreated control level (0 h) and are normalized against actin values. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from water-infiltrated controls (*, $P < 0.05$).

boxylic acid (ACC), and hydrogen peroxide (H_2O_2). Total RNA was isolated 12, 24, and 48 h after treatment. Expression of *Ntshsp17* was induced by treatment with all chemicals tested (Fig. 4). H_2O_2 (3 mM) induced expression of *Ntshsp17* 12 to 24 h after treatment, and expression subsequently declined at 48 h after treatment. *Ntshsp17* was also induced 24 h after treatment with 0.03 and 0.3 mM H_2O_2. ACC and MeJA also induced *Ntshsp17* expression in a dose-dependent manner and the peak of expression was observed 24 h after treatment with each chemical. Among these chemicals, SA was the most effective in inducing expression of *Ntshsp17*. Expression of *Ntshsp17* was increased between 12 to 48 h after SA treatment in a dose-dependent manner and the expression level after 48-h treatment with 500 μM of SA was increased over 300 times in comparison to nontreated tobacco plants (Fig. 4).

Expression of *Ntshsp17* Is Independent of Induction of Cell Death

Expression of *Ntshsp17* was significantly induced in tobacco leaves during development of the HR and appearance of necrotic lesions. To examine the relationship between cell death and induction of *Ntshsp17*

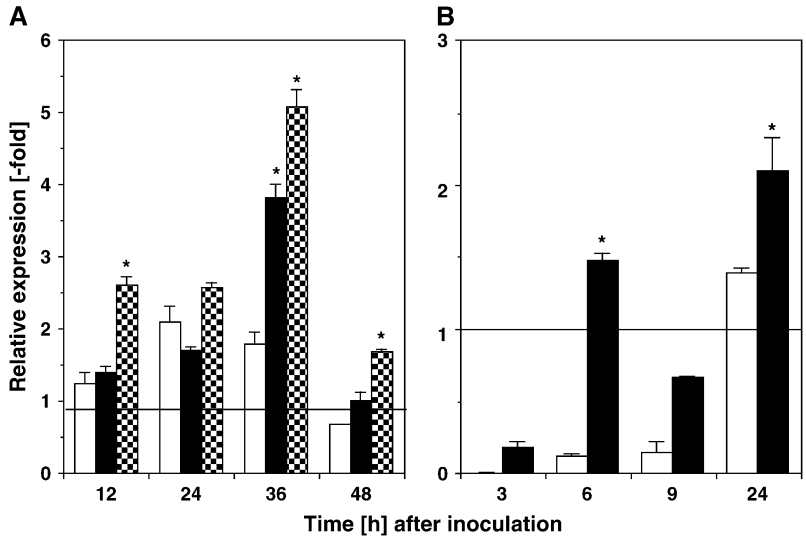

Figure 5. Expression pattern of *Ntshsp17* in response to INF1 and the *hrp* mutant of *R. solanacearum* 8107. A, Total RNA was isolated from tobacco 'Samsun NN' leaves infiltrated with water (white bars), INF1-expressing (black bars), or GUS-expressing (black and white bars) *Agrobacterium* at indicated time points. B, Total RNA was isolated from tobacco 'Samsun NN' leaves infiltrated with water (white bars) or the *hrpY* mutant of Rs8107 (black bars) at indicated time points. Quantitative real-time PCR was carried out as described in "Materials and Methods" with A6rtpF and A6rtpR primers. Expression values are relative to the absolute nontreated control level and are normalized against actin values. Line indicates value of nontreated control level. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from water-infiltrated controls (*, $P < 0.05$).

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 *Ntshsp17* induced by inoculation with a *hrpY* (encoding Hrp pilus) mutant of Rs8107 (Rs8107ΔY) that is not able to induce a HR. Because the peaks of *Ntshsp17* expression were observed at 3 to 6 HAI and 24 HAI with wild-type Rs8107, total RNA was isolated from tobacco 3, 6, 9, and 24 HAI with Rs8107ΔY. As shown in Figure 5A, expression of *Ntshsp17* was induced in tobacco plants inoculated with INF1-expressing *A. tumefaciens*, as well as GUS-expressing control *A. tumefaciens*. *Ntshsp17* was also up-regulated in tobacco leaves inoculated with the Rs8107ΔY, two expression peaks being observed 6 and 24 h after inoculation in a similar pattern to wild-type Rs8107 (Fig. 5B). Induction of *Ntshsp17* by *Agrobacterium* containing the GUS reporter and by the nonvirulent Rs8107ΔY demonstrated that expression of the *Ntshsp17* gene was not directly related to cell death.

VIGS of *Nbshsp17* in *N. benthamiana*

Expression of *Ntshsp17* was observed in *N. tabacum* plants inoculated with *R. solanacearum* and those treated with well-known signaling molecules, such as ACC, H_2O_2, MeJA, and SA. This information prompted us to test the function of *Ntshsp17* in plant defense responses using a VIGS approach in *N. benthamiana*. In these experiments, the A6 sequence was used to silence a *N. benthamiana* homolog of *Ntshsp17* using the PVX vector (Baulcombe et al., 1995; Fig. 6A). 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-shsp17 (data not shown). Analysis with quantitative RT-PCR confirmed that the *Nbshsp17* gene was silenced (Fig. 6B).

Silencing of *Nbshsp17* Compromised Expression of Defense-Related Genes

To test the influence of silencing of *Nbshsp17* on expression of defense-related genes, we selected a marker gene for SA signaling (*PATHOGENESIS-RELATED1* [*PR1*]), a marker gene for MeJA signaling (*PR4*), and a marker gene for ET signaling (*ET-RESPONSIVE ELEMENT-BINDING PROTEIN* [*EREBP*]). Total RNA was extracted from *Nbshsp17*-silenced and control leaves 6, 9, 12, and 24 HAI with Rs8107. As shown in Figure 7, expression of *EREBP* showed two phase peaks in control plants 6 and 12 HAI with Rs8107. However, expression of *EREBP* was greatly reduced in *Nbshsp17*-silenced leaves throughout the experiment. In the case of *PR1* and *PR4*, the highest level of expression was observed in control plants 24 HAI with Rs8107. Expression levels of both PR genes were also reduced in *Nbshsp17*-silenced leaves. The reduction in *PR1*, *PR4*, and *EREBP* expression levels was consistent with the *Ntshsp17* responses to the intracellular signaling molecules SA, MeJA, and ACC. In contrast to these defense-related genes, expression of a housekeeping gene, *ELONGATION FACTOR1α* (*EF1α*), was scarcely affected by *Nbshsp17* silencing. These results suggested that *Nbshsp17* may have important roles in expression of defense-related, inducible genes.

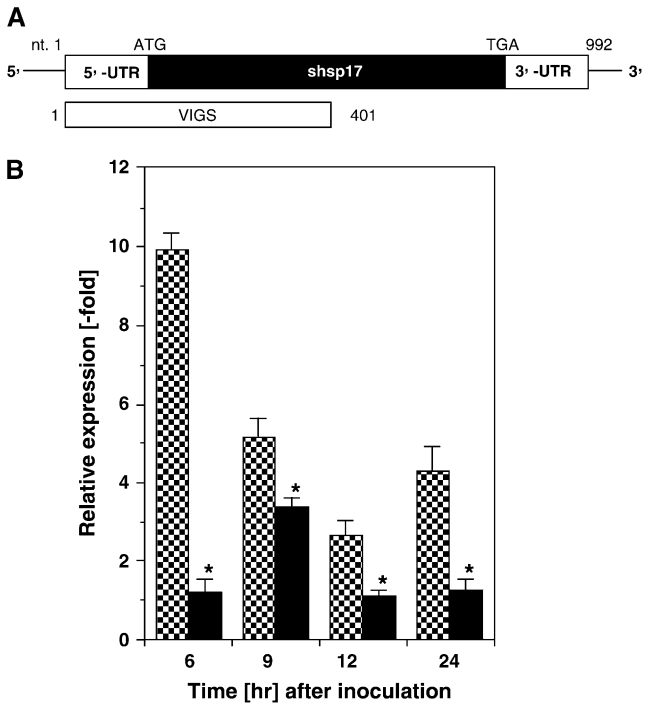

Figure 6. VIGS of *Nbshsp17*. A, Diagram illustrates the SHSP cDNA and the fragments used for VIGS. Length and position are indicated for the corresponding nucleotide sequence. B, Relative abundance of *Nbshsp17* transcripts in *Agrobacterium* carrying either empty PVX (black and white bars) or PVX:shsp17 (black bars) infected plants were analyzed using quantitative real-time PCR with A6rtpF2 and A6rtpR2 primers. Expression values are relative to the absolute nontreated control level and are normalized against actin values. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*, $P < 0.05$).

HR Caused by *R. solanacearum*, *Pseudomonas cichorii*, and INF1 Is Not Affected by Silencing of *Nbshsp17*

The HR is one of the best-characterized plant defenses against pathogens. To examine the response of *Nbshsp17*-silenced plants to HR-triggering agents, nonpathogenic bacteria Rs8107, *Pseudomonas cichorii* (Kanzaki et al., 2003), and INF1-expressing *A. tumefaciens* were inoculated into *Nbshsp17*-silenced plants as well as control plants. A HR developed in both the control and *Nbshsp17*-silenced plants 48 HAI with all bacteria (Fig. 8).

Silencing of *Nbshsp17* Accelerates Growth of *R. solanacearum* and Disease Development of Bacterial Wilt

Because reduction of defense-related gene expression was observed in *Nbshsp17*-silenced plants, this raised

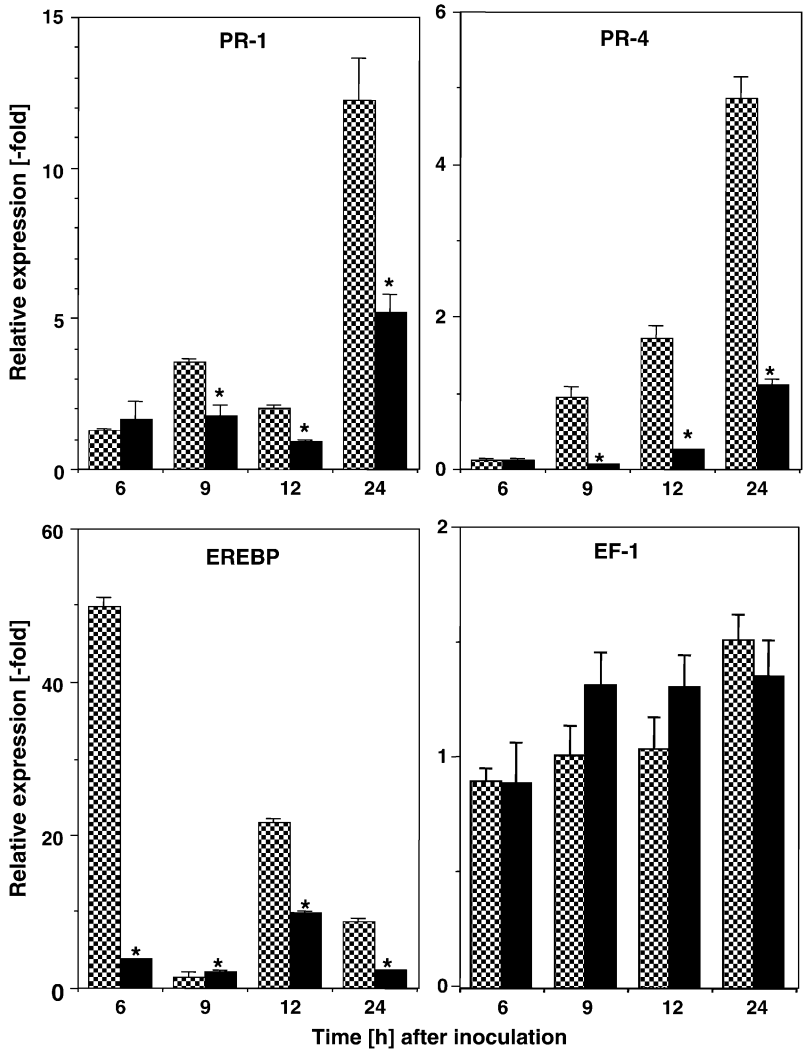

Figure 7. Effect of silencing of *Nbshsp17* on expression of defense-related genes. *N. benthamiana* plants were infected with *Agrobacterium* carrying either PVX (black and white bars) or PVX:shsp17 (black bars). Three weeks later, the fourth leaves above the primary *Agrobacterium*-infected leaves were infiltrated with Rs8107 and total RNA was isolated at indicated time points. The relative abundance of *PR1α*, *PR4*, *EREBP*, and *EF1α* transcripts was 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 are relative to the absolute nontreated control level and are normalized against actin values. Values represent the mean and SD of results from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*, $P < 0.05$).

the possibility that disease resistance to nonpathogenic bacteria is compromised and disease susceptibility to pathogenic bacteria may increase in silenced plants. To address whether silencing of *Nbshsp17* would affect growth of an avirulent strain of *R. solanacearum*, Rs8107 was inoculated into *Nbshsp17*-silenced leaves and control leaves. The bacterial population was determined 18 and 24 HAI. As shown in Figure 9A, growth of Rs8107 was significantly enhanced in *Nbshsp17*-silenced plants 24 HAI, showing an approximately 10-fold increase in comparison with control plants. Next, we confirmed the effect of *Nbshsp17* silencing on growth of a virulent strain of *R. solanacearum*, RsOE1-1. Enhancement of growth of RsOE1-1 was also observed in *Nbshsp17*-silenced plants 1 and 2 d after inoculation (Fig. 9B). However, acceleration of RsOE1-1 growth (about 5-fold) was not remarkable in comparison with the growth of Rs8107 (about 10-fold). These results might reflect the expression level of *Ntshsp17* in response to the two bacterial strains. We also observed the phenotype of *Nbshsp17*-silenced and control plants challenged with RsOE1-1. In control plants, bacterial wilt was first observed at 10 d and plants were completely wilted at 14 d after inoculation with RsOE1-1. When challenged with RsOE1-1, *Nbshsp17*-silenced plants started to wilt at 7 d and were completely wilted at 10 d (Fig. 9, C and D).

DISCUSSION

HSPs are highly conserved proteins expressed in cells that have been subjected to elevated temperatures or various environmental stresses (Cooper, 2000). HSPs include HSP100/ClpB, HSP90/HtpG, HSP70/DNAK, and HSP60/GroEL (Kotak et al., 2007). HSPs act as molecular chaperones to stabilize or facilitate refolding of proteins that have been denatured during stress events. This allows cells to adapt to changes in their environment and to survive in otherwise lethal conditions (Didelot et al., 2006). Another group of HSPs with a molecular mass of 15 to 42 kD is designated as the SHSPs. SHSPs are usually undetectable in vegetative tissues under normal growth conditions,

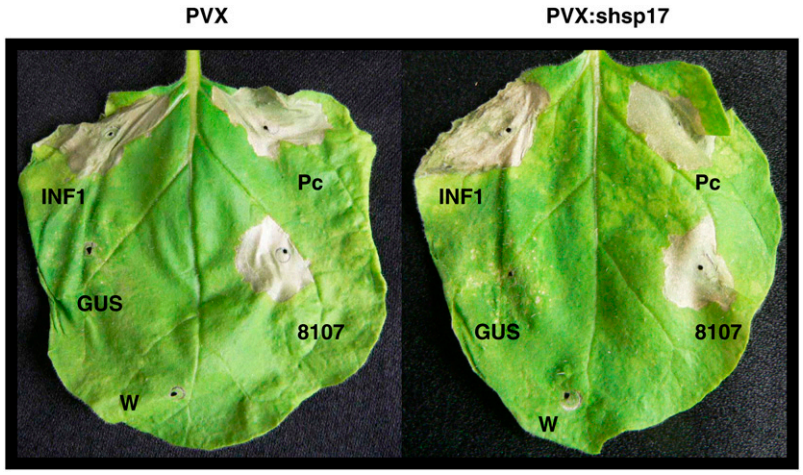

Figure 8. Effect of *Nbshsp17* silencing on induction of HR. *N. benthamiana* plants were infected with *Agrobacterium* carrying either PVX or PVX:shsp17. Three weeks later, the fourth leaves above the primary *Agrobacterium*-infected leaves were infiltrated with *P. cichorii* (Pc), Rs8107 (8107), or *Agrobacterium* harboring 35S-GUS (control; GUS) or 35S-INF1 (INF1). Photographs were taken 4 d after infiltration of each bacterium.

but can be induced by environmental stresses and developmental stimuli. Plant SHSPs are divided into six classes. Three classes (CI, CII, and CIII) of SHSPs are localized in the cytosol or in the nucleus and the other three in the plastids/chloroplast, the endoplasmic reticulum, and the mitochondria (Sun et al., 2002). Some SHSPs have been demonstrated to act as molecular chaperones in vitro (Lee et al., 1997) and in vivo (Forreiter et al., 1997) and bind to and stabilize an unstable conformation of another protein. By controlled binding and release, SHSPs facilitate folding, oligomeric assembly, transport to specific subcellular compartments, or disposal by degradation (Hendrick and Hartl, 1995). Expression of SHSPs is reportedly induced not only by heat shock, but also by various cellular stresses, such as osmotic stress (Almoguera and Jordano, 1995), oxidative stress (Banzet et al., 1998), cold stress (Sabehat et al., 1998), heavy metal treatment (Györgyey et al., 1991), and ozone exposure (Eckey-Kaltenbach et al., 1997). Recent studies have also demonstrated induction of SHSPs in response to viral infection (Whitham et al., 2006). The correlation between the synthesis of SHSPs and the induction of a variety of stress responses and their chaperone activity led to the hypothesis that SHSPs protect cells from the detrimental effects of stress. However, little is known about the role of SHSPs in plant-phytopathogenic bacteria interactions. In this study, we isolated a member of the SHSPs from tobacco (*Ntshsp17*) that showed greatly increased expression in tobacco in response to

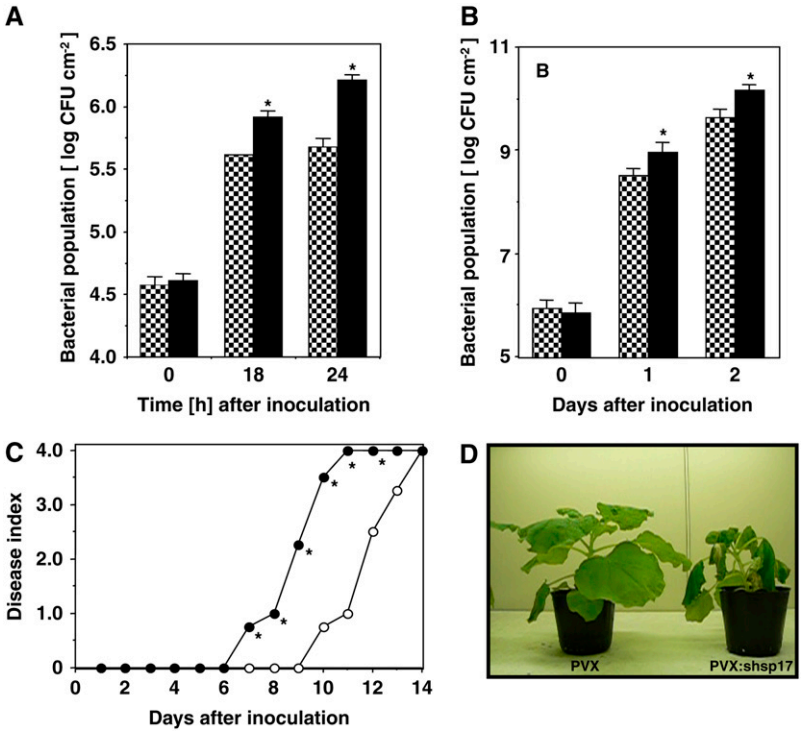

Figure 9. Effect of *Nbshsp17* silencing on growth of *R. solanacearum* and development of bacterial wilt. A, *N. benthamiana* leaves were preinoculated with *Agrobacterium* either carrying empty PVX (black and white bars) or PVX:shsp17 (black bars). Three weeks later, the fourth leaves above the primary *Agrobacterium*-infected leaves were infiltrated with a bacterial suspension of Rs8107. B, Three weeks later, the fourth leaves above the primary *Agrobacterium*-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:shsp17 (black circles). 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 *Nbshsp17*-silenced (PVX:shsp17) *N. benthamiana*. Photograph was taken 10 d after inoculation with RsOE1-1.

an avirulent strain of *R. solanacearum* (Rs8107) in comparison to the virulent strain RsOE1-1 (Fig. 2). Inoculation with *P. cichorii*, a bacterium that is nonpathogenic in tobacco, also induced *Ntshsp17* expression (data not shown). These results suggested that SHSPs may have a role in plant protection against both abiotic and biotic stress.

Growth of *R. solanacearum* (Rs8107) was accelerated in *N. benthamiana* plants in which *Nbshsp17* (an ortholog of *Ntshsp17*) was silenced, suggesting a crucial role of *Ntshsp17* in disease resistance (Fig. 9). This observation is consistent with the reduction in expression of defense-related genes, including *PR1*, *PR4*, and *EREBP* observed in *Nbshsp17*-silenced plants (Fig. 7). Generally, host resistance responses to phytopathogenic bacteria are extremely complex and are likely to involve myriad cellular processes in addition to expression of PR proteins (Maleck et al., 2000; Mysore et al., 2002; Tao et al., 2003). Reduction of disease resistance seems to be partially caused by a reduction in expression of defense-related genes. Taken together, the results presented here suggest essential roles of SHSPs in induction of defense responses. As far as we know, this is the first report of direct involvement of a SHSP in plant disease resistance.

Plant innate immune responses are divided into two categories, HR-based defense accompanied by programmed cell death and symptomless basal defense. HR-based defense is the most characteristic plant defense against phytopathogens. In the case of pathogenic bacteria, the type III secretion apparatus encoded by *HRP* genes enables effector proteins to be injected into plant cells. Effector proteins are recognized by plant cells after which the HR-based defense develops. Lack of type III secretion apparatus (the *hrp* mutant) results in loss of pathogenicity and the ability to induce a HR (Kanda et al., 2003a; Szatmari et al., 2005). However, *hrp* mutant bacteria still induce basal resistance (Klement et al., 1999; Szatmari et al., 2005). Basal resistance is triggered by recognition of general conserved elicitors, so-called pathogen-associated molecular patterns, and defends all plant species against most microbes (Nurnberger and Brunner, 2002). Expression of *Ntshsp17* was shown to be induced in tobacco leaves inoculated with *hrp* mutants of Rs8107 (Rs8107ΔY) and *P. cichorii* (Fig. 5; data not shown). An HR elicitor (INF1) did not show significant effects on *Ntshsp17* induction in comparison with the control GUS-expressing control *Agrobacterium* (Fig. 5). Therefore, *Ntshsp17* may be induced by pathogen-associated molecular pattern recognition. In addition to evidence from the expression pattern of *Ntshsp17*, silencing of *Nbshsp17* did not compromise HRs induced by INF1 and wild-type strains of Rs8107 and *P. cichorii* (Fig. 8). In *Nbshsp17*-silenced plants, development of bacterial wilt was drastically accelerated in comparison to that in control plants (Fig. 9). Taken together, these results also suggest that Ntshsp17 (Nbshsp17) may be required for basic immune responses other than HR-based resistance in plants.

In plant cells, HSP families, including HSP70 and HSP90, have a role in signal transduction leading to plant defenses. HSP90 reportedly interacts with a SA-induced protein kinase, which acts as a signaling component during plant defense. Silencing of HSP90 and HSP70 in *N. benthamiana* compromises not only induction of the HR, but also nonhost resistance (Kanzaki et al., 2003; Lu et al., 2003). It has also been reported that HSP90 interacts with RAR1 and SGT and modulates innate immune responses involving gene-for-gene specific interactions, including *N* gene-mediated resistance to *Tobacco mosaic virus* in tobacco and *RPS2*-mediated and *RPM1*-mediated resistance to *Pseudomonas syringae* in Arabidopsis (Hubert et al., 2003; Schulze-Lefert, 2004). HSP90 acts as a scaffold protein in a protein complex that mediates signal transduction (Schulze-Lefert, 2004). In contrast, the biological function of SHSPs in plant defense responses remains unresolved. Ntshsp17 was shown to have typical molecular chaperone activity in a substrate nonspecific manner (Fig. 3). Expression of *Ntshsp17* was nonspecifically induced by oxidative stress (H_2O_2) and by treatment with intracellular signaling molecules (SA, MeJA, and ACC; Fig. 4). Weak expression of *Ntshsp17* was also observed in water-infiltrated leaves (Figs. 2, 4, and 5), possibly in response to osmotic and/or wounding stress. Moreover, expression of *PR1*, *PR4*, and *EREBP* was reduced, whereas expression of *EF1α* was scarcely affected in *Nbshsp17*-silenced plants (Fig. 7). HSPs are reported to be involved in nonspecifically stabilizing stress-sensitive proteins (Minton et al., 1982). A possibility is that Ntshsp17 (Nbshsp17) acts as a molecular chaperone and nonspecifically stabilizes intracellular proteins, including signaling-related proteins, resulting in maintenance of the cellular conditions suitable for inducible defense responses in plants.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

Ralstonia solanacearum isolates OE1-1, 8107, and *Pseudomonas cichorii* SPC9018 were grown for 16 h at 30°C in peptone-yeast (*Saccharomyces cerevisiae*) extract medium. An *hrpY* mutant of *R. solanacearum* 8107 was cultured in peptone-yeast medium containing 50 μg mL^{-1} spectinomycin. The bacterial population was measured spectrophotometrically at OD_{600} and the suspension was adjusted to 10^8 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 (*Nicotiana tabacum*) plants against *R. solanacearum* strains when compared to 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 OE1-1, 8107, and a mutant strain of the bacteria (Kanda et al., 2003a, 2003b; Kiba et al., 2003; A. Kiba, unpublished data).

Plant Growth Conditions

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

Isolation of RNA

Total RNA was isolated from tobacco 'Samsun' NN and *N. benthamiana* leaves by the method described previously (Kiba et al., 2003). RNA samples were treated with DNase I (Rnase free; TaKaRa Shuzo) to degrade contaminating genomic DNA, according to the manufacturer's instructions.

Construction of Equalized cDNA Libraries

Construction of equalized cDNA libraries was performed following the procedure described by Kouchi et al. (1995), with slight modifications (Kiba et al., 2007). mRNA was purified from 1 mg of total RNA using the PolyATract system (Promega). cDNA was synthesized using reverse transcriptase RAV-2 (TaKaRa) with oligo(dT) primers (Supplemental Table S1). Lone linkers (LL-SseIA and LL-SseIB; Supplemental Table S1) were annealed and ligated to both ends of the cDNA and amplified by PCR using the LL-SseIA primer as follows: 25 cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 4 min, and one cycle of 94°C for 2 min, 50°C for 2 min, and 72°C for 10 min. The amplified cDNA library (20 μg mL^{-1}) was suspended in 50 μL of equalization buffer containing 0.3 M sodium phosphate buffer (pH 7.0), 0.4 mM EDTA, and 0.04% SDS, denatured in boiling water for 5 min, and reassociated at 65°C for 24 h. Single-stranded cDNA was separated from the double-strand cDNA by hydroxyapatite column chromatography (Bio-Rad) at 65°C. Single-stranded cDNA was then amplified by PCR under the same conditions as for cDNA amplification. This equalization cycle was repeated three times, thus constructing the equalized cDNA libraries.

Differential Display and Selection of *R. solanacearum*-Responsive Gene Fragments

Isolation of *R. solanacearum*-responsive genes was carried out by the method described previously (Kiba et al., 2007). The random 12-mer primers shown were used for differential display using the equalized cDNA libraries as templates according to Yoshida et al. (1994) with a slight modification (Kiba et al., 2007). The PCR product was analyzed on a 1% agarose gel stained with ethidium bromide. Amplified DNA fragments of interest were isolated from the gels and DNA extraction from the gel was performed using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad) according to the manufacturer's instructions. PCR-amplified fragments were then cloned into a pGEM-T vector (Promega) according to the manufacturer's specifications. False-positive clones were eliminated by reverse northern hybridization by the method described previously (Zegzouti et al., 1997) with a slight modification. Respective cDNA inserts were amplified with pGEMT-S and pGEMT-A primers (Supplemental Table S1). Equal amounts of the amplified fragments were run in duplicate on the same 1% agarose gel. The separated cDNA inserts were transferred onto a Hybond N+ membrane. Digoxygenin-labeled total cDNA probes were created by PCR amplification with LLSseIA and nonequalized original cDNA libraries from water- or RsOE1-1-infiltrated tobacco, according to the manufacturer's instructions. Duplicate membranes were then hybridized overnight, one to a digoxygenin-labeled total cDNA probe from water-infiltrated tobacco, and the other to that from RsOE1-1-infiltrated tobacco (Kiba et al., 2007).

Sequencing

The PCR products were sequenced using M4 and RV primers (Supplemental Table S1) with the reagents for the Big Dye terminator cycle sequencing kit (Applied Biosystems) and Applied Biosystems 3100 Avant automated sequencer (Applied Biosystems) according to the manufacturer's instructions. 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).

Isolation of Full-Length cDNA

For isolation of the complete cDNA of A6, a modified RACE method was performed (Frohman et al., 1988). The A6S primers were designed based on internal nucleotide sequences of the A6 (Supplemental Table S1). PCR amplification was performed with a primer combination of A6F and oligo(dT)-AD as listed in Supplemental Table S1. Cycling parameters were as follows: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Full-length cDNA was cloned into the vector pGEMT-Easy (Promega) and pGEMA6 was created.

Quantitative Real-Time PCR

RT was carried out with 1 μg 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 cDNA stock and 10 pM of the respective primers (Supplemental Table S1) using the SYBR premix Ex Taq (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. Specificity of the primers in the amplifying PCR conditions was initially verified by agarose gel electrophoresis, which yielded single products at the expected molecular size. Amplified DNA fragments were also checked by direct sequencing with the upper primer (Supplemental Table S1) of each respective gene and matched with the original sequence of the RsRGs. Melting curve runs were also performed at the end of each PCR reaction to verify the specificity of 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, as described previously (Szatmari et al., 2005), and were shown relative to the absolute 0 time control level. Expression analyses were carried out with at least two biological replications to ensure that expression patterns were reproducible. We showed characteristic data in respective figures. Standard deviations and differences between expression ratios of nontreated controls and other samples were tested for statistical significance using the *t* test.

Overexpression of Recombinant Ntshsp17

The open reading frame of *Ntshsp17* was amplified with A6-Nde and A6-Bam (Supplemental Table S1). Cycling parameters were as follows: 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified cDNA fragment was digested with *Nde*I and *Bam*HI and cloned into the pET16b vector (Novagen) digested with the same restriction enzymes (pET-Ntshsp17). pET-Ntshsp17 was transformed into *Escherichia coli* (BL21). Bacteria carrying pET-Ntshsp17 were grown overnight in Luria-Bertani medium and the OD_{600} was measured and adjusted to 0.1. Bacteria were transferred to fresh Luria-Bertani medium and grown at 37°C with 50 μg mL^{-1} ampicillin until the population reached an OD_{600} of 0.6. Bacterial cultures were induced with isopropylthio-β-galactoside at a final concentration of 1 mM and incubated for 3 h. Bacterial cells were harvested by centrifugation at 4°C for 5 min at 12,500*g*. Bacterial pellets were resuspended in BE-PER reagent (Pierce) and the solubilized protein fraction was harvested by centrifugation at 4°C for 5 min. Purification of recombinant Ntshsp17 was carried out using a HiTrap Ni^{2+}-chelating column (Amersham) according to the manufacturer's instructions. Purified Ntshsp17 was subjected to 12% SDS-PAGE according to Laemmli (1970) and stained with Coomassie Blue to confirm the purity.

Chaperone Activity of Recombinant Ntshsp17

Analysis of the thermal stability of *E. coli* cellular proteins was carried out according to a modified protocol provided by Yu et al. (2005). An overnight culture of *E. coli* (BL21) carrying pET16B and pET-Ntshsp17 (3 mL) in Magic Media *E. coli* expression medium was centrifuged for 5 min at 12,500*g* and resuspended in BE-PER reagent (Pierce). Bacterial cell debris was removed by centrifugation at 4°C for 5 min at 12,500*g*. The supernatant was transferred to a new tube and the protein concentration determined using Quick Start Bradford dye reagent (Promega). One milligram of protein fractions was treated with varying temperatures: 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C for 15 min. Heat-treated proteins were then centrifuged at 12,500*g* for 10 min and the supernatant (nondenatured protein fraction) was separated from the pellet (heat-denatured protein fraction). Protein content of supernatant fractions was determined using Quick Start Bradford dye reagent.

Aggregation protection of firefly (*Photinus pyralis*) LUC (Promega) was assessed using the method of Basha et al. (2004) as follows: LUC at 1 μM was heated in the absence or presence of an appropriate amount of Ntshsp17 in

50 mM sodium phosphate, pH 7.5, for 15 min at 42°C. After heating, samples were centrifuged for 15 min at 16,250*g* and the supernatant fractions (nondenatured protein fraction) were separated from the pellet (heat-denatured protein fraction). The supernatant and pellet fractions were subjected to 15% SDS-PAGE and stained with Coomassie Blue.

Treatment with Intracellular Signaling Molecules

Tobacco 'Samsun NN' plant leaves were treated by leaf infiltration using a syringe. Concentrations of chemicals used in the experiment were as follows: 0.03, 0.3, and 3 mM H_2O_2 (Nakarai Tesuque Co. Ltd); 5, 50, and 500 μM SA (sodium salicylate; Sigma); 0.5, 5, and 50 μM MeJA (Nakarai Tesuque); and 1, 10, and 100 μM ACC (Sigma).

Transient Expression of INF1

For agroinfiltration experiments, we used the binary vector p35S-INF1 containing a fusion between the signal peptide of tobacco *PR1α* and the *Phytophthora inf1* gene driven by the 35S promoter of the *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 *A. tumefaciens* strain GV3101, which harbors the transformation helper plasmid pSoup (Hellens et al., 2000) and inoculated into tobacco or *N. benthamiana* leaves as described previously (Katou et al., 2003).

DNA Constructs and Seedling Infection for VIGS

A 315-bp cDNA fragment responsible for the initial A6 sequence was amplified with primers pGEMTS-Pst and pGEMTA-Sal (Supplemental Table S1) using pGEMA6 as a template. This cDNA fragment was subcloned into the TA cloning site of pGEM-T-Easy and pGEMA6PVX was created. The pGEMA6PVX plasmid was digested with *Pst*I and *Sal*I and ligated into the PVX vector pPVX201 digested with *Sse*8387I and *Sal*I (Baulcombe et al., 1995). The construct containing this insert in the antisense orientation was designated pPVX-shsp17. Plasmid pPVX201 that does not contain any insert was used as a control. These binary plasmids were transformed into *A. tumefaciens* strain GV3101 and inoculated into *Nicotiana benthamiana* leaves as described previously (Katou et al., 2003). Three weeks after initial *Agrobacterium tumefaciens* inoculation, Rs8107, RsOE1-1, and *Agrobacterium* were inoculated into a *N. benthamiana* leaf three to four leaves above the *Agrobacterium*-inoculated leaf as a challenge inoculation.

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. Plants were coded and inspected daily for wilting symptoms for 14 d. Each assay was repeated in at least six successive trials and the disease index was recorded as described previously (Kanda et al., 2003a, 2003b; Shinohara et al., 2005).

Nucleotide sequences reported in this article have been submitted to DDBJ, EMBL, and GenBank under the accession numbers AB290185 and AB290186 (*Ntshsp17* and *Nbshsp17*, respectively).

Supplemental Data

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

Supplemental Table S1. Primers used in this study.

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