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Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*

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SUMMARY

Mitogen-activated protein kinases (MAPKs) play pivotal roles in the signal transduction pathway of plant defence responses against pathogens. A search for MAPK-interacting proteins revealed an interaction between a *Nicotiana benthamiana* MAPK, SIPK (NbSIPK) and cytosolic Hsp90 (NbHsp90c-1) in yeast two-hybrid assay. To study the function of Hsp90 in disease resistance, we silenced *NbHsp90c-1* in *N. benthamiana* by virus-induced gene silencing (VIGS) with *Potato virus X* (PVX). *NbHsp90c-1* silenced plants exhibited: (1) a stunted phenotype, (2) no hypersensitive response (HR) development after infiltration with the *Phytophthora infestans* protein INF1 and a non-host pathogen *Pseudomonas cichorii* that normally triggers HR in *N. benthamiana*, (3) compromised non-host resistance to *P. cichorii*, and (4) consistently reduced transcription levels of PR (pathogenesis related) protein genes. Similar phenotypes were observed also for plants in which a cytosolic *Hsp70* (*NbHsp70c-1*), a gene for another class of molecular chaperon, was silenced. Hsp90 was isolated as a MAPK-interacting protein in yeast two-hybrid assay, therefore we tested the effect of *NbHsp90c-1* silencing as well as *NbHsp70c-1* silencing on the HR development caused by infiltration of a hyperactive potato MAPKK ($StMEK1^{DD}$). No difference in the timing or extent of HR was found among *NbHsp90c-1* silenced, *NbHsp70c-1* silenced and control plants. This result indicates that observed impairment of INF1- and *P. cichorii*-mediated HR development in *NbHsp90c-1* silenced and *NbHsp70c-1* silenced plants was not caused by the abrogation in MAPK function downstream of active MAPKK that leads to HR. These findings suggest essential roles of Hsp90 and Hsp70 in plant defence signal transduction pathway upstream or independent of the MAPK cascade.

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INTRODUCTION

Heat-shock proteins (Hsps) are highly conserved proteins expressed in cells that have been subjected to elevated temperatures or other forms of environmental stress (Cooper, 2000). They are thought to stabilize and facilitate the refolding of proteins that have been denatured during exposure to elevated temperature and stress. Recent studies, however, have revealed essential cellular functions of Hsps under normal cell growth conditions as well. They are required for folding of nascent proteins and intracellular transportation in addition to the stress responses. Hsp families, consisting of Hsp70, Hsp60 and Hsp90 proteins, therefore came to be collectively called molecular chaperons. The roles of Hsp70 and Hsp60 in protein folding are well established (Bukau and Horwich, 1998). Recent studies indicate the interactions of Hsp90 to a wide variety of signalling proteins, including steroid receptors, transcription factors and serine/threonine kinases (Smith, 2001). Hsp90 and Hsp70 frequently function together, as shown by the example of progesterone receptor signalling (Lassle *et al.*, 1997). In contrast to the wealth of knowledge accumulated in prokaryote and animal systems, little is known about the function of plant molecular chaperones.

We are interested in the plant defence signal transduction through mitogen-activated protein kinase (MAPK) cascades. MAPK cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses in yeast and animal cells (Davis, 2000; Widman *et al.*, 1999) as well as in plants (Tena *et al.*, 2001). Key roles of MAPKs in plant defence signalling have recently been revealed (Zhang and Klessig, 2001). For instance, hyperactivation of a MAPKK caused hypersensitive response (HR)-like cell death in tobacco leaf (Yang *et al.*, 2001). An *Arabidopsis* mutant *mpk4* deficient in a functional MAPK exhibited constitutive systemic acquired resistance (SAR) (Petersen *et al.*, 2000).

In the present study, we show that SIPK, a MAPK of *Nicotiana benthamiana* interacts with a cytosolic Hsp90 (NbHsp90c-1) in yeast two-hybrid assay. *N. benthamiana* is a dicot species in which

virus-induced gene silencing (VIGS) can readily be applied to rapidly knockout genes using *Potato virus X* (Baulcombe, 1999; Saitoh and Terauchi, 2002). Function of Hsp90 relevant to plant disease resistance was studied by silencing *NbHsp90c-1* in *N. benthamiana* plants. Interestingly, *NbHsp90c-1* silencing as well as silencing of *NbHsp70c-1*, a gene for another member of cytosolic molecular chaperone Hsp70, caused an impairment of most of the defence responses tested. This result suggests that cytosolic Hsp90 and Hsp70 are essential components of plant defence signalling pathways.

RESULTS

NbHsp90c-1 and NbSIPK interact in yeast two-hybrid assay

Yeast two-hybrid screen identified an interaction between a rice cytosolic Hsp90 (OsHsp90c-1: GenBank accession no. AB111810) and a MAPK (OsMPK2: GenBank no. AF332873), which is induced upon fungal elicitor treatment of rice suspension cultured cells (see Experimental procedures). We subsequently tested whether a similar interaction exists between *N. benthamiana* MAPKs and Hsp90. Amino acid sequence of OsMPK2 has a high homology to SIPK and WIPK of *N. tabacum* (Ichimura *et al.*, 2002). Thus, we isolated homologs of *SIPK* (*NbSIPK*: GenBank accession no. AB098730) and *WIPK* (*NbWIPK*: GenBank no. AB098729) from *N. benthamiana* (Sharma *et al.*, in press). Similarly, a *N. benthamiana* homolog of *OsHsp90c-1* was isolated and named *NbHsp90c-1* (GenBank no. AB105429). OsHsp90c-1 and NbHsp90c-1 have a high amino acid sequence homology (> 90%) to each other.

Using yeast two-hybrid assay, we tested the interactions between NbSIPK and NbHsp90c-1 as well as between NbWIPK and NbHsp90c-1. This experiment showed that NbSIPK, but not NbWIPK, interacts with NbHsp90c-1 (Fig. 1).

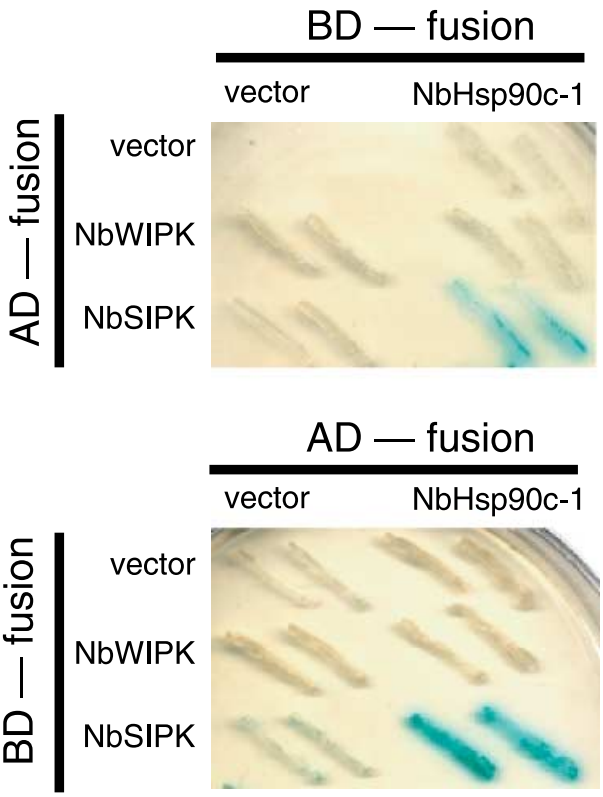

Fig. 1 NbSIPK, but not NbWIPK interacts with NbHsp90c-1 in GAL4 yeast two-hybrid assay. NbSIPK and NbWIPK were expressed as GAL4-activation domain fusion protein and tested for the interaction with expressed NbHsp90c-1 protein fused with GAL4 DNA binding domain protein (top). NbSIPK and NbWIPK were expressed as GAL4-DNA binding domain-fusion protein, and tested for the interaction with expressed NbHsp90c-1 protein fused with GAL4-activation domain protein (bottom). Protein interaction was visualized by the activation of *MEL1* gene located downstream of *MEL1*-UAS promoter.

Gene silencing of *NbHsp90c-1* and *NbHsp70c-1* caused stunted phenotypes in *N. benthamiana* plants

Activation of SIPK and WIPK by constitutively active MAPKK caused cell death in *N. tabacum* (Yang *et al.*, 2001). Subsequently, over-expression of *SIPK* alone was found to cause HR in *N. tabacum* (Zhang & Liu, 2001). NbHsp90c-1 was identified as an NbSIPK interacting protein, therefore we became interested in its function with respect to HR signalling. To address the function of NbHsp90c-1 in HR development, we silenced the gene in *N. benthamiana* using VIGS by inoculating PVX.NbHsp90c-1, a PVX vector harbouring a partial fragment of *NbHsp90c-1*. In animal systems, many examples are reported in which Hsp90 functions together with Hsp70, another class of molecular chaperons, in cell signalling including steroid receptor regulation (Smith, 2001). Involvement of both Hsp90 and Hsp70 in the regulation of animal apoptosis has also been demonstrated (Beere, 2001). This information prompted us to test the function of Hsp70 in parallel with Hsp90. Thus, a cDNA encoding cytosolic Hsp70 was isolated from *N. benthamiana* (*NbHsp70c-1*: GenBank no. AB105430). Its partial fragment was cloned into PVX (PVX.NbHsp70c-1) and used for VIGS of *NbHsp70c-1*. Surprisingly, both PVX.NbHsp90c-1- and PVX.NbHsp70c-1-infected plants started exhibiting stunted phenotypes (Fig. 2A). Twenty-one days after inoculation, three to four leaves above the inoculated one were used for confirmation of gene silencing of *NbHsp90c-1* and *NbHsp70c-1*, respectively. Reverse-transcription (RT)-PCR confirmed that gene silencing of *NbHsp90c-1* and *NbHsp70c-1* was established (Fig. 2B). Note that *NbHsp90c-1* and *NbHsp70c-1* are constitutively expressed genes with slight heat-induction (Fig. 2B). To address the specificity of gene silencing, we selected each three ESTs corresponding to cytosolic Hsp90 and Hsp70, respectively, and their expression tested in *NbHsp90c-1* and *NbHsp70c-1* silenced plants by RT-PCR using gene-specific primers (Fig. 2B). *NbHsp90c-2* (GenBank no. AB113028) and *NbHsp90c-3* (GenBank

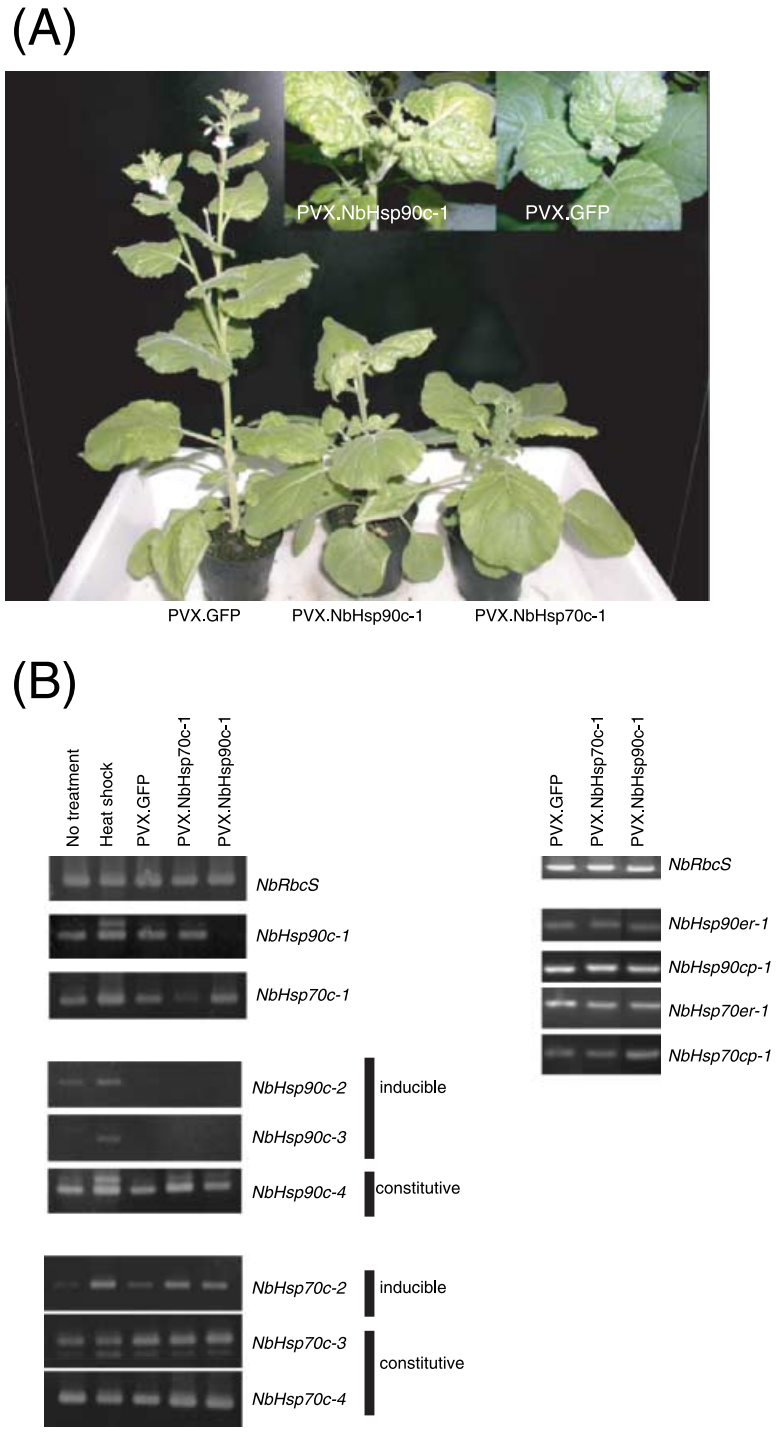

Fig. 2 VIGS of *NbHsp90c-1* and *NbHsp70c-1* in *Nicotiana benthamiana*. Gene silencing of *NbHsp90c-1* and *NbHsp70c-1* caused stunted phenotypes in *N. benthamiana*. The upper leaves showed abnormality (inlet, A). RT-PCR results show that inoculation of PVX.NbHsp90c-1 to *N. benthamiana* plants silenced the expression of *NbHsp90c-1*, and PVX.NbHsp70c-1 silenced *NbHsp70c-1* expression (B, left). Expression of another cytosolic Hsp90 gene (*NbHsp90c-4*) and three cytosolic Hsp70genes (*NbHsp70c-2*, *-3* and *-4*) were not affected by PVX.NbHsp90c-1 and PVX.NbHsp70c-1 inoculation, respectively. *NbHsp90c-1*, *NbHsp90c-4*, *NbHsp70c-3* and *NbHsp70c-4* are constitutively expressed genes, whereas *NbHsp70c-1*, *NbHsp90c-2*, *NbHsp90c-3* and *NbHsp70c-2* are heat (40 °C, 1 h)-inducible genes. Gene expression of Hsps at endoplasmic reticulum (*NbHsp90er-1* and *NbHsp70er-1*) and chloroplast Hsps (*NbHsp90cp-1* and *NbHsp70cp-1*) were not affected (B, right) in PVX.NbHsp70c-1 and PVX.NbHsp90c-1 inoculated plants, respectively.

no. AB112813) are heat inducible genes sharing 85% and 92% amino acid (aa) sequence similarity and 86% and 91% nucleotide (nt) sequence similarity with *NbHsp90c-1*, respectively. *NbHsp90c-4* (GenBank no. AB113029) is a constitutively expressed gene showing 90% aa and 91% nt sequence similarity to *NbHsp90c-1*. *NbHsp90c-2* and *NbHsp90c-3* showed very low basal levels of expression without heat shock treatment, and we could not test whether their expression was affected by *NbHsp90c-1* silencing. Expression of *NbHsp90c-4* was not affected by *NbHsp90c-1* silencing. *NbHsp70c-2* (GenBank no. AB112814) is an inducible gene with 77% aa and 78% nt sequence similarity to *NbHsp70c-1*. *NbHsp70c-3* (GenBank no. AB112815) and *NbHsp70c-4* (GenBank

no. AB112816) are constitutively expressed genes sharing 80% and 76% aa sequence similarity and 76% and 73% nt sequence similarity with *NbHsp70c-1*, respectively. *NbHsp70c-1* silencing did not affect expression of any of these *NbHsp70c* genes, showing that gene silencing is specific to *NbHsp70c-1*. Gene silencing of *NbHsp90c-1* did not affect the expression levels of *Hsp90* from endoplasmic reticulum (*NbHsp90er-1*) or chloroplast (*NbHsp90cp-1*) (Fig. 2B). Likewise, *NbHsp70c-1*-silencing did not affect the expression levels of genes for endoplasmic reticulum or chloroplastic Hsp70s. Stature of the *NbHsp90c-1* silenced plant (PVX.NbHsp90c-1) and *NbHsp70c-1* silenced plant (PVX.NbHsp70c-1) 21 days after the inoculation was reduced to about half of the control (PVX.GFP-infected) plants (Fig. 2A). This is caused by the shortening of internodes of stems. The upper leaves of *NbHsp90c-1* and *NbHsp70c-1* silenced plants showed abnormality including asymmetry, upward curling of the leaf blade in the region between veins (Fig. 2A).

INF1-mediated HR is compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced plants

To see the responses of *NbHsp90c-1* and *NbHsp70c-1* silenced plants to HR-triggering signal, INF1, a secreted protein of *Phytophthora infestans*, was infiltrated to the *NbHsp90c-1* silenced (PVX.NbHsp90c-1), *NbHsp70c-1* silenced (PVX.NbHsp70c-1) as well as the control (PVX.GFP) plants. INF1 is a known elicitor of HR in *N. benthamiana* (Kamoun *et al.*, 1997; 1998). Thirty-six hours after the infiltration of 100 nM INF1, HR developed in the control plants, whereas HR was totally absent from *NbHsp90c-1* silenced as well as *NbHsp70c-1* silenced (Fig. 3A) plants.

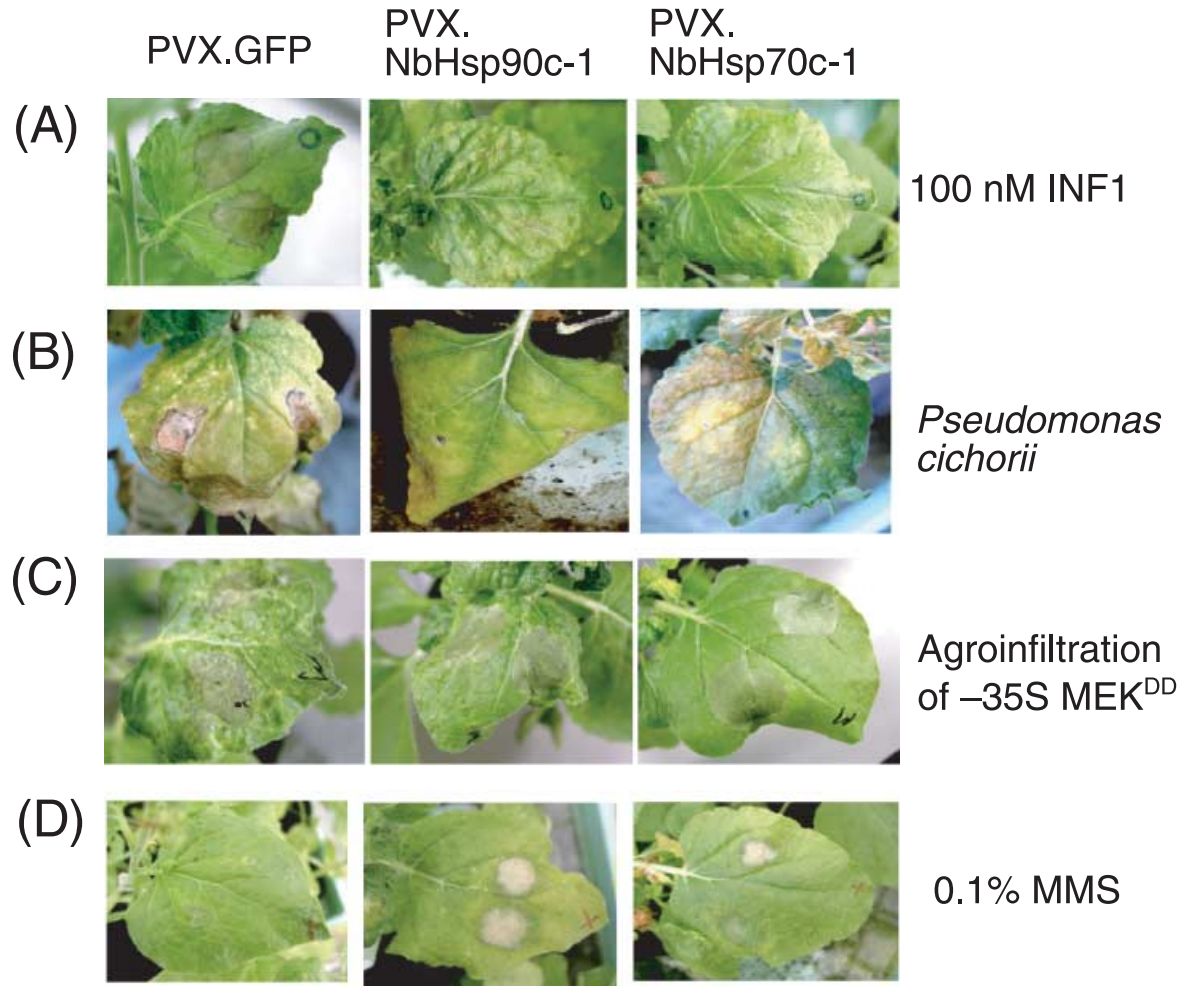

Fig. 3 HR and cell death are altered in *NbHsp90c-1* and *NbHsp70c-1* silenced plants. After the establishment of gene silencing, leaves of the control (PVX.GFP-inoculated, left), *NbHsp90c-1* silenced (PVX.NbHsp90c-1-inoculated, centre) and *NbHsp70c-1* silenced (PVX.NbHsp70c-1-inoculated, right) plants were infiltrated with 100 nM *Phytophthora infestans* elicitor INF1 (A), *Pseudomonas cichorii* (B), *Agrobacterium tumefaciens* harbouring 35S-StMEK1^{DD} (C) or 0.1% methyl methane sulphonate (MMS, D).

HR caused by a non-host pathogen, *Pseudomonas cichorii*, is compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced plants

Pseudomonas cichorii, a bacterial pathogen of lettuce, causes HR and non-host resistance to *N. benthamiana* (Y. Hikichi, personal communication). To test the effect of *NbHsp90c-1* as well as *NbHsp70c-1* silencing to HR caused by non-host resistance, *P. cichorii* was infiltrated to the *NbHsp90c-1* silenced (PVX.NbHsp90c-1), *NbHsp70c-1* silenced (PVX.NbHsp70c-1) as well as the control (PVX.GFP) plants. No HR was induced in *NbHsp90c-1* silenced as well as *NbHsp70c-1* silenced plants, whereas strong HR was induced in the control plant 48 h after the inoculation (Fig. 3B).

StMEK1^{DD}-mediated HR is not affected by silencing of *NbHsp90c-1 and NbHsp70c-1*

Constitutively active MAPKK can be made by replacing Ser/Thr residues at the two phosphorylation sites (S/TxxxS/T) with Asp (D) (Mansour *et al*., 1994). Such mutagenized MAPKK is known to activate downstream MAPKs. Yang *et al*. (2001) showed that transient expression of NtMEK2^{DD}, a constitutively active *Nicotiana tabacum* MAPKK, activates both the SIPK and WIPK in *N. tobacco* leaf, and eventually causes HR-like cell death. StMEK1 is a potato (*Solanum tuberosum*) homolog of NtMEK2, and StMEK1^{DD} also can cause cell death in *Nicotiana* species by activating SIPK and WIPK (Katou *et al*., 2003). To see the effect of *NbHsp90c-1* silencing and *NbHsp70c-1* silencing in MEK^{DD}-mediated cell death, *NbHsp90c-1* silenced as well as *NbHsp70c-1* silenced plants were infiltrated with *Agrobacterium tumefaciens* expressing *StMEK1^{DD}*. HR-like cell death developed invariably in the *NbHsp90c-1* silenced, *NbHsp70c-1* silenced as well as in the control plants 48 h after the infiltration (Fig. 3C).

MMS-mediated cell death is accelerated in *NbHsp90c-1* and *NbHsp70c-1* silenced plants

An alkylating agent methane methyl sulphonate (MMS) is known to cause genotoxic stress to organisms. In *Arabidopsis thaliana*, this stress was shown to be mediated by the activation of a MAPK, AtMPK6 (Ulm *et al*., 2002), which is an ortholog of tobacco SIPK (Ichimura *et al*., 2002). To see the effect of *Hsp90c-1* and *Hsp70c-1* silencing on the genotoxic stress, 0.1% MMS was infiltrated to the *NbHsp90c-1*, *NbHsp70c-1* silenced plants as well as the control plants. Eighteen hours after the MMS treatment, the infiltrated area became dry and cell death was obvious in *NbHsp90c-1* and *NbHsp70c-1* silenced plants, whereas no visible change was observed in the control plants (Fig. 3D).

Non-host disease resistance is compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced plants

Absence of HR in *NbHsp90c-1* silenced and *NbHsp70c-1* silenced plants after INF1 treatment and *P. cichorii* inoculation raised a possibility that general defence responses are compromised in these plants. Thus, we tested the resistance of *NbHsp90c-1* silenced, *NbHsp70c-1* silenced and the control plants to a non-host pathogen by inoculating *P. cichorii* (Fig. 4A). One, two and three days following the inoculation, titre of the bacterium in the leaf was measured. Disease resistance was compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced plants as compared to the control.

Defence gene expression is reduced in *NbHsp90c-1* and *NbHsp70c-1* silenced plants

Compromised disease resistance of *NbHsp90c-1* and *NbHsp70c-1* silenced plants against a non-host pathogen may be caused by the reduced expression of defence-related genes. To address this possibility, kinetics of expression of selected genes (*PR1a*, *PR2a*, *PR2b*, *PR5b*, *PI-II*, *Myb1*, *EREBP* and *WRKY*) after INF1 infiltration was examined by RT-PCR in *NbHsp90c-1* and *NbHsp70c-1* silenced as well as in the control plants. As expected, expression of most of the tested PR-protein genes were similarly reduced in *NbHsp90c-1* and *NbHsp70c-1* silenced plants (Fig. 4B), demonstrating that compromised disease resistance in *NbHsp90c-1* and *NbHsp70c-1* silenced plants are in part explained by the reduction in the expression of defence genes including PR-protein genes.

DISCUSSION

MAPK cascades are major components downstream of receptors and sensors that transduce extracellular stimuli into intracellular responses in eukaryotes (Davis, 2000; Widman *et al*., 1999). The roles of MAPKs in plant defence responses have been a focus of intensive research (Romeis, 2001; Zhang and Klessig, 2001). The present study showed that a *N. benthamiana* MAPK, NbSIPK interacts with a cytosolic Hsp90 (NbHsp90c-1) in yeast two-hybrid assay. To understand the functions of MAPKs and their interacting proteins, we employed the virus-induced gene silencing (VIGS) system using *N. benthamiana* and PVX (Baulcombe, 1999; Sharma *et al*., in press). Silencing of a gene for cytosolic Hsp90, *NbHsp90c-1*, and a gene for another major molecular chaperone Hsp70, *NbHsp70c-1,* both caused a strikingly stunted phenotype of the plants and abnormal development of leaves. These are similar to the phenotypes reported in *A. thaliana* plants that were treated with geldanamycin, a potent inhibitor of Hsp90 (Queitsch *et al*., 2002). It is obvious that Hsp90 and Hsp70 are necessary for normal plant development.

When infiltrated with INF1 and a non-host pathogen, *P. cichorii*, no HR was induced in *NbHsp90c-1* and *NbHsp70c-1*

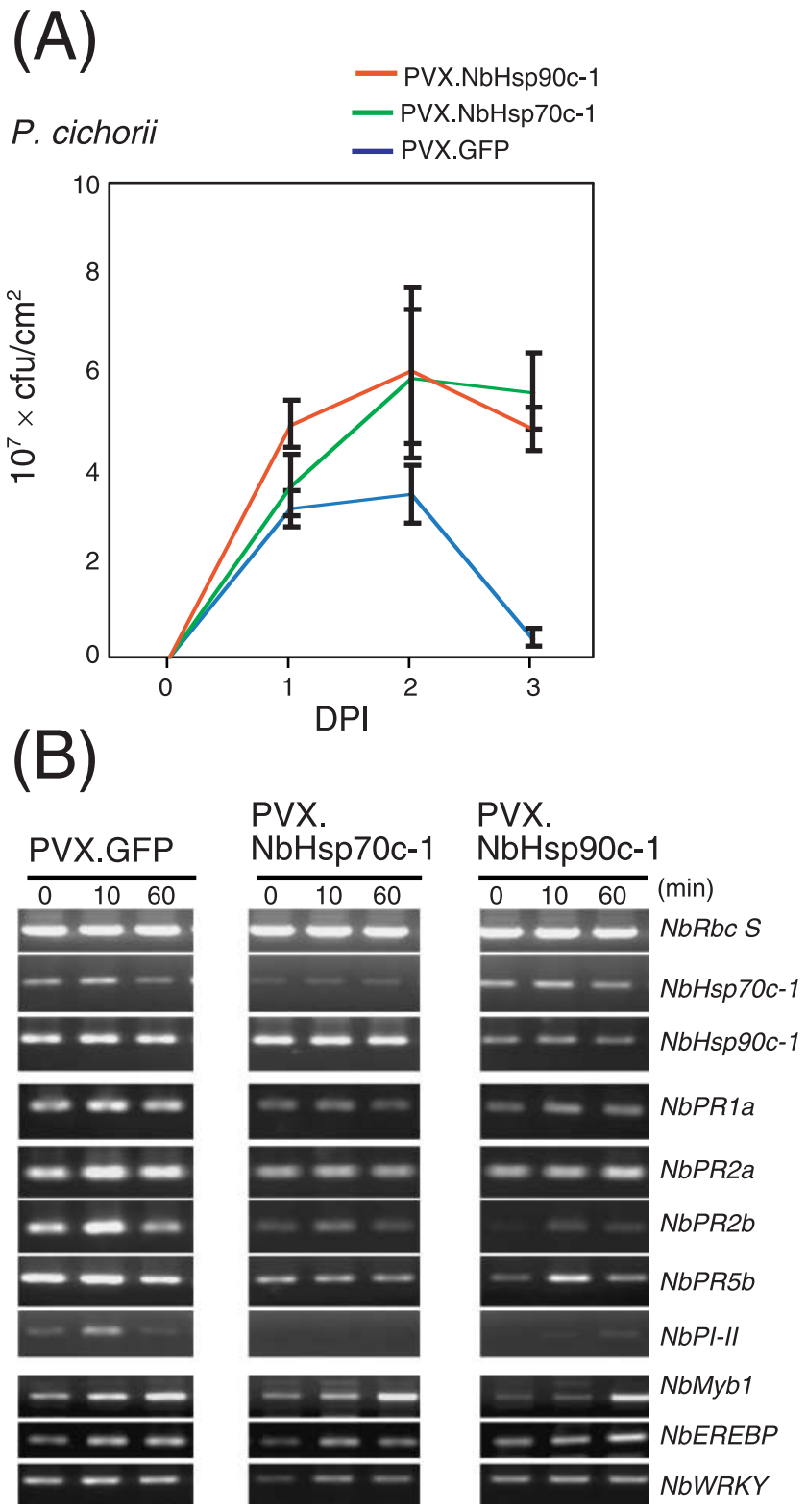

Fig. 4 Disease resistance and defence gene expression are compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced *Nicotiana benthamiana* plants. *Pseudomonas cichorii*, a non-host pathogen of *N. benthamiana*, was inoculated to *N. benthamiana* plants that were pre-inoculated with PVX.NbHsp90c-1 and PVX.NbHsp70c-1 as well as PVX.GFP (control). Leaf spots were punched out from the leaves at the given days post-inoculation (dpi) of the bacterium, and the titre of bacteria in the leaves measured (A). Leaf spots were punched out at 0, 10 and 60 min following infiltration of 100 nM INF1 from *N. benthamiana* plants that were pre-inoculated with PVX.NbHsp90c-1 and PVX.NbHsp70c-1 as well as PVX.GFP (control). Using RNAs isolated from them, RT-PCR was carried out to study the gene expression of PR-protein genes (*PR1a*, *PR2a*, *PR2b*, *PR5b* and *PI-II*) as well as defence-related transcription factor genes (*Myb1*, *EREBP* and *WRKY*). RT-PCR results of the *rbcS* gene (top) demonstrate the equal amount of total RNA used for RT and the equal efficiency of RT-reaction across the samples.

silenced plants. Challenge with *P. cichorii* revealed that HR and non-host disease resistance were severely compromised in *NbHsp90c-1* and *NbHsp70c-1* silenced plants. This reduction in disease resistance seems to be partly caused by reduction in the expression of defence-related genes including PR protein genes. Taken together, these results suggest the essential roles of cytosolic Hsp90 and Hsp70 in signal transduction of HR development and defence responses in *N. benthamiana*. To our knowledge, this is the first report of direct involvement of Hsps in plant defence signalling pathways.

The cytosolic Hsp90 was first isolated as OsMPK2- and NbSIPK-interacting protein in yeast two-hybrid assay, thus we hypothesized that Hsp90, and presumably Hsp70 as well, was required for the effective signalling in MAPK cascades. To address this possibility, *NbHsp90c-1* and *NbHsp70c-1* silenced plants were treated with StMEK1^{DD}, the hyperactive MAPKK, as an input signal to activate the downstream MAPKs, SIPK and WIPK (Katou *et al.*, 2003; Yang *et al.*, 2001). StMEK1^{DD} caused HR-like cell death invariably in *NbHsp90c-1* and *NbHsp70c-1* silenced plants as well as in the control. This result implies that the cell death signal emanating from hyperactive MEK is efficiently transduced via MAPK regardless of the reduced amounts of NbHsp90c-1 or NbHsp70c-1. The impairment of INF1- and *P. cichorii*-mediated HR in *NbHsp90c-1* and *NbHsp70c-1* silenced plants therefore seems not to be caused by abrogation in MAPK signal transduction downstream of activated MEK. From this result, we envisage that NbHsp90c-1 and NbHsp70c-1 are indispensable components of INF1- and *P. cichorii*-mediated HR signal transduction, exerting their effects either upstream of MAPK cascade or in an independent pathway of MAPK such as CDPK pathway (Romeis *et al.*, 2001).

Then what is the biological meaning of the interaction between SIPK and Hsp90? One possibility is that Hsp90 is actually regulating SIPK activity and/or stability in other circumstances than that addressed by the StMEK1^{DD} expression experiment. An alkylating agent, MMS, caused enhanced cell death in *NbHsp90c-1* and *NbHsp70c-1* silenced plants as compared to the control (Fig. 3D). MMS is known to activate an *A. thaliana* MAPK, AtMPK6 (Ulm *et al.*, 2002) that is an ortholog of *Nicotiana* SIPK (Ichimura *et al.*, 2002). A mutant deficient in a dual-specificity phosphatase AtMKP1 is hypersensitive to MMS, and AtMKP1 was shown to negatively regulate the activity of AtMPK6 (Ulm *et al.*, 2002). By analogy, these findings suggest a possibility that MMS treatment in *N. benthamiana* activates SIPK. Hsp90c-1 and Hsp70c-1 may have functions in negatively regulating SIPK activity. If this is the case, SIPK activation should be caused by yet unidentified MAPKK that is different from NtMEK2. Extrapolating from the observation in yeast and mammals, it is possible that StMEK1^{DD} and a genotoxic stress-derived signal separately employ SIPK by virtue of different scaffolding proteins (Schaeffer and Weber, 1999) to exert different outputs. To test such a possibility, SIPK activation after MMS treatment should be studied by MBP kinase assay in *NbHsp90c-1* and *NbHsp70c-1* silenced plants in a future study.

EXPERIMENTAL PROCEDURES

Yeast two–hybrid screen and interaction assay

A full-length OsMPK2 (GenBank no. AF332873) was isolated from a cDNA made from mRNA extracted from rice suspension cultured cells 15 min after *Magnaporthe grisea* elicitor-treatment (Matsumura *et al.*, 2003), and cloned into pT7B plasmid vector (Novagen). OsMPK2 was mutagenized by PCR so that Lys-65 was replaced by Arg using a kit (QuickChange mutagenesis kit, Stratagene) resulting in OsMPK2mut. This mutation renders MAPK catalytically inactive and enhances the binding of interacting proteins (Tanoue *et al.*, 1999). The bait vector containing GAL4 DNA binding domain was prepared by cloning OsMPK2mut into a plasmid pGBKT7 (Clontech). MATCHMAKER Library construction & screening kit (Clontech) was used to construct rice cDNA library from rice suspension-cultured cells 15 min after elicitor-treatment. Yeast strain AH109 competent cells was transformed with pGBKT7 containing OsMPK2mut, pGADT7-Rec and the rice cDNA library using PEG/LiCl method, and plated on selective agar plates containing minimal medium without Leu, Trp, His and Ade, and supplemented with 10 mM 3-amino-1,2,4-triazole (3-AT) and 20 mg/L of X-α-gal. cDNAs in the library were transferred to pGADT7-Rec vector harbouring GAL4 activation domain (AD) by homologous recombination in yeast cells. Positive yeast transformants were streaked to minimal medium agar plate without Leu and Trp and used for interaction assay. This assay identified an Hsp90 protein, OsHsp90c-1, as the OsMPK2-interacting protein among others. For interaction assay of isolated cDNAs, pGBKT7 bait vector containing the GAL4 DNA binding domain and pGADT7 prey vector containing the AD domain were prepared by cloning full-length cDNAs of OsMPK2, OsHsp90c-1, NbHsp90c-1, NbSIPK and NbWIPK.

PVX constructs and PVX-mediated gene silencing in *N. benthamiana*

Partial cDNA regions of *NbHsp90c-1* (position no. 242–555 setting the first nucleotide of the start codon as no. 1) and *NbHsp70c-1* (position no. 419–718) genes of *N. benthamiana* were inserted into *Eco*RV and *Sal*I sites of *Potato virus X* (PVX) vector, resulting in pPVX.NbHsp90c-1 and pPVX.NbHsp70c-1, respectively. A PVX vector harbouring GFP (pTXS.GFP; Baulcombe *et al.*, 1995) was used as control. pPVX.NbHsp90c-1, pPVX.NbHsp70c-1 as well as pTXS.GFP were linearized by restriction endonuclease *Spe*I, and *in vitro* runoff transcripts were synthesized by T7 RNA polymerase. The transcripts were inoculated on

to leaves of *N. benthamiana* as described (Saitoh and Terauchi, 2002). Confirmation of gene silencing of *NbHsp90c-1* and *NbHsp70c-1* in *N. benthamiana* was performed using reverse-transcription (RT)-PCR as described.

RT-PCR assay

For the analysis of gene expression, RT-PCR was carried out using gene specific primer sets to amplify the partial fragments of cDNAs:

NbHsp90c-1 (5′-GATATTTACTACATTACTGGTGAGAGCAAGAAG-3′; 5′-CAACACGGTCAGAAACAATGACCTTTTC-3′), *NbHsp70c-1* (5′-GTTATGACCACCTTGATTCCAAGGAAC-3′; 5′-CTTCATCTTCAG-CTTTGTACTTCTCAGC-3′), *NbRbcS* (5′-CCTCTGCAGCAGTTGCCACC-3′; 5′-CCTGTGGGTATGCCTTCTTC-3′), *NbHsp70c-2* (5′-GAGAAGAT-GGTCCAGGAAGCTGAAAAGTACAAGG-3′; 5′-CCAACTAGTATCTTT-ATCAACCGCTTAATCAACCTCCTC-3′), *NbHsp70c-3* (5′-GCCAAGAT-GTACCAAGGTGCTGGCGG-3′;5′-GGCATAGATAGATCAAACGGTA-CAGCCGAGC-3′), *NbHsp70c-4* (5′-GGATGATAAGATTAGTTCTAAG-CTTAGTGCAGACGACAAG-3′; 5′-GAGCCATCAACATTCAACAG-CTTAATAAATAGACGAACCG-3′), *NbHsp90c-2* (5′-GGCATTAT-GGAGGAGTTGAGGAAGAGAGCTG-3′; 5′-GATATACGGATTT-GCTGCTTACACAACATGACACTACTG-3′), *NbHsp90c-3* (5′-GAGAGCTGATGCTGATAAGAATGACAAGTCCGTC-3′; 5′-CATATCAT-GTCCAGATGGTGGAGCTGAGTAGAAG-3′), *NbHsp90c-4* (5′-GTCTAGCAAGAAGACCATGGAGATCAACCCAG-3′; 5′-CCCTAACATTGAACTGCCATTCTAAAAGAACGGCATTAAAAC-3′), *NbHsp90er-1* (5′-GAAGATGTCAAGATAAGCAGCCGTTTGGCCG-3′; 5′-CCTCTTCTGCCTTGGTTTCAGTCTCAGGTTC-3′), *NbHsp90cp-1* (5′-CGTTTAAGCTCATCACCATGTGTGC-3′; 5′-CCTTACTCAGAT-GGCTCGACG-3′), *NbHsp70er-1* (5′-GTCAAGGCTAATTTACATT-TCTCACTTAGTAGAAG-3′; 5′-GCAGCAAGTTCTTTATGTCTGAAA-AATATGGGA-3′), *NbHsp70cp-1* (5′-GTGGAGTCATGACCAAAAT-TATCCCAAG-3′; 5′-GAAGTCTGCATCGATAACTTCTCCATC-3′), *NbPR1a* (5′-GTTCTCTTTTCACAATTGCC-3′; 5′-CGTAGGTCGTTTCAATTAGT-3′), *NbPR2a* (5′-TCAGGGATCTTAGCGAATAC-3′; 5′-AGTTGATATTTT-GCCCTCTG-3′), *NbPR2b*(5′-AGACATGACCTTGATTGGAA-3′; 5′-CAACTGAACTGTCCCAAACT-3′), *NbPR5b* (5′-ATTTCTGGGA-CATTTCTTTA-3′; 5′-CTTAGCCACTTCATCACTTC-3′), *NbPI-II* (5′-ATAGCGCTAATGGAACTTTT-3′; 5′-GGCAACTTATGGTAGCAACT-3′), *NbMYB1* (5′-CCTCCTCAGAACTCCAAAAGACAC-3′; 5′-GCAGCAACCATAACATGATCAGTACTAC-3′), *NbWRKY4* (5′-CCTCAACTTTAGTGGATGATGGCC-3′; 5′-CCACAAAATCAACAGT-TCCCTCCATC-3′), and *NbEREBP* (5′-GGGAAAACGGGTTTGGTT-GGG-3′; 5′-CGTCAAAGTCAAACTCGCCGAATTC-3′).

HR assay and titration of *P. cichorii* in *N. benthamiana* leaves

For studying HR, *Phytophthora infestans* elicitor (INF1), prepared according to Kamoun *et al*. (1998) was infiltrated into leaves of *N. benthamiana* at a concentration of 100 nM. Constitutively active potato MAPKK (StMEK1^{DD}) gene driven by a CaMV35S promoter (Katou *et al*., 2003) was transiently transformed into *N. benthamiana* leaves by *Agrobacterium* infiltration as described (Yang *et al*., 2001). For the inoculation of phytopathological bacterium, *Pseudomonas cichorii* SPC9201 were suspended in 10 mM $MgCl_2$ and infiltrated into leaves using a syringe. At a given time period after the inoculation, the leaf was punched out, homogenized, and the titre of bacteria in it was monitored by plating the dilution series of homogenates on the LB agar plate containing 10 μg/mL of ampicillin. MMS was dissolved in water adjusting the concentration to 0.1%, and infiltrated into leaves with a needleless syringe.

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