Molecular Chaperone Hsp90 Associates with Resistance Protein N and Its Signaling Proteins SGT1 and Rar1 to Modulate an Innate Immune Response in Plants*

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SGT1 and Rar1 are important signaling components of resistance (R) gene-mediated plant innate immune responses. Here we report that SGT1 and Rar1 associate with the molecular chaperone Hsp90. In addition, we show that Hsp90 associates with the resistance protein N that confers resistance to tobacco mosaic virus. This suggests that Hsp90-SCF-Rar1 and R proteins might exist in one complex. Suppression of Hsp90 in Nicotiana benthamiana plants shows that it plays an important role in plant growth and development. In addition, Hsp90 suppression in N plants compromises N-mediated resistance to tobacco mosaic virus. Our results reveal a new role for SGT1- and Rar1-associated chaperone machinery in R gene-mediated defense signaling.

In their natural environment plants are subject to attack by a variety of microbial pathogens and by insect and nematode pests. Plants have therefore evolved a diverse repertoire of defense mechanisms that allow them to evade these assaults. The specific gene-for-gene interaction between plant resistance (R) genes and pathogen avirulence genes is one such mechanism (1). Recognition of pathogen avirulence products by plant R proteins initiates a signaling cascade leading to a type of localized cell death at the site of pathogen entry known as the hypersensitive response, rapid oxidative burst, ion fluxes, strengthening of cell walls, protein phosphorylation, and the activation of various defense response genes (2).

In recent years a number of R genes have been cloned, providing insight into their structure and the nature of host-pathogen interactions (2). Much progress has been made in deciphering the signaling pathways involved in R gene-mediated resistance (3). Recent evidence suggests that most known

R genes require two signaling components, Rar1 and/or SGT1. Rar1 is present in all eukaryotes examined so far except yeast and possesses a pair of 60-amino acid zinc finger motifs termed CHORD-I and CHORD-II (cysteine- and histidine-rich domains) (4). Metazoan Rar1 has an additional C-terminal domain called the CS domain, which is found in yeast and plant SGT1 and human SIP proteins (5–10). Interestingly, Rar1 interacts directly with SGT1 in plants (8, 9). In yeast, plants, and mammals SGT1/SIP associates with the SCF (Skp1/Cullin/F-box protein)-type E3 ubiquitin-ligase complex (5, 6, 8, 9). Components of the SCF complex are also known to interact with the COP9 signalosome (11), which functions in protein degradation via the 26 S proteosome (12). Accordingly, plant SGT1 and Rar1 associate with subunits of the COP9 signalosome (8, 9). In addition, silencing of CSN3 and -8, two components of the COP9 signalosome, compromises N gene-mediated resistance to tobacco mosaic virus (TMV) (9). Association of SGT1 with the SCF ubiquitin ligase and the COP9 signalosome suggests that plant SGT1 and Rar1 might play some role in ubiquitination of resistance-regulating proteins via a specific SCF complex.

The CS domain of SGT1 is similar to the human p23 protein, which is known to interact with heat shock protein 90-kDa (Hsp90) and to participate in the folding of different regulatory proteins (13). SGT1 proteins also contain a tetra-tricopeptide repeat (TPR) domain, which is an Hsp90-binding domain in mammalian SGT1/SIP (5–10). Interestingly, Rar1 in

Hsp90 is an abundant, highly conserved, ATP-dependent molecular chaperone that is essential for eukaryotic cell viability (see review, Ref. 14). Studies, primarily with animals, have revealed that Hsp90 plays roles in a diverse array of cellular processes including protein folding, stress responses, signal transduction, and genomic silencing. Recent studies show that Hsp90 also plays a critical role in the ATP-dependent assembly of the 26 S proteosome (17). Other studies suggest that Hsp90 acts as a buffer against genetic variation as a capacitor of phenotypic variation in plants as in fruit flies (18, 19). Although Hsp90 genes have been isolated from several plant species (see review, Ref. 20), understanding of the function and regulation of Hsp90 in plants is limited. Furthermore, the role of Hsp90 in plant defense remains obscure, although there has been recent progress in this area (16, 21).

Here we report that the Hsp90 that interacts with NbRar1 also interacts with the TIR-NB-LRR protein N that mediates resistance to TMV (22). We show that N, NbRar1, and NbSGT1
interact with Hsp90 in yeast two-hybrid and in itro pull-down assays. In addition, we show that Hsp90 forms a complex with N, NbRar1, and NbSGT1 in itvo. Knockdown of Hsp90 expression using virus-induced gene silencing (VIGS) suggests that it is required for plant survival. Moreover, the suppression of Hsp90 compromises N-mediated resistance to TMV. Our results suggest that SGT1 and Rar1 associate with Hsp90 as co-chaperones in the assembly or the conformational regulation of R protein recognition complexes.

EXPERIMENTAL PROCEDURES

Plasmid Construction—NbSGT1 and NbRar1 fusion to LexA-BD are described in Ref. 9. NbRar1 CHORD-I and N-LRR domain fusions to LexA-BD were generated by cloning cDNA PCR products into pTBS1 downstream of the LexA-BD. pTBS1 is a derivative of pESC-His (Stratagene) and contains a NLS-LexA-BD under the control of the GAL10 promoter. The NLS-p50 PCR product was cloned into pTBS1 downstream of the GAL1 promoter. NbHsp90-1 fusion to AD was generated by cloning the full-length PCR product of NbHsp90-1 into pG4J-5(23).

To express GST fusion proteins in Escherichia coli, the LRR domain of N was amplified by PCR and recombined into pDEST15 using the GATEWAY system (Invitrogen). GST-NbSGT1 and -NbRar1 expression constructs were described in Ref. 9. To synthesize [35S]Met-labeled in itro translation products, PCR products of NbHsp90-1 were recombined into pDEST14 (Invitrogen).

A tobacco Hsp90 cDNA fragment (nucleotides 587–1482) was amplified and inserted into pTrpV2 (24) and pPVX (9). A tomato Hsp90 cDNA fragment (nucleotides 1–717) was inserted into pTRV2.

The vector pYL436, contains the tandem affinity purification (TAP) tag consisting of MYC 9-His6-3C protease cleavage site-IgG 2 binding domain. The TAP tag was fused to the C terminus of the N coding sequence upstream of the 3'-untranslated region. The entire genomic clone, consisting of the 5'-regulatory sequence, the N coding sequence with the TAP tag, and the 3'-untranslated region was then placed into pCAM-BIA2300 (GenBank™ accession number AF234315) to generate pTBS1-N, TAP. GFP PCR product was cloned into pYL436 upstream of the TAP tag to generate 35 S::GFP-TAP.

Yeast Two- and Three-hybrid Screens and Interaction Assays—The yeast two-hybrid library used has been described (9). Yeast two- and three-hybrid screens and interaction assays were performed as described (23).

GST Pull-down Assay—GST-NbSGT1, GST-NbRar1, and GST-N-LRR fusion proteins were produced in BL21 (DE3) codon plus RIL cells (Stratagene) and affinity purified using glutathione-Sepharose beads. GST-tagged fusion proteins were expressed in Escherichia coli and GST alone served as control were used to pull down [35S]Met-labeled in itro translated (Tst, Promega) NbHsp90-1 as described in Ref. 9.

Generation of Antibodies—Anti-SGT1 antisera against the peptide KAEILDFSMSKYALKR corresponding to residues 61–76 of NbSGT1 (9) and anti-NbRar1 antisera against the peptide TEDDNPENSCTYKAIELDPSMSKAYLRK corresponding to residues 61–76 of NbSGT1 (24) were generated in rabbits and affinity purified using custom service from Invitrogen.

Co-immunoprecipitation—Total protein was extracted from N. benthamiana plants containing the N gene (NN plants) using the protocol described in Ref. 9, and immunoprecipitations were performed using the ProFound co-immunoprecipitation kit (Pierce). Anti-NbRar1, anti-NbSGT1 antibodies, and respective preimmune serum were coupled to rabbits and affinity purified using custom service from Invitrogen.

Identification of NbHSP90 as NbRar1-interacting Partner—Previous studies from our laboratory had identified 13 clones that represented the ATPase domain of two classes of tomato Hsp90, LeHsp90-1 and LeHsp90-2, as an NbRar1-interacting partner in a yeast two-hybrid screen (9) (Fig. 1). Although Rar1 possesses two N-terminal CHORD domains, the CHORD-II domain of Rar1 is responsible for interaction with SGT1 (8). We were therefore interested in determining the proteins interacting with the CHORD-I domain of Rar1. We used this domain as bait to screen a tomato cDNA library and identified several interacting proteins including LeHsp90-1 and the smaller chaperones Hsp70 and 40.

We cloned full-length Hsp90 cDNAs from tomato and N. benthamiana by reverse transcription with primers based on the LeHsp90-1 sequence followed by PCR (RT-PCR). The sequence analysis of six independent cDNA clones suggests that there are two sequences showing high similarity to LeHsp90-1 in N. benthamiana (NbHsp90-1 and -2). Both encode open reading frames of 669 amino acids and are highly homologous (Fig. 1).

Northern Analysis—Transgenic SR1 tobacco plants carrying N (22) at the four-leaf stage were incubated at 32 °C overnight before being infected with TMV by rubbing virus onto every leaf. The plants were kept at 32 °C for 3 days, then placed at room temperature, and samples collected at the following time points: 0, 0.5, 1, 2, 4, 8, 16, 24, and 48 h. RNA was extracted from samples using RNAwiz (Ambion) according to the manufacturer’s instructions. Samples were then analyzed by Northern blot using standard molecular biology protocols. The probe used was derived from NbHsp90-1 using the same primers used in RT-PCR analysis.

RESULTS

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FIG. 1. Comparison of the *N. benthamiana* Hsp90 amino acid sequences with orthologs from other organisms. Alignment of the predicted *N. benthamiana* Hsp90 protein sequences with its orthologs in tomato (*LeHSP90*), *Arabidopsis* (*AtHsp90-1–4*), and humans (*HsHsp90α* and *-β*). The alignment was generated using ClustalW. Numbers at left indicate amino acid residue positions. Identical residues are shaded in black, and similar residues are in gray.
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trol of galactose-inducible promoters. Yeast transformed with LexA-NbSGT1 and AD-NbHsp90-1 grew on Leu− selection medium containing galactose and turned blue on X-gal plates containing galactose. Control strains transformed with LexA-BD and AD only showed no growth on Leu− selection medium containing galactose and remained white on X-gal plates. Glucose-containing media were used as a control. Interactions were carried out at 28 °C for 4 days. B, for in vitro binding assays, 1 μg of GST-NbSGT1 (lane 2), GST-NbRar1 (lane 3), or GST (lane 4) was incubated with 35S-labeled NbHsp90-1. Beads were washed and proteins analyzed by SDS-PAGE followed by fluorography. As a control, 10% of input 35S-labeled NbHsp90-1 was loaded directly onto the same gel (lane 1). C, yeast strains transformed with bait LexA-BD-N-LRR (first row, top and bottom panels) or LexA-BD-N-LRR+p50 (first row, top and bottom panels) and prey AD-NbHsp90-1 grew on Leu− selection medium containing galactose and turned blue on X-gal plates containing galactose. Control strains transformed with LexA-BD and AD alone was unable to grow on selection plates or turn X-gal blue on glucose or galactose (Fig. 2A, bottom panels). NbSGT1 therefore interacts directly with NbHsp90-1.

Using a similar assay, we examined the interaction between full-length NbHsp90-1 and NbRar1. Yeast transformed with LexA-NbRar1 and AD-NbHsp90-1 was able to grow on selection media containing galactose but not on glucose (Fig. 2A, top panels). Control yeast containing LexA-BD or AD alone was not able to grow on selection plates or turn X-gal blue on glucose or galactose (Fig. 2A, bottom panels). NbSGT1 therefore interacts directly with NbHsp90-1.

We confirmed these interactions in vitro by GST pull-down assays. NbRar1 and NbSGT1 were individually expressed in E. coli as GST fusions and purified using glutathione-Sepharose beads. The identity of these proteins was confirmed by Western blot with GST antibodies (data not shown). 35S-Radiolabeled NbHsp90-1 was in vitro translated and mixed with GST fusions proteins. Analysis of the bound fraction by fluorography indicates that NbHsp90-1 binds directly to GST-NbRar1 and GST-NbSGT1 (Fig. 2B). GST alone control did not interact with in vitro translated NbHsp90-1 (Fig. 2B).

The LRR Domain of N Interacts with Hsp90-1—The LRR domain of R proteins has been implicated in recognition of pathogens and downstream signaling (2). Therefore, to identify protein factors that may interact with the LRR domain of N (N-LRR), we performed a three-hybrid screen using N-LRR as bait in the presence of its avirulence determinant, TMV-p50. In this screen full-length LeHsp90-1, protein phosphatase 5, and plant-specific transcription factors were identified.

Interaction of NbHsp90-1 with N-LRR was confirmed by yeast two-hybrid analysis and GST pull-down assay. Yeast containing both LexA-N-LRR+TMV-p50 and AD-NbHsp90-1 grew on Leu− selection medium containing galactose and turned blue on X-gal plates containing galactose. Control strains transformed with LexA-BD and AD alone was unable to grow on selection plates or turn X-gal blue on glucose or galactose (Fig. 2C). They turned blue on media containing X-gal and galactose but remained white on media with glucose (Fig. 2C). We then tested the interaction between N-LRR and NbHsp90-1 in the absence of TMV-p50. Our results indicate that the interaction between N-LRR and NbHsp90-1 is independent of TMV-p50 (Fig. 2C). Control yeast expressing LexA-BD and AD alone was unable to activate transcription of reporter genes on media containing either glucose or galactose (Fig. 2C).

For in vitro pull-down assays, N-LRR was expressed in E. coli as a GST fusion (GST-N-LRR) and purified using glutathione-Sepharose beads. 35S-Radiolabeled NbHsp90-1 was in vitro translated and mixed with GST-N-LRR fusion protein. Analysis of the bound fraction indicates that GST-N-LRR binds directly to NbHsp90-1 (Fig. 2D). A GST alone control fails to interact with in vitro translated NbHsp90-1 (Fig. 2D). The association of Hsp90-1 with N protein in addition to its interaction with NbRar1 and NbSGT1 suggested that this protein is critical in N-mediated resistance signaling.
**Fig. 3. Hsp90 association with N, NbRar1, and NbSGT1 in vivo.**

A–C, wild type *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying the *N*- or GFP-TAP constructs. Expression of N-TAP fusion was confirmed by immunoblot analysis using anti-MYC antibodies (A, lane 3). Tissue from wild type plants was used as control (A, lane 2). N- and GFP-TAP protein extracts were incubated with IgG-Sepharose beads, and then the beads were washed, and the proteins were separated by SDS-PAGE. Immunoblot analysis was carried out with anti-Hsp90 antibodies that immunoreact with *N. benthamiana* Hsp90 (B, lanes 2 and 3). Hsp90 was detected in N-TAP immune complex (C, lane 2) but not in GFP-TAP (C, lane 3). Lanes 1 in A, B, and C represent MagicMark Western Standard from Invitrogen; molecular size is shown in kilodaltons (kDa). D, protein was extracted from *N. benthamiana* plants and incubated with anti-NbRar1 (lane 4) or anti-NbSGT1 (lane 5) antibodies coupled to AminoLink plus resin. As a control, protein extract was incubated with preimmune serum of NbRar1-injected rabbit (lane 2) or NbSGT1-injected rabbit (lane 3) coupled to AminoLink plus resin. Proteins were eluted and analyzed by SDS-PAGE followed by immunoblot analysis using anti-Hsp90 antibodies. Whole cell extract from *N. benthamiana* was included as a control (lane 1).

*Fig. 4. NbHsp90 RNA levels remain constant upon infection with TMV.*** Tissue from plants systemically infected with TMV-GFP was collected at different time points after being shifted from the restrictive to permissive temperature for *N* function. Northern blot analysis was performed using a probe that anneals to NbHsp90-1. Lanes 1–11 represent tissue collected before any treatment, overnight at restrictive temperature, and 0, 0.5, 1, 2, 4, 8, 16, 24, and 48 h at permissive temperature. The ethidium bromide-stained gel shown below the Northern blot indicates equal loading of RNA.

*Fig. 5. NbHsp90 is required for normal growth and development.*** *N. benthamiana* NN and wild type plants were infiltrated with *Agrobacterium* cultures carrying TRV1 and TRV2-NbHsp90-1 or TRV2 alone. A, comparison of TRV-VIGS vector only infiltrated plant (left) with TRV-NbHsp90-1 infiltrated plant (right) 14 days after infiltration. B–D, TRV2-NbHsp90-1-infected plants die gradually. B, TRV2-NbHsp90-1-infected plant 14 days after infiltration and 26 days after infiltration (C and D). E, PVX-NbHsp90-1 has the same effect as TRV2-NbHsp90-1. Plant is shown 26 days after infiltration.

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*NbHsp90 Interacts Directly with N, NbRar1, and NbSGT1 in Vivo*—We tested whether NbHsp90 physically associates with N, NbRar1, and NbSGT1 in vivo. Wild type *N. benthamiana* plants were infiltrated with *A. tumefaciens* containing an N-TAP construct. In this construct, *N* is fused to a tandem affinity purification tag that contains the MYC epitope. In addition, expression of the N-TAP fusion is under the control of its native N promoter and N-3′ sequence. *N. benthamiana* tissue infiltrated with this functional N-TAP was collected 6 days postinfiltration. Immunoblot analysis of total protein extracts from the N-TAP-transfected tissue detected a single protein band of the expected N-TAP fusion size (Fig. 3A, lane 3) compared with the control non-transfected tissue (Fig. 3A, lane 2). To test whether N associates with Hsp90, we incubated total protein extracts from the N-TAP and control GFP-TAP-transfected tissue with IgG-Sepharose 6 Fast Flow beads. Bound fraction was analyzed by immunoblotting using anti-Hsp90 antibodies (25) that immunoreact with NbHsp90 (Fig. 3B, lanes 2 and 3). These results suggest that *N* exists in a complex with Hsp90 (Fig. 3C, lane 2). Control immunoprecipitations with a GFP-TAP fusion did not detect Hsp90 (Fig. 3C, lane 3).

To test whether NbRar1 and NbSGT1 associate with Hsp90, we used tissue from transgenic NN plants (24) to immunoprecipitate the protein complexes using anti-NbRar1 and anti-NbSGT1 antibodies. Immune complexes were analyzed by immunoblotting using anti-Hsp90 antibodies. Our results suggest that NbRar1 and NbSGT1 immune complexes contain Hsp90 (Fig. 3D, lanes 4 and 5). Control immunoprecipitations did not detect Hsp90 (Fig. 3D, lanes 2 and 3). Taken together, these results indicate that NbHsp90 interacts with N, NbRar1, and NbSGT1 in vivo.

*NbHsp90-1 Expression after TMV Infection in N-containing Plants*—Hsp90 is induced upon heat and cellular stress (see review, Ref. 14). We therefore examined the effects of TMV infection on Hsp90-1 mRNA expression levels. Northern blot analysis was performed using total RNA isolated from NN plants collected at 0, 0.5, 1, 2, 4, 8, 16, 24, and 48 h after infection with TMV (see “Experimental Procedures” for details). There was no change in Hsp90-1 mRNA levels in infected tissue (Fig. 4). We also determined whether there was any change in the protein levels of Hsp90 after infection using anti-Hsp90 antibodies (25). Hsp90 levels remained unchanged over the course of infection (data not shown). From these results, we conclude that Hsp90 is not induced in NN plants upon infection with TMV.

**Suppression of Hsp90 Leads to Plant Developmental Phenotypes**—We used a tobacco rattle virus (TRV)-based VIGS system (24) to determine the biological role of NbHsp90 in N-mediated resistance to TMV. A fragment of NbHsp90-1 was cloned into pTRV2 and introduced into *A. tumefaciens* strain GV2260. *Agrobacterium* harboring pTRV2-NbHsp90-1, pTRV2 alone, and pTRV-N controls were then separately mixed with a culture of *A. tumefaciens* carrying pTRV1 and infiltrated into NN plants at the four-leaf stage. Wild type and NN plants that had been infiltrated with pTRV2-NbHsp90-1 resembled nonsilenced control plants until about 7 days after introduction of the Hsp90-silencing construct. Later these plants stopped growing and became chlorotic and severely stunted because of death of the meristem and young leaves compared with TRV-VIGS vector alone infiltrated control plants (Fig. 5A). Plants were completely dead ~24–26 days after introduction of the
Fig. 6. **NbHsp90 is required for N-mediated resistance to TMV.** 
A–E, N. benthamiana NN plants were infiltrated with TRV-VIGS vector carrying a LeHsp90-1 fragment to silence NbHsp90-1 or with TRV-VIGS vector-only controls. After 4 days, plants were infiltrated with TMV-GFP to monitor resistance or susceptibility responses. The spread of the virus to the upper leaves from the inoculated leaf indicates loss of resistance to TMV. A and C, virus-inoculated leaf of control and NbHsp90-silenced plant, respectively. B, upper leaves of control plants. D and E, upper leaves of NbHsp90-1-silenced plants. F, RT-PCR analysis was used to confirm the suppression of NbHsp90. First-strand cDNA was synthesized using total RNA from NbHsp90-silenced and non-silenced NN plants. PCR was performed with gene-specific primers. Typical PCR products for EF-1α (upper panel) derived from non-silenced (left) and silenced plants (right). Lanes 1–6 correspond to products from PCR cycles 15, 18, 21, 24, 27, and 30. Lane C represents the control reaction in which the RT mix without reverse transcriptase was used as template. PCR products of NbHsp90 (lower) from non-silenced (left) and NbHsp90-silenced plant (right). Lanes 1–6 correspond to products from PCR cycles 12, 14, 16, 18, 20, and 22. Lane C is the no RT control. G, Western blot analysis was used to confirm the suppression of NbHsp90. Total protein extracted from non-silenced (lane 1) and Hsp90-silenced (lane 2) plants was analyzed by SDS-PAGE followed by immunoblot analysis using anti-Hsp90 antibodies. As a loading control, Coomassie staining of the membrane is shown. H, RT-PCR analysis was used to confirm the presence of TMV-GFP RNA in NbHsp90-silenced tissue. First-strand cDNA was synthesized using silencing vector (Fig. 5, B–D). Similar results were obtained with a potato virus X (PVX)-based VIGS vector (26) carrying the NbHsp90-1 sequence (Fig. 5E). These results show that NbHsp90 is required for the normal growth and development of *N. benthamiana* and is therefore an essential gene.

**Suppression of NbHsp90 Compromises N-mediated Resistance to TMV—** We believed that the severe phenotype we had observed because of the VIGS of NbHsp90 was the result of silencing not only NbHsp90-1 but also other closely related isoforms. Because LeHsp90-1 is the corresponding ortholog of NbHsp90-1 and -2, LeHsp90-1 should have higher homology with these orthologs than other NbHsp90 isoforms. We therefore decided to use the LeHsp90 sequence to silence NbHsp90-1 as we hypothesized that this would avoid the severe plant phenotypes observed with TRV-NbHsp90-1. Wild type and NN plants infiltrated with pTRV2-LeHsp90-1 showed only a mild, if any, plant phenotype (data not shown). These plants were infected with TMV-GFP 4 days after infiltration to avoid the severe phenotype of NbHsp90-silenced plants. In control NN plants where *N* had been silenced by infiltration with pTRV2-N and subsequently infected with TMV-GFP after 4 days, the virus was able to spread to the upper parts of the plants (data not shown), indicating that silencing was effective at least as early as 4 days after infiltration with VIGS vectors. In NN control plants infiltrated with the empty TRV-VIGS vector TMV-GFP was unable to spread, and the virus remained restricted to the inoculated leaf (Fig. 6, A and B). In NbHsp90-silenced NN plants, TMV-GFP was able to overcome N-mediated resistance to TMV and spread from the inoculated leaf (Fig. 6C) to the upper leaves of plants (Fig. 6, D and E). These results demonstrate that NbHsp90 is required for N-mediated resistance to TMV.

We confirmed the suppression of NbHsp90 by semiquantitative RT-PCR and Western blot analysis. The level of NbHsp90 mRNA (Fig. 6F) and protein (Fig. 6G) was reduced by ~70% in silenced plants compared with non-silenced TRV-VIGS vector alone-infected control plants. EF-1α levels were monitored to serve as an internal control and no obvious change was observed in silenced or non-silenced plants (Fig. 6F). We also confirmed the spread of TMV-GFP in NbHsp90-silenced plants with RT-PCR using primers that anneal to the viral movement protein (Fig. 6H, lane 1). There was no TMV-RNA in the upper uninoculated leaves of the control non-silenced NN plants (Fig. 6H, lane 2).

**DISCUSSION**

The Role of Hsp90 in Plant Defense and Development—We report here that Rar1, SGT1, and the resistance protein *N* interact with the molecular chaperone Hsp90 in yeast two-hybrid assays as well as *in vitro* pull-down and *in vivo* immunoprecipitation assays. Because Rar1 and SGT1 are involved in plant resistance, including N-mediated resistance to TMV, this suggested that Hsp90 might play a central role in plant defense as well. To confirm this, we knocked down the expression of Hsp90 using a VIGS approach. Silencing of NbHsp90-1 using VIGS vectors carrying fragments of NbHsp90-1 sequence resulted in severe plant developmental defects and ultimately in plant death. In *Arabidopsis*, there are four Hsp90 homologs (20) that show high homology to NbHsp90-1 (Fig. 1). Therefore, we suspected that because of high homology among NbHsp90 isoforms, we may have effectively silenced other isoforms along with NbHsp90-1 and -2. However, this result indicates that total RNA from NbHsp90-silenced NN plants and non-silenced controls. This was used as the template in PCR to amplify the TMV movement protein gene. Lane 1, NbHsp90-silenced plants; lane 2, control non-silenced NN plants; lane C, no RT control.
Hsp90 is essential for plant growth and survival. This plant death phenotype prevented us from determining the role of Hsp90 in plant defense. Therefore, to silence only Hsp90-1 to understand its function in plant defense, we used the tomato ortholog of NbHsp90-1, LeHsp90-1, that we identified in our yeast screens. We hypothesized that LeHsp90-1 would have enough homology to generate efficient silencing of NbHsp90-1 and/or NbHsp90-2 but not other Hsp90 homologs, because we isolated only these two clones from N. benthamiana using primers based on the LeHsp90-1 sequence. Indeed, silencing using LeHsp90-1 has minimal effects on plant development. Moreover, this allowed us to assess the role of Hsp90 in N-mediated resistance to TMV. However, because of the high homology between NbHsp90-1 and -2 we cannot definitely determine which of the two was silenced. Despite this our RT-PCR as well as Western analysis data confirm the silencing of the NbHsp90-1, and our results suggest that suppression of NbHsp90-1 compromises N-mediated responses to TMV. This clearly indicates that Hsp90 plays an important role in R gene-mediated resistance. Further evidence for the role of Hsp90 in R gene signaling is given by the requirement of Hsp90 for the function of another R gene, RPS2 (16). Here, both the knock-out of Hsp90 and its chemical inhibition using geldanamycin-compromised resistance mediated by this R gene.

The Possible Function of Hsp90, SGT1, and Rar1 in Plant Defense—In this report we show the association of Hsp90 with SGT1 and Rar1. This is in agreement with the recently reported findings of Takahashi et al. (16). These authors found that both barley Rar1 and SGT1 interact with cytosolic HvHsp90 in yeast two-hybrid and in vitro pull-down assays. Previous studies have shown that SGT1 and Rar1 associate with the SCF complex and COP9 signalosome involved in protein degradation (8, 9). Silencing of Skp1, a key component of SCF complexes, and two components of the COP9 signalosome compromised N-mediated resistance to TMV (9). These data suggest that SGT1 may be an important component of the SCF complex. However, work in yeast suggests that SGT1 might not be an integral component of SCF complexes because SGT1 is found at substoichiometric levels in the SCF complex (5). This is further supported by the fact that human SCF components expressed from E. coli can be assembled in vitro without SGT1 (27). We therefore predict that SGT1 and Rar1 are also not integral components of plant SCF complexes. Instead, they may associate with the SCF complex and/or the Hsp90/Hsp70 chaperone complex to take part in R gene-mediated resistance.

Hsp90 is a highly conserved stress protein in all eukaryotic cells. It is an ATP-dependent chaperone that has critical functions in the folding, activation, and assembly of client proteins that are typically involved in signal transduction, cell cycle control, or transcriptional regulation (14, 28–30). To date, ~100 proteins are known to be regulated by Hsp90 (Ref. 31 and www.picard.ch/DPDphome.html). Our identification of NbHsp90 as an interactor with NbSGT1 and NbRar1, as well as with the R protein N, suggests that NbHsp90 acts as a chaperone in the formation of active signaling complexes involved in R gene-mediated disease resistance. SGT1 might also function as a co-chaperone component in the Hsp90-substrate complex because the TPR domain and the CS domain of SGT1 are structurally similar to the TPR region of the Sti/Hsp co-chaperone and p23, respectively (13). Furthermore, two known components of the Hsp90-substrate complex, LeHsp70 and -40, interact with NbRar1 in yeast two-hybrid analysis (data not shown). These results suggest that Hsp90, Hsp70, Hsp40, SGT1, and Rar1 exist in a complex that participates in the regulation of protein complexes involved in R gene-mediated resistance, including an E3 ligase or R protein complex. This might explain the differential dependence of various R genes on SGT1 and Rar1. Some R proteins may be able to independently assemble into recognition complexes whereas others are reliant on a chaperone machinery that includes SGT1 and/or Rar1.

It is also interesting that Hsp90 has recently been implicated in broader plant defense. Kanazaki et al. (21) have shown that a constitutively expressed cytosolic NbHsp90 isoform interacts with a mitogen-activated protein kinase, SIPK, and that loss of Hsp90 function leads to compromised non-host resistance and INP1-mediated hypersensitive response (21). SIPK is a known component of N-mediated disease signaling (32). Thus, Hsp90 may be recruited in more than one situation in plants to function in defense signaling. It will be exciting to determine which and how complexes, if any, involved in this branch of defense signaling function.

Role of Hsp90 in N Protein Complex—The association of N with Hsp90 hints at a role for chaperones in the activation of this R protein. Indeed, our silencing experiments have confirmed the requirement of Hsp90 for N signaling to bring about resistance. There is a precedent for the involvement of HSPs in disease resistance through interaction with a receptor. In mammalian systems, HSPs are able to stimulate both innate and adaptive immune systems (33). The ability to stimulate the innate immune system is conferred by the toll-like receptors (TLRs) that recognize highly conserved features of pathogens termed pathogen-associated molecular patterns (34).

Plant TIR-NB-LRR proteins, including N, have homology to TLRs. Gp96, an endoplasmic reticulum Hsp90 paralog, Hsp70, and Hsp60 are shown to activate TLR2- and TLR4-mediated signaling (35, 36). Gp96 is known to assist in the folding and export of soluble and membrane-bound oligomeric proteins. In the absence of Gp96, TLR1, -2, and -4 are retained intracellularly and hence fail to respond to microbial stimuli (35). TLR9 is known to recognize unmethylated bacterial CpG DNA to initiate defense (37). Recently, Hsp90 has been implicated in facilitating this recognition (38). Therefore, Hsp90 probably acts as a ligand transfer molecule bridging the receptor-ligand gap in this system (38). We envisage a similar role for Hsp90 in N-mediated resistance to TMV and in broader R protein signaling. In addition, Hsp90, like Gp96, might play a crucial role in the intracellular trafficking of an N protein complex. Hsp90 and immunophilins play a major role in glucocorticoid receptor intracellular trafficking (39). In this regard, it is interesting that protein phosphatase 5 was also isolated in our N-LRR+p50 screen. Mammalian protein phosphatase 5 is a TPR domain-containing immunophilin that has roles in signal transduction (40). The TPR domain of protein phosphatase 5 interacts with Hsp90 within glucocorticoid receptor-Hsp90 complex and also with the kinase domain of the ANP-guanylate cyclase receptor in vivo (see review, Ref. 40). Additional screens or direct biochemical analysis of N-containing complexes will help to identify additional protein partners of N and shed light on the mechanism of pathogen recognition.

We have yet to determine whether the dependence of Hsp90 on N-mediated resistance is due in part to loss of SGT1 and Rar1 complexes or if it is solely because of the loss of N-Hsp90-containing complexes. This question arises from the fact that the same Hsp90 sequence was found in both the Rar1 screen and the N-LRR screen. In the future we will determine whether N, SGT1, and Rar1 are constituents of a larger complex that contains Hsp90. This would provide good insights into N-mediated resistance to TMV as it would shed light on the functions of Rar1 and SGT1 as part of a larger E3 ubiquitin ligase and as components of a chaperone complex.
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