RESEARCH PAPER

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Molecular chaperons and co-chaperons, Hsp90, RAR1, and SGT1 negatively regulate bacterial wilt disease caused by *Ralstonia solanacearum* in *Nicotiana benthamiana*

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Keywords: Hsp90, *Nicotiana benthamiana*, plant immunity, required for Mla12 resistance 1, suppressor-of-G2-allele-of-skp1, virus-induced gene silencing

Abbreviations: HR, hypersensitive response; Hsp, heat shock protein; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative real time polymerase chain reaction; RAR1, required for Mla12 resistance 1; SGT1 suppressor-of-G2-allele-of-skp1; TTSS, type III secretion system; VIGS, virus-induced gene silencing.

Ralstonia solanacearum is the causal agent of bacterial wilt disease. To better understand the molecular mechanisms involved in interaction between *Nicotiana benthamiana* and *R. solanacearum*, we focused on Hsp90, RAR1 and SGT1. Appearances of wilt symptom were significantly suppressed in *Hsp90*, *RAR1* and *SGT1*-silenced plants compared with control plants. In *RAR1*-silenced plants, population of *R. solanacearum* increased in a similar manner to control plants. In contrast, multiplication of *R. solanacearum* was significantly suppressed in *Hsp90* and *SGT1*-silenced plants. In addition, expression of PR genes were increased in *Hsp90* and *SGT1*-silenced plants challenged with *R. solanacearum.* Therefore, RAR1 might be required for disease development or suppression of disease tolerance. These results also suggested that Hsp90 and/or SGT1 might play an important role in suppression of plant defenses leading to disease susceptibility and disease development.

Introduction

Plants have evolved sophisticated defense mechanisms that are activated in response to pathogen attacks. In most cases, plants resist infection through active defense mechanisms. The front line of induced defense is triggered by pathogen-associated molecular patterns (PAMPs), also known as PAMP-triggered immunity (PTI). PAMPs are generally conserved compounds, like chitin in fungi and flagellins in bacteria, and PAMP-triggered immunity is induced by all invading pathogens. In contrast, some adapted pathogens suppress PTI by evolving effector molecules. The second line of plant defense is activated via recognition of pathogen effectors by Resistance gene products, followed by triggering of effector-triggered immunity.[1–3]

Ralstonia solanacearum is a devastating, soil-borne pathogen with a global distribution and wide host range.[4] It causes bacterial wilt in several economically important solanaceous crops. In the tomato, resistance to *R. solanacearum* is controlled by several loci.[5,6] In contrast, resistance is monogenic and is conferred by the *RRS1-R* gene that encodes a R protein in *Arabidopsis thaliana*, and this resistance is dependent upon salicylic acid and the *NDR1* signaling pathway.[7] PopP2, type III effector of *R. solanacearum* was identified and shown to interact with the RRS1-R.[8] Recent studies showed that ethylene-, salicylic acid- and MAP kinase-related defense signaling pathways are involved in the resistance of tomato to *R. solanacearum.*[7] We previously reported that cellular components, such as asparagine-rich protein, S-glycoprotein-like protein and translationally controlled tumor protein, have important role in regulation of plant defenses against *R. solanacearum.*[10-12] In addition, our previous report showed that molecular chaperon, Nbshsp17, has a crucial role in regulation of plant defenses, suggesting involvement of molecular chaperons and co-chaperons in plant and *R. solanacearum* interaction.[13]

In this study, we focused on Hsp90, RAR1 and SGT1. Although, these chaperons and co-chaperons have been well-known to have important role in regulation of plant immune responses, the role of Hsp90, RAR1 and SGT1 on plant and *R. solanacearum* interaction is largely unknown. Therefore, we carried out functional analysis of Hsp90, RAR1 and SGT1 using

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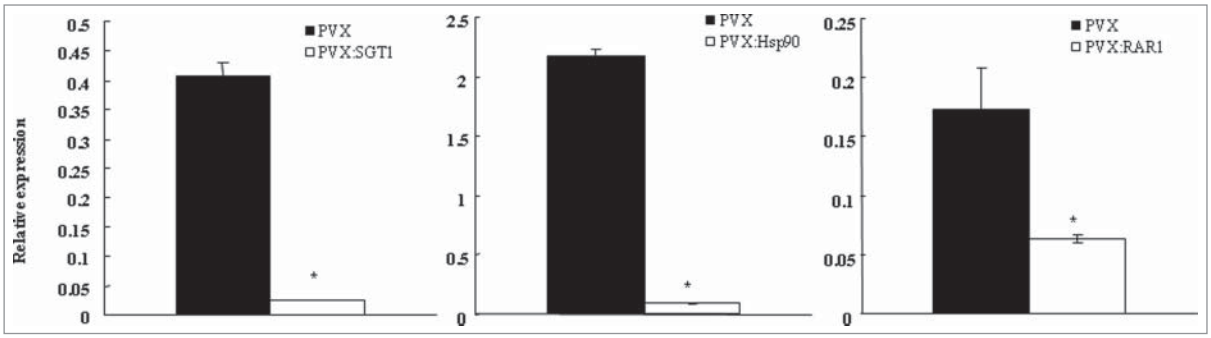

Figure 1. Creation of gene-silenced plants. Total RNA was isolated from Control (PVX), *Hsp90*-(PVX:Hsp90), *RAR1*-(PVX:RAR1) and *SGT1*-(PVX:SGT1) silenced plants. Expression values of *Hsp70, Hsp90, RAR1* and *SGT1* were estimated by qRT-PCR, and expressed as [Qty] after normalization with actin. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*; $P < 0.05$).

N. benthamiana, and discussed a possible mechanism by which Hsp90, RAR1 and SGT1 affects bacterial wilt disease.

Results

Plant molecular chaperons and co-chaperons such as Hsp70, Hsp90, RAR1 and SGT1, play a critical role in plant-pathogen interaction. Then, we created *Hsp70-*, *Hsp90-*, *RAR1-* and *SGT1*-silenced plants (**Fig. 1**), and observed the effect of silencing of these genes on development of bacterial wilt disease. As shown in **Figure 2**, the control plants showed wilt symptom from 6 day after inoculation and completely died 12 d after inoculation with *R. solanacearum*. In *Hsp70*-silenced plant, wilt symptom was slightly accelerated 6–8 d after inoculation (**Fig. S1**). Therefore, Hsp70 might play a role in plant defenses similar to previous report.[14] In contrast, we could observe significant delay of wilt symptom in *Hsp90-*, *RAR1-* and *SGT1*-silenced plants compared to those in the control plants. *RAR1*-silenced plants showed suppression of wilt symptom 6–16 d after inoculation. Significant suppression of wilt symptom was also observed 6–19 d after inoculation in *Hsp90*-silenced plants. Most drastic suppression of wilt symptom was observed in *SGT1*-silenced plants, and wilt symptom was suppressed during the experiment period up to 20 d after inoculation. Generally, it has been reported that Hsp90, RAR1 and SGT1 have a crucial role in induction of plant defenses. In contrast, our present results suggested that Hsp90, RAR1 and SGT1 might have roles in suppression of defense responses and/or wilt symptom.

Since suppression of wilt symptom was observed in *Hsp90*, *RAR1* and *SGT1*-silenced plants, this raised the possibility that disease resistance to *R. solanacearum* may increase in these

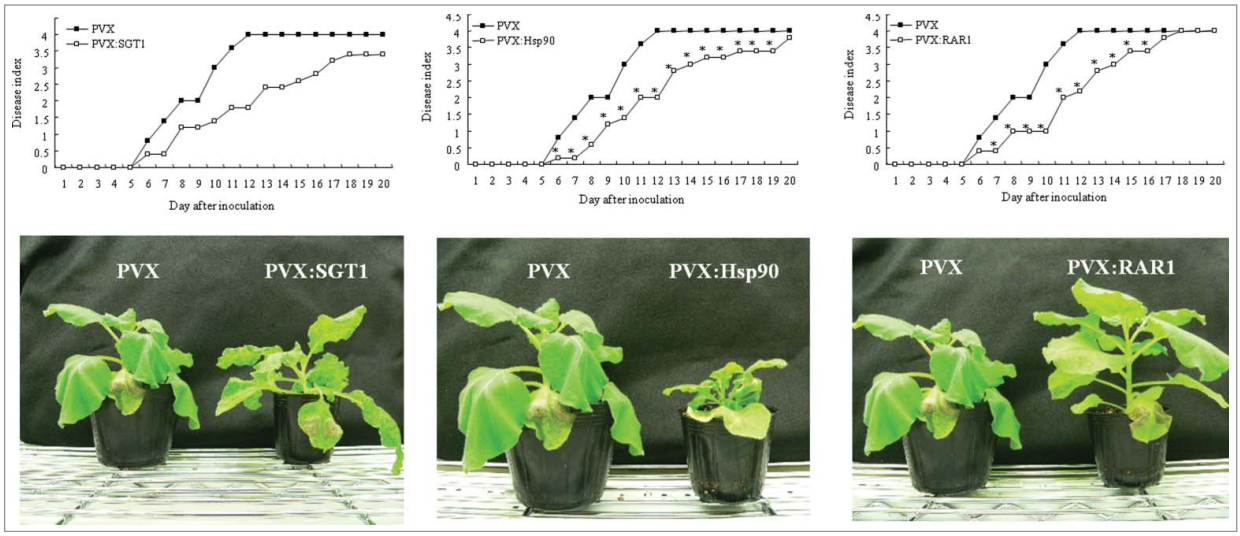

Figure 2. Effect of *Hsp90, RAR1 and SGT1*-silencing on bacterial wilt disease by inoculation with *R. solanacearum*. Control (PVX), *Hsp90-* (PVX:Hsp90), *RAR1-* (PVX:RAR1) and *SGT1-* (PVX:SGT1) silenced plants were infiltrated with *R. solanacearum*. (**A**) Disease development of bacterial wilt was rated daily on a 0–4 disease index in control (open squares) or silenced (solid squares) plants. Asterisks denote values significantly different from those of control plants (*; $P < 0.05$, *t*-test). (**B**) Characteristic symptoms in control and silenced plants. Photograph was taken 12 d after inoculation with *R. solanacearum*.

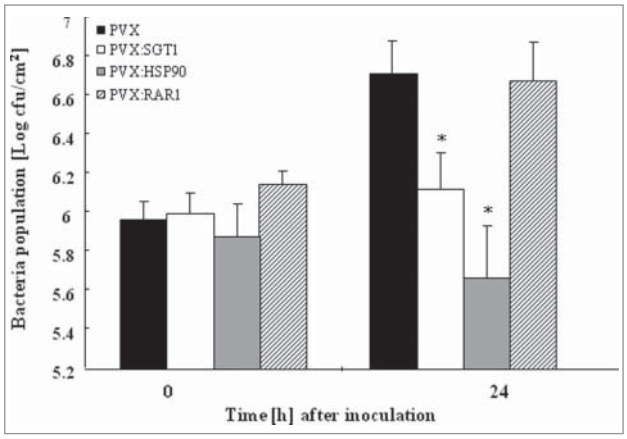

Figure 3. Growth of Ralstonia solanacearum in Hsp90, RAR1 and SGT1-silenced plants Control (PVX), *Hsp90*- (PVX:Hsp90), *RAR1*- (PVX:RAR1) and *SGT1*- (PVX:SGT1) silenced plants were infiltrated with *R. solanacearum* (10^8 CFU/ml). Bacterial population was determined by plating at specified time points. Values are means of 4 replicate experiments with SD. Asterisks denote values significantly different from those of empty PVX controls (*; $P < 0.05$, *t*-test).

silenced plants. To address whether silencing of *Hsp90*, *RAR1* and *SGT1* would affect disease resistance, we estimated multiplication of *R. solanacearum* in *Hsp90*, *RAR1* and *SGT1*-silenced and control plants. As shown in **Figure 3**, growth of *R. solanacearum* was scarcely affected by *RAR1*-silencing. In contrast, growth of *R. solanacearum* was significantly reduced in *Hsp90* and *SGT1*-silenced plants 24h after inoculation in comparison with control plants. Intriguingly, population of type III secretion system (TTSS)-deficient mutant of *R. solanacearum* increased in control plants as well as *Hsp90* and *SGT1*-silenced plants (**Fig. S2**).

We could observe growth inhibition of *R. solanacearum* in *Hsp90* and *SGT1*-silenced plants, suggesting the up-regulation of defense responses in *Hsp90* and *SGT1*-silenced plants. To further determine the role of Hsp90 and SGT1 in defense responses against *R. solanacearum*, we analyzed salicylic acid-dependent *PR-1a* expression and jasmonic acid-dependent *PR-4* expression. In control plants inoculated with *R. solanacearum*, expression of *PR-1a* and *PR-4* increased at 24-72 hours after inoculation. Expression levels of *PR-1a* and *PR-4* transcript were greatly enhanced in *Hsp90*- and *SGT1*-sielnced plants (**Fig. 4**).

Discussion

It has been shown that molecular chaperones act as a critical component of plant defense responses. Hsp90 is reportedly required for HR mediated by the resistant genes, *Pto* and *Rx*.[14] Another Hsp, *Hsp70*, is required not only for induction of HR in response to INF1 elicitin and *Pseudomonas cichorii*, but also basal resistance against *P. syringae*.[15,16] The *RAR1* gene is reportedly required for *N*-mediated HR.[14] *SGT1* is also a critical signaling component required for *R* gene-mediated HR in several plant species against various plant pathogens, including fungi, bacteria and viruses.[17-20] Recent study showed that pepper SGT1 act as a host interactor of AvrBsT during the HR induction.[21] Our present result showed that acceleration of bacterial wilt symptom was observed in *Hsp70*-silenced plant, but bacterial manipulation was not affected (**Fig. S1**). Therefore, Hsp70 might be required for suppression of bacterial wilt disease and/or induction of disease tolerance.

Recent studies also showed that plant molecular chaperons and co-chaperons play important roles not only in disease resistance but also disease development and disease susceptibility. Mutations in 2 Hsp90 genes lead to heightened accumulation of immune receptors, including SNC1, RPS2 and RPS4. Hsp90s, and enhanced disease resistance, suggesting involvement of Hsp90 in the negative regulation of immune receptor accumulation.[22] SGT1 has been shown to be involved in cell death that promotes the pathogenesis of *Botrytis cinerea*[23] and *Fusarium*

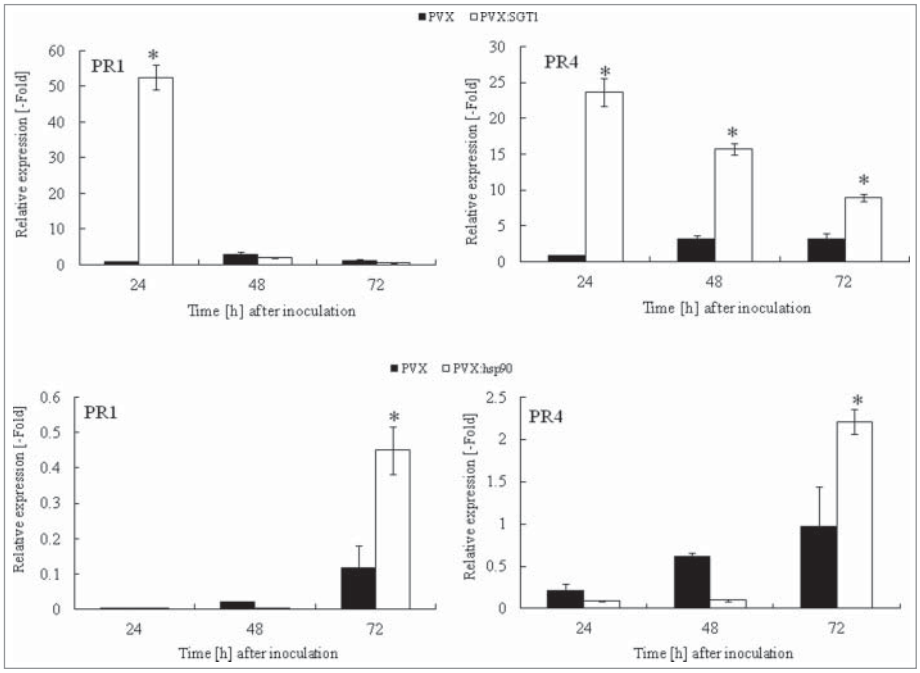

Figure 4. Acceleration of PR genes expression in *Hsp90 and SGT1*-silenced plants in response to *Ralstonia solanacearum* infection. Total RNA was isolated from control (PVX) and *Hsp90* (PVX:Hsp90) and *SGT1* (PVX:SGT1)-silenced plants inoculated with *R. solanacearum* (10^8 CFU/ml). Relative expression of *PR-1a* and *PR-4* transcripts were normalized with actin and calculated as relative to the non-treated control. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*; $P < 0.05$).

culmorum.[24] SGT1 was also reportedly to be required for coronatine-induced chlorosis and cell death during bacterial speck disease on tomato.[25] Development of disease-associated cell death caused by the *P. syringae* pv. *tabaci,* require for SGT1.[26] Our previous report showed Hsp70 is required for tabtoxinine-β-lactum induced cell death and wildfire disease development by *P. syringae* pv *tabaci*.[27] Previous report showed SGT1 tightly interacts with RAR1 is required for early stages of *Agrobacterium*-mediated plant transformation, suggesting that RAR1, along with SGT1 is important for virulence function.[28] Recent reports also suggested that the plant chaperon and co-chaperon act as type III effector targets during establishment of disease susceptibility. The major target of HopI1, a virulence type III effector from *P. syringae*, was reported as plant heat shock protein Hsp70.[16] *P. syringae* effector protein AvrB enhances plant susceptibility by interaction with Hsp90 and/or RAR1.[29,30] *Salmonella typhimurium* type III effector-mediated phenotypes required its catalytic E3 ubiquitin ligase activity and interaction with the conserved host protein SGT1 in both plants and mammals.[31]

Our present data showed the requirement of plant chaperon and co-chaperon in bacterial wilt. Silencing of *RAR1* significantly reduced bacterial wilt symptom, but not affected on bacterial manipulation (**Figs. 2**,**3**). We previously reported that PopA-mutant of *R. solanacearum*, which grew in intercellular spaces and systemically infected into tobacco plants similarly to wild type *R. solanacearum*, did not cause wilt on tobacco plants, suggesting suppression for disease development or induction of disease tolerance.[32] Therefore, RAR1 might be required for disease development or suppression of disease tolerance. In contrast, hyper-induction of defense related *PR*-gene expression and suppression of *R. solanacearum* growth were observed in *Hsp90* and *SGT1*-silenced plants (**Figs. 2–4**). Intriguingly, silencing of *Hsp90* and *SGT1* did not affected on multiplication of TTSS-mutant of *R. solanacearum* (**Fig. S2**). These results suggested that Hsp90 and SGT1 might be type III effector targets for during the pathogenesis of *R. solanacearum*, and play an important role in suppression of plant defenses leading to disease susceptibility.

In conclusion we found out that in addition to positive regulation of plant immune responses by SGT1, RAR1 and Hsp90, they are also involved in the negative regulation of immune responses. Further research about plant intracellular proteins related to SGT1, RAR1 and Hsp90, and cognate bacterial effectors of Hsp90 and SGT1 will be required to clarify molecular mechanisms of plants-*R. solanacearum* interactions.

Materials and Methods

Plant materials, bacterial isolates and chemicals

Nicotiana benthamiana was grown in a growth room.[13] *Ralstonia solanacearum* strain OE1–1 and the corresponding *hrpY* (encoding Hrp pilus)-mutant of *R. solanacearum* strain OE1–1 was cultured in PY medium containing 20 μg/mL rifampicin or 20 μg/mL kanamycin, respectively.[12] Bacterial suspension were infiltrated into *N. benthamiana* leaves as described previously.[13]

Bacterial Population and Disease Index

The population of *R. solanacearum* and TTSS-deficient mutant of *R. solanacearum* was determined by plating on Hara-Ono plates. Plants inoculated with *R. solanacearum* were coded and inspected daily for wilting symptoms for 20 d For each plant, a disease index on a scale of 0 to 4 was calculated as described elsewhere.[13]

Isolation of RNA and cDNA synthesis

Total RNA was isolated from *N. benthamiana* leaves with RNAiso (Takara), and RNA samples were treated with DNase I (RNase-free; Takara) to degrade contaminating genomic DNA as described previously.[13] cDNA (cDNA) was synthesized by PrimeScript RT reagent Kit (Takara).

Vector constructs and seedling infection for virus-induced gene silencing

Construction of virus vectors for VIGS experiments were described previously.[27] The VIGS experiment was carried out with *Agrobacterium tumefaciens* strain GV3101[33] and inoculated into *N. benthamiana* leaves as described previously.[3] We observed characteristic dwarf phenotypes of *SGT1*-, *Hsp70*- and *Hsp90*-silenced plants as shown previously.[27]

Quantitative real time PCR

Quantitative real time PCR was carried out according to the method of Maimbo et al.[13] Reverse transcription was carried out with 1 μg total RNA using the PrimeScript RT reagent Kit (Takara). Quantitative reverse-transcription PCR (qRT-PCR) was carried out with a 20 μL reaction mixture containing 1 μL of the cDNA stock and 0.4 μL of the respective primers (10 pM; Supplemental **Table 1**), using the SYBR GreenER qPCR Reagent System (Invitrogen, Tokyo Japan), with an Applied Biosystems 7300 real time PCR system. Standard deviations and differences between expression ratios of non-treated controls and other samples were tested for statistical significance using the t-test.

Control (PVX) and *Hsp70* (PVX:Hsp70)-silenced plants were infiltrated with *R. solanacearum* (10^8 CFU/ml). (*A*) Total RNA was isolated from Control (PVX) and *Hsp70*-(PVX:Hsp70) silenced plants. Expression values of *Hsp70* was estimated by qRT-PCR, and expressed as [Qty] after normalization with actin. Values represent the means and SD from triplicate experiments. Asterisks denote values significantly different from empty PVX controls (*; $P < 0.05$). (*B*) Disease development of bacterial wilt was rated daily on a 0–4 disease index in control or silenced plants. Asterisks denote values significantly different from those of control plants (*; $P < 0.05$, *t*-test). (*C*) Characteristic symptoms in control and silenced plants. Photograph was taken 8 d after inoculation with *R. solanacearum*. (*D*) Bacterial population was determined by plating at specified time points. Values are

means of 4 replicate experiments with SD. Asterisks denote values significantly different from those of empty PVX controls (*; $P < 0.05$, *t*-test).

Control, *Hsp90* (PVX:Hsp90)- and *SGT1* (PVX:SGT1)-silenced plant were inoculated with type III secretion system-deficient *R. solanacearum* (10^8 CFU/ml). Bacterial population was determined by plating at specified time points. Values are means of 4 replicate experiments with SD. Asterisks denote values significantly different from those of empty PVX controls.

Table S1 List of primers used for qRT-PCR in this study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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