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# SGT1 interacts with the Prf resistance protein and is required for Prf accumulation and *Prf*-mediated defense signaling

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ABSTRACT

The highly conserved eukaryotic co-chaperone SGT1 (suppressor of the G2 allele of *skp*1) is an important signaling component of plant defense responses and positively regulates disease resistance conferred by many resistance (R) proteins. In this study, we investigated the contribution of SGT1 in the *Prf*-mediated defense responses in both *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*). SGT1 was demonstrated to interact with Prf in plant cells by co-immunoprecipitation. The requirement of SGT1 in the accumulation of Prf or autoactive $Prf^{D1416V}$ was determined by the degradation of these proteins in *N. benthamiana*, in which SGT1 was repressed by virus-induced gene silencing (VIGS). *Pseudomonas* pathogen assay on the SGT1-silenced tomato plants implicates SGT1 is required for the *Prf*-mediated full resistance to *Pseudomonas syringae* pv. tomato (*Pst*). These results suggest that, in both *N. benthamiana* and tomato, SGT1 contributes to the *Prf*-mediated defense responses by stabilizing Prf protein via its co-chaperone activity.



## 1. Introduction

In tomato (*Solanum lycopersicum*), the resistance to the bacterial speck disease caused by *Pseudomonas syringae* pv *tomato* (*Pst*) is determined by the resistance gene Prf [1]. The *Prf*-mediated resistance is initiated by recognition of *Pst*-secreted effectors AvrPto or AvrPtoB by the Prf recognition partner Pto kinase [2–4], which is detected by Prf and consequently activates the defense signaling [5]. Prf belongs to the largest family of plant R proteins that contain a central nucleotide binding (NB) domain and C-terminal leucine rich repeat (LRR) domain [1]. It is generally thought that the NB domain serves as a molecular switch for the activation of R proteins and the LRR domain is responsible for effector recognition or/and signal transduction [6,7]. Consistent with this notion, we have recently found that the D1416V mutation in an I–H–D sequence of the Prf NB domain activates defense signaling in the absence of pathogen, and the LRR domain has a role in defense signal transduction [8]. Significantly, overexpression of the unattached LRR domain can suppress the HR cell death caused by the autoactive $Prf^{D1416V}$ mutant by apparently triggering the degradation of $Prf^{D1416V}$ protein. Thus, it is reasonable to speculate that LRR may be important for maintaining Prf in a proper conformation via interaction with other factor(s) (such as chaperones), and overexpression of unattached LRR can titer out the interaction of intact Prf protein with the factor(s), thereby leading to the destabilization of Prf.

The highly conserved eukaryotic protein SGT1 (suppressor of the G2 allele of *skp*1) was originally identified in yeast as a high copy suppressor of *skp1–4*, a mutation causing defects in kinetochore function [9]. SGT1 associates with the heat shock protein 90 (HSP90) chaperone to facilitate client protein assembly and maturation [10], meaning SGT1 is generally required for the accumulation of its client proteins. In plants, SGT1 and another co-chaperone RAR1 (required for Mla12 resistance) interact with HSP90 to form a chaperone complex that positively regulates disease resistance signaling conferred by many R proteins [11]. A model for the ATP-dependent NB–LRR protein maturation by the HSP90–SGT1–RAR1 complex has been proposed by Kadota and Shirasu: RAR1 first binds to the HSP90 dimer to form an open conformation that facilitates assembly of SGT1 through association with RAR1. SGT1 finally recruits client NB–LRR protein to form a HSP90–RAR1–SGT1–NB–LRR intermediate complex, followed by dissociation of this complex and release of the mature NB–LRR protein [11]. Consistent with this model, it has been demonstrated that SGT1 is required for the accumulation of several NB–LRR proteins, including Rx [12], I2 [13], Mi [13], N [14] and Rsv [15], despite that none of them has been shown to interact with SGT1 *in vivo*.

*Nicotiana benthamiana*, a close relative of tomato in the Solanaceae family, contains a functional *Prf*-mediated defense signal pathway and has been widely used as a model plant to study the

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*Prf*-mediated defense signaling. Transient co-expression of Pto with AvrPto or AvrPtoB [2,3], or expression of the Pto autoactive mutant $Pto^{Y207D}$ [16], trigger the HR-associated cell death on *N. benthamiana* leaves. Significantly, the cell death is dependent on the endogenous *N. benthamiana* Prf gene *NbPrf*, since silencing of *NbPrf* completely compromises the cell death signaling [16–18]. It has been demonstrated that SGT1 is required for the *Prf*-mediated defense signaling in *N. benthamiana*, as manifested by the abolishment of the AvrPto/Pto-triggered cell death when SGT1 is silenced in *N. benthamiana* [19]. However, the mechanistic basis of how SGT1 contributes to the *Prf*-mediated defense signaling and whether SGT1 is required for resistance to *Pseudomonas* pathogen in tomato remains unknown. In this study, we show SGT1 is required for the accumulation of Prf and interacts with Prf through the LRR domain and plays a role in *Prf*-mediated disease resistance to *Pst* in tomato.

## 2. Materials and methods

### 2.1. Plasmid construction

Assembly of C-terminal HA-tagged and FLAG-tagged constructs used for transient expression in *N. benthamiana* leaves were made through the process of PCR amplification of SGT1 cDNAs (*S. lycopersicum* or *N. benthamiana*) and ligation into the *Kpn*I–*Stu*I and *Kpn*I–*Sal*I sites of pBTEX, respectively. The resulting constructs would express fusion proteins with a C-terminal HA tag under the control of the CaMV 35S promoter. All constructs were confirmed by DNA sequencing. Primers used in the cloning process are listed in Supplementary Table S1.

### 2.2. Agrobacterium-mediated transient expression in N. benthamiana and co-immunoprecipitation

*Agrobacterium*-mediated transient assay was conducted according to previous publication by Sessa and colleagues [20]. *N. benthamiana* leaf tissues (2 $cm^2$ disc) were collected at 48 h after *Agrobacterium* infiltration and ground with liquid nitrogen and then resuspended with 300 μl of extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 1% polyvinylpolypyrrolidone, plant protease inhibitor cocktail (Sigma–Aldrich, St. Louis, Missouri)). After centrifugation at 15,000g/4 °C for 10 min, 40 μl of the supernatant was subjected to standard Western blotting using α-HA antibody or α-FLAG antibody (Sigma–Aldrich, St. Louis, USA). To determine the protein–protein interaction by Co-IP assay, 1 g leaf tissue was ground with liquid nitrogen, suspended in 1.5 ml extraction buffer and centrifuged at 15,000g/4 °C for 10 min to get rid of the cell debris. One tenth of supernatant (V/V) was saved as the input material. 10 μl of α-FLAG agarose beads (Sigma–Aldrich, St. Louis, USA) or 15 μl of α-HA agarose beads (Roche, USA) were added to the remaining supernatant and incubated at 4 °C for 2 h. The agarose beads harboring the immunocomplex were washed four times with the wash buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA) and then resuspended with 5× SDS–PAGE loading buffer to release the immunocomplex, followed by Western blotting using the alternate antibody (α-FLAG or α-HA) as the primary antibody.

### 2.3. Virus-induced gene silencing (VIGS)

The TRV (*Tobacco rattle virus)* vector system was used for gene silencing in both *N. benthamiana* and tomato plants as described previously [21,22]. Acetosyringone-induced *Agrobacterium* cultures containing TRV constructs *SlSGT1–2* [22] or *Nb*SGT1 [19] were used for the inoculation of 2-week–old *N. benthamiana* or tomato seedlings. *SlSGT1-2*-silenced tomato plants were used for the *Pseudomonas* inoculation 4 weeks after *Agrobacterium* inoculation.

### 2.4. Pseudomonas inoculation into plants

The *P. syringae* pv tomato *DC3000* was prepared as described previously [23]. Bacteria were collected and suspended in 10 mM $MgCl_2$ containing 0.05% Silwet-77 at a final concentration of $2 \times 10^4$ cfu/ml. The *Pst DC3000* bacterial suspension was then used to vacuum infiltrate the *SlSGT1-2*-silenced RG-*PtoR* tomato plants and fully expanded leaves were selected for disease assays 4 days later. Three leaf discs (1 $cm^2$) were collected, homogenized and serially diluted in 10 mM $MgCl_2$ to measure bacterial populations *in planta.*

## 3. Results

### 3.1. SGT1 is required for the accumulation of both steady-state and active-state Prf protein and the Prf-mediated cell death signaling in N. benthamiana

Previous studies have demonstrated that SGT1 is required for the *Prf*-mediated defense signaling in *N. benthamiana*. For example, silencing of SGT1 in *N. benthamiana* abolishes cell death caused by the co-expression of Pto and AvrPto [19]. The current model proposed by Kadota and Shirasu emphasizes that SGT1, together with HSP90 and RAR1, functions as a chaperone complex to maintain the NB–LRR type R proteins in a stable steady-state [11]. We asked whether SGT1 is also required for the accumulation of and cell death by the active form of R protein. If SGT1 is only required for the stabilization of the pre-activation state of R protein, depletion of SGT1 may not affect cell death signaling caused by the autoactive R mutant, such as $Prf^{D1416V}$ [8]. To answer this question, we assessed the cell death triggered by the hemagglutinin (HA) epitope-tagged autoactive $Prf^{D1416V}$ mutant in the SGT1-silenced *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression. As shown in Fig. 1(A), the $Prf^{D1416V}$ mutant was not able to trigger cell death on *SGT1*-silenced *N. benthamiana* leaves (TRV2::*Nb*SGT1), whereas cell death was observed on the control non-silenced *N. benthamiana* leaves (TRV2::00). To further determine whether SGT1 contributes to the accumulation of $Prf^{D1416V}$ or plays a role at a further downstream point in the cell death signaling pathway, we monitored the protein level of $Prf^{D1416V}$-HA by Western blotting analysis using anti-HA antibody. $Prf^{D1416V}$-HA protein was detected in the control *N. benthamiana* leaves but not in the *Nb*SGT1-silenced *N. benthamiana* leaves, suggesting SGT1 contributes to the cell death signaling via stabilization of $Prf^{D1416V}$-HA rather than other activity further downstream in the signaling pathway. Moreover, we tested the requirement of SGT1 for the wild type (WT) Prf accumulation. As expected, the silencing of SGT1 also destabilized the WT Prf protein (Fig. 1B). Thus, we conclude that SGT1 is required for the stabilization of both pre-activation and active Prf in plant cells, thereby it is essential for the *Prf*-mediated cell death signaling.

### 3.2. NbSGT1 interacts with Prf in plant cells

The fact that silencing of SGT1 results in the degradation of Prf in *N. benthamiana*, together with previous results showing that SGT1 interacts with some of the R proteins *in planta*, prompted us to determine whether Prf interacts with SGT1 *in vivo*. To this end, we co-expressed the FLAG-tagged Prf (Prf-FLAG) with the HA-tagged *Nb*SGT1 (*Nb*SGT1-HA) or empty vector in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression. The association of Prf-FLAG with *Nb*SGT1-HA was examined by

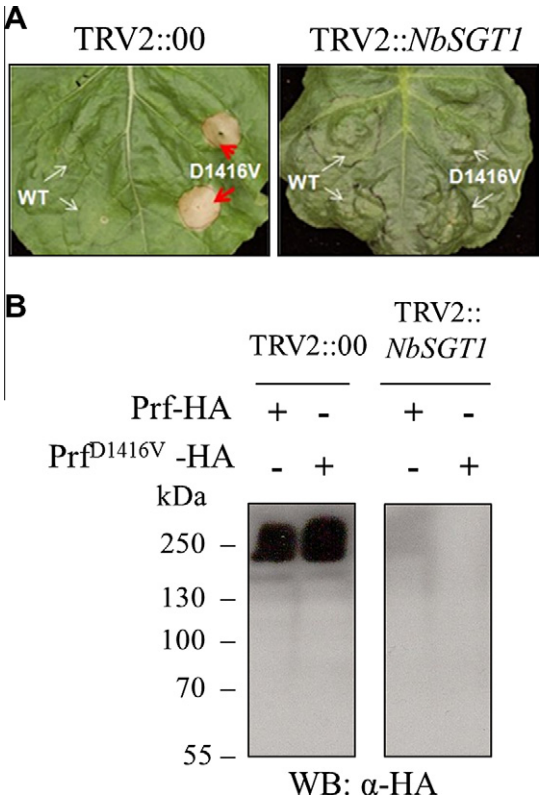


**Fig. 1.** SGT1 is required for the accumulation of Prf protein and the *Prf*-mediated cell death signaling in *N. benthamiana*. *A. tumefaciens* GV2260 strains harboring the CaMV 35S promoter-driven wild type (WT) Prf-HA or autoactive $Prf^{D1416V}$-HA constructs were syringe-infiltrated into the SGT1-silenced (TRV2::*NbSGT1*) or control (TRV2::00) *N. benthamiana* leaves at a concentration of $OD_{600}$ = 0.4. (A) $Prf^{D1416V}$ elicited cell death on the control, but not the SGT1-silenced, *N. benthamiana* leaves. Photographs were taken 7 days after *Agrobacterium* injections. (B) Western blotting analysis indicated the accumulation of Prf-HA and $Prf^{D1416V}$-HA in the control, but not the SGT1-silenced N. *benthamiana* leaves. Leaf tissues were collected 2 days after *Agrobacterium* infiltration. Proteins were extracted and separated by SDS–PAGE, followed by standard Western blotting using anti-HA antibody.

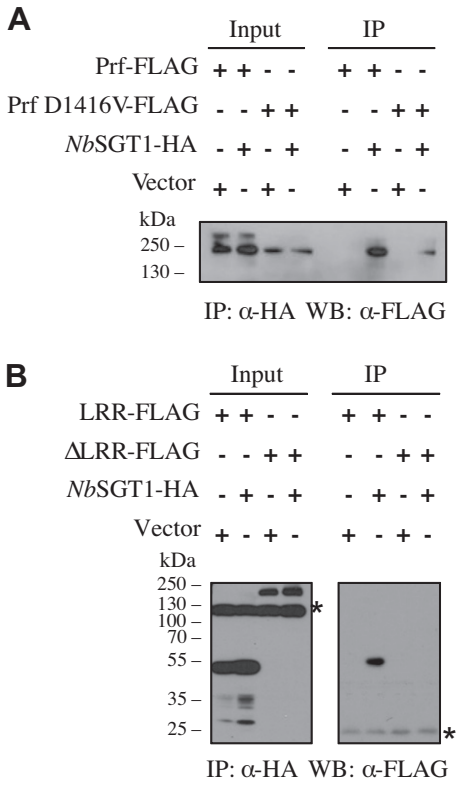


**Fig. 2.** Prf interacts with *Nb*SGT1 through the LRR domain in plant cells. *A. tumefaciens* GV2260 strains containing the CaMV 35S promoter-driven epitope-tagged Prf constructs (Prf-FLAG, $Prf^{D1416V}$-FLAG, LRR-FLAG, or ΔLRR-FLAG) or *Nb*SGT1 (*Nb*SGT1-HA) were syringe-infiltrated into *N. benthamiana* leaves at a concentration of $OD_{600}$ = 0.8, 0.8, 0.3, 0.8 and 0.2, respectively. The *A. tumefaciens* GV2260 strain containing the empty vector was used as a negative control. Two days after *Agrobacterium* infiltration, proteins were extracted for immunoprecipitation with anti-HA affinity matrix, followed by Western blotting using the anti-FLAG antibody to determine the association of full length Prf, LRR or ΔLRR with *Nb*SGT1. The asterisk indicates a non-specific cross-reaction band detected by antibody. (A) Prf and $Prf^{D1416V}$ interacts with *Nb*SGT1. (B) *Nb*SGT1 specifically interacts the LRR domain of Prf.

co-immunoprecipitation (co-IP) assay. After immunoprecipitation with anti-HA antibody matrix (Roche, USA), the immunoprecipitated complex was verified by Western blotting using anti-FLAG antibody. As shown in Fig. 2, the Prf-FLAG protein was detected by anti-FLAG antibody in the anti-HA antibody-immunoprecipitated complex from tissue with co-expression of Prf-FLAG and *Nb*SGT1-HA, but not in the immunoprecipitated complex from tissue with co-expression of Prf-FLAG and vector, suggesting that Prf interacts with SGT1 *in vivo*. Moreover, we determined whether the autoactive $Prf^{D1416V}$ mutant also associates with SGT1 *in vivo* by co-IP assay and the result indicated that $Prf^{D1416V}$ interacts with SGT1. This result suggests that the active form of Prf still requires SGT1 to remain in a stable state (note that due to non-specific protein degradation caused by the $Prf^{D1416V}$-triggered cell death, the accumulation of $Prf^{D1416V}$ protein was much less than that of Prf).

The next outstanding question was which domain of the Prf protein is responsible for the interaction with SGT1. We first targeted the LRR domain since its slender and arc-shaped structure provides an ideal protein–protein interaction platform [24]. Co-IP analysis indicated that the LRR-FLAG was able to co-immunoprecipitate with *Nb*SGT1-HA after *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves (Fig. 2B). We further tested whether the LRR is the only domain of Prf involved in interaction with SGT1. Similar co-IP assay was carried out using the Prf truncation mutant with the LRR being removed (ΔLRR) and the result indicated that there is no interaction between SGT1 and ΔLRR (Fig. 2B). Thus, we conclude that Prf interacts with SGT1 through the LRR domain.

### 3.3. Prf interacts with two tomato paralogs SlSGT1-1 and SlSGT1-2

Tomato contains two SGT1 paralogs, *SlSGT*1-1 and *SlSGT*1-2, which share 89.73% and 96.76% identity respectively with *Nb*SGT1 at the amino acid level [22]. We next determined the possible interaction of the Prf and *SlSGT*1-1/1-2. To this end, Prf-FLAG was co-expressed with *SlSGT*1-1-HA or *SlSGT*1-2-HA in *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression, and the *in vivo* association of Prf-FLAG with *SlSGT*1-1-HA or *SlSGT*1-2-HA was examined by co-IP assay, with GFP-HA serving as a negative control. As shown in Fig. 3, both *SlSGT*1-1-HA and *SlSGT*1-2-HA were co-immunoprecipitated with Prf-HA but not the GFP-HA control, indicating that *SlSGT*1-1 and *SlSGT*1-2 interact with Prf in plant cells.

### 3.4. SlSGT1-2 is required for the full resistance to Pst in tomato

We next sought to investigate whether *SlSGT1* is required for the *Prf*-mediated resistance to *Pst* in tomato plants. Taking advantage of virus-induced gene silencing (VIGS), we intended to silence the *SlSGT1-1* or *SlSGT1-2* in resistant RG-*PtoR* tomato seedlings and

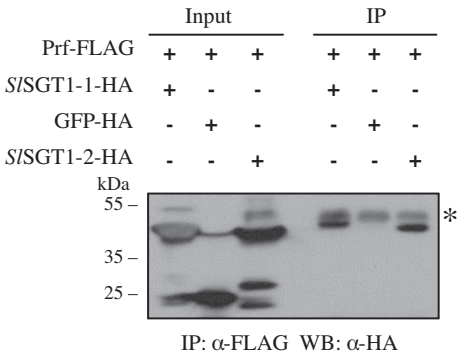


**Fig. 3.** Two tomato SGT1 paralogs, *SlSGT*1-1 and *SlSGT*1-2, interact with Prf *in planta*. The FLAG-tagged Prf protein was transiently co-expressed with HA-tagged *SlSGT*1-1 or *SlSGT*1-2 in *N. benthamiana* leaves, with the HA-tagged GFP serving as a negative control. Proteins were extracted 2 days after Agrobacterium infiltration and subjected to immunoprecipitation with α-FLAG agarose beads. The immunoprecipitated complex was separated by SDS–PAGE, The presence of *SlSGT*1-1 and *SlSGT*1-2 in the immunoprecipited complex was then determined by Western blotting using α-HA antibody. The asterisk indicates a non-specific cross-reaction band detected by the antibody.

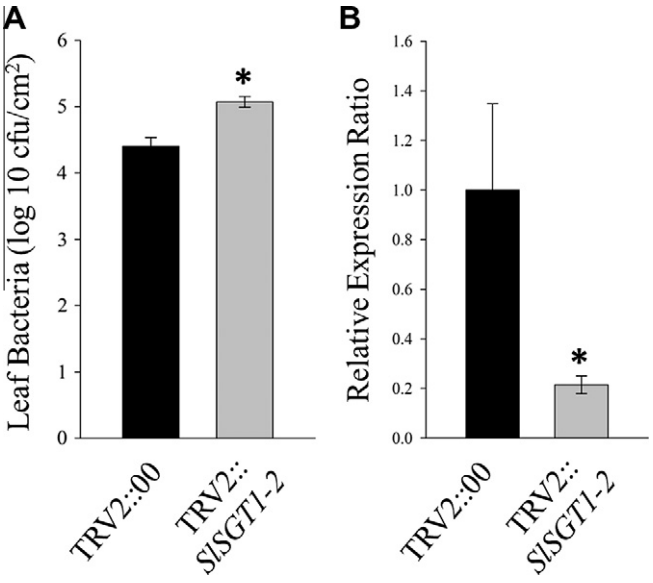


**Fig. 4.** *SlSGT1-2* is required for the *Prf*-mediated full resistance to *Pst* DC3000. Resistant tomato RT-*PtoR* (expressing Prf gene) plant seedlings were silenced by VIGS using the TRV2::*SlSGT1-2* silencing construct [22] or TRV2 vector only (TRV2::00). 4 weeks after VIGS, plants were vacuum infiltrated with *Pst* DC3000 (expressing AvrPto and AvrPtoB) at a concentration of $2 \times 10^4$ cfu/ml. Bacterial numbers were examined by plating serial dilutions of leaf extracts 4 days after infiltration. (A) The bacterial populations in the *SlSGT1-2*-silenced (TRV2::*SlSGT1-2*) or control (TRV2::00) tomato plants. Error bars represent the standard deviation of three replicates. The asterisk indicates a significant difference at $p < 0.005$ using Student's *t*-test. (B) The suppression of *SlSGT1-2* in the *SlSGT1-2*-silenced tomato plants was verified by real-time RT-PCR. The relative quantification of PCR products was calculated by the comparative CT method (ΔΔCT) using the tomato *actin* gene as an internal control for equal cDNA amounts.

assess the possible altered resistance to *Pst*DC3000 at 3–4 weeks after VIGS. Silencing of *SlSGT1-1* resulted in lethality and all VIGS-treated tomato seedlings died 7–10 days after VIGS treatment, whereas silencing of *SlSGT1-2* did not cause any morphological alternation (data not shown). These different phenotypic features caused by silencing of *SlSGT1-1* or *SlSGT1-2* in RG-*PtoR* were consistent with the previous observations from different tomato cultivars [22]. Two to three weeks after VIGS treatment, *SlSGT1-2*-silenced tomato plants were challenged with *Pst* DC3000 and the altered resistance was assessed by speck disease symptoms and bacterial populations at 4 days post-inoculation (dpi). Despite that no speck disease symptoms were observed in plants inoculated with *Pst*DC3000 (data not shown), there was a 5 to 10-fold increase in the population of *Pst* DC3000 in *SlSGT1-2*-silenced plants compared to the non-silenced control plants (Fig. 4A), indicating that resistance to *Pst* DC3000 in *SlSGT1-2*-silenced plants was partially compromised. Real-time RT-PCR analysis indicated the *SlSGT1-2* gene was significantly repressed (Fig. 4B). Thus, we conclude that *SlSGT1-2* is required for the *Prf*-mediated full resistance to *Pst* DC3000.

## 4. Discussion

Since SGT1 is a co-chaperone for proper protein folding and assembly, it is generally thought that SGT1 contributes to plant defense signaling through the HSP90–SGT1–RAR1 chaperone complex-mediated R protein maturation [11]. In the absence of functional HSP90–SGT1–RAR1 chaperone complex, e.g. in the case of genetic impairing of SGT1, the R protein may not fold correctly, thereby resulting in degradation. In fact, among the twelve R proteins that require SGT1 for their function, four (Rx, I2, N and Mi) need SGT1 for their accumulation in plant cells [12,13]. Here we add Prf to the list of R proteins that require SGT1 for their functionality and stability, and, significantly, Prf is the only one in this group whose interaction with SGT1 has been determined. Moreover, we have demonstrated that SGT1 is required for the accumulation of the autoactive $Prf^{D1416V}$ mutant and $Prf^{D1416V}$-triggered cell death in *N. benthamiana*, suggesting that SGT1 is required for maintaining the stability of not only the steady-state of the pre-activated signaling-prone R proteins, but also signaling-active R proteins after activation by the recognition of pathogen effectors.

Our results can also explain why silencing of SGT1 in *N. benthamina* resulted in the abolishment of the *Prf*-mediated defense signaling and resistance to *Pseudomonas* bacteria, including the compromise of the HR cell death triggered by co-expression of AvrPto and Pto in the wild type *N. benthamiana* and attenuation of disease resistance to *P. syringae* pv. *tabaci* (AvrPto) in transgenic *N. benthamiana* expressing tomato *Pto* gene [19]. We speculate this is because the *N. benthamiana* endogenous Prf (*NbPrf*) protein is likely destroyed when SGT1 is silenced in *N. benthamiana*, thereby resulting in the interruption of Pto/AvrPto-trigged *Prf*-dependent defense signaling and disease resistance. Further experiments such as Western blotting analysis using the *NbPrf*-specific antibody will help to determine the disruption of *NbPrf* in the SGT1-silenced *N. benthamiana*.

The current model for the maturation of R protein by the HSP90–SGT1–RAR1 chaperone suggests that R protein is recruited to the chaperone complex through interaction with SGT1 [11]. So far, two R proteins have been shown to interact with SGT1 in yeast or plant cells. Significantly, it has been demonstrated that the LRR domain of Bs2 and Mla1 mediates the interaction with SGT1 [25,26]. The LRR domain is rich in hydrophobic leucine/isoleucine residues which are buried inside the hydrophobic core. It is likely that SGT1 bridges LRR-containing protein to the chaperone complex during maturation and/or activates events connected with Prf conformational changes upon pathogen recognition. Thus, it is possible that SGT1 may play an important role in Prf proper folding by delivering Prf to HSP90 and/or is involved in the assembly of the Prf-containing protein complex. We have recently found that over-expression of the unattached Prf LRR domain can suppress the *Prf*-mediated HR cell death signaling in *N. benthamiana* by triggering degradation of Prf protein [8]. The finding that Prf interacts with the co-chaperone SGT1 through its LRR domain (Fig. 2) would explain this phenomena. We hypothesize that in the normal condition, Prf is folded in a stable conformation by the HSP90–SGT1–RAR1 chaperone complex, relying on the SGT1-interaction through

its LRR domain. When the unattached LRR domain is over-expressed, it competitively binds to the chaperoning complex via SGT1 and titrates out the intact Prf so that it can no longer bind these chaperones, consequently resulting in the destruction of Prf and abolishment of the *Prf*-mediated defense signaling.

Given the fact that the HSP90–SGT1–RAR1 chaperone complex is essential for the correct folding of many client proteins, especially those playing important roles in various physiological processes, it is no surprise to see silencing of the SGT1 gene results in a significant effect on plant growth in *N. benthamiana* [19] or even lethality in the early development in tomato [22]. Tomato has two SGT1 paralogs, *SlSGT*1-1 and *SlSGT*1-2, which apparently play distinct and/or additive roles in plant defense and development. We found that silencing of *SlSGT1-2* in tomato RG-*PtoR* cultivar attenuates disease resistance to *Pst* DC3000, whereas silencing of *SlSGT1-1* results in seedling lethality, which is consistent with the results of silencing of *SlSGT1-1* in tomato Money-Maker cultivar [22]. We speculate that *Sl*SGT1-1 is also required for the *Prf*-mediated disease resistance since *SlSGT*1-1 and Prf interact *in vivo*. It is worthy to note that silencing of *SlSGT1-2* did not attenuate the Mi-mediated resistance, whereas conditional silencing of both *SlSGT1-1* and *SlSGT1-2* in tomato cultivar Motelle resulted in abolishment of the *Mi*-mediated resistance [22], again suggesting *SlSGT*1-1 and *SlSGT*1-2 play an additive role in the R-mediated disease resistance.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.028.

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