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SGT1 contributes to maintaining protein levels of MEK2^{DD} to facilitate hypersensitive response-like cell death in *Nicotiana benthamiana*

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ABSTRACT

Gene silencing revealed that the mitogen-activated protein kinase (MAPK) cascade in *Solanaceae* consisted with MEK2-WIPK/SIPK, is required for R protein-induced hypersensitive response (HR) cell death and/or resistance. Overexpression of MEK2^{DD} results in HR-like cell death. MEK2^{DD} is a phospho-mimic and constitutive active form harboring mutations at putative phosphorylation sites of upstream MAPKKK. The molecular mechanism that induces HR-like cell death is unknown. Here we report SGT1 is required for the accumulation of MEK2^{DD} protein, not MEK2^{WT}. Virus-induced gene silencing of *SGT1* resulted in low protein accumulation of MEK2^{DD}. This result suggests that SGT1 has a positive role in the accumulation of the MEK2 active form protein to facilitate signal transduction.

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1. Introduction

Plants have a layered surveillance system to detect and eliminate pathogenic microbes. Firstly, plasma membrane pattern recognition receptors (PRRs) recognize microbe-associated molecular patterns (MAMPs) that commonly exist in bacteria, fungi, oomycetes, and other pathogens. The archetypal PRRs are often encoded by receptor-like kinases with different extracellular domains [1]. Upon MAMPs recognition, PRRs are activated and induce pattern-triggered immunity (PTI), accompanied by mitogen-activated protein (MAP) kinase activation, transient production of reactive oxygen species (ROS), activation of plasma membrane ion channels, expression of defense-related genes, callose deposition, reinforcement of cell walls, and phytoalexin biosynthesis [2,3]. The second layer of the innate immune system is initiated upon pathogen effector recognition by host cells, resulting in effector-triggered immunity (ETI). Pathogen effectors secreted into host cells are specifically recognized by corresponding resistance (R) proteins. This causes infected cells to elicit a hypersensitive response (HR) that involves rapid cell death in accordance with ROS production and prolonged MAP kinase activation. Once the HR occurs, salicylic acid (SA) is produced in the tissue surrounding the HR site, resulting in systemic acquired resistance [4].

The MAP kinase (MAPK) pathway is a universal module for signal transduction in response to extracellular stimuli, not only in plants but also in eukaryotes. The MAPK pathway plays a crucial role in biotic and abiotic stress responses, hormone responses, and growth and development in plants. The MAPK pathway comprises MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Stimulated MAPKKK activates MAPKK through phosphorylation of serine or threonine residues at the P-loop region in the protein kinase domain. A MAPKK in turn activates MAPK by phosphorylation of the TXY motif at the same region. An activated MAPK phosphorylates many kinds of substrates, such as transcription factors, metabolic enzymes, and structural proteins, and results in changes in gene expression and cellular responses [5].

Plant MAPK pathways have a crucial role in innate immunity signaling during PTI and ETI. Loss-of-function analyses revealed that a specific *Nicotiana* MAPK pathway, MEK2-wound-induced protein kinase (WIPK) and SA-induced protein kinase (SIPK), is involved in HR cell death induced by several R proteins, including heterologous expression of effector (avirulence factor) and R protein combinations [6]. Virus-induced gene silencing (VIGS) of MEK2

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in *Nicotiana benthamiana* suppressed HR cell death triggered by Pto, NRC1, N, RPS2, RPP13, Rx2, and Gpa2 [6]. These results suggested that these R proteins require the MEK2-WIPK, SIPK pathway to exert HR cell death. In addition, in a gain-of-function analysis, overexpression of constitutively active $MEK2^{DD}$ was consistently able to induce HR-like cell death [7,8]. The $MEK2^{DD}$ contains the "phospho mimic" aspartate amino acid substitution at the conserved serine or threonine residue in the putative phosphorylation sites by MAPKKK. MEK2 belongs to the group C plant MAPKK [9], whose constitutive active form exhibits induction of HR-like cell death, which is a common feature of the group [10].

SGT1, suppressor of the G2 allele of *skp1*, is also commonly required for HR induction by many R proteins. In *N. benthamiana*, VIGS of *SGT1* results in suppression of HR or HR-like cell death by Rx, Pto, Cf-4, Cf-9, RPW8, AvrRpt2, and INF1 [11]. A major molecular function of SGT1 is as a component of a chaperon complex for R proteins. SGT1 binds to RAR1 (required for *Mla12* resistance) and HSP90, and these proteins form a chaperone complex for R proteins [12]. Another molecular function of SGT1 is as a component of the SCF ubiquitin E3 ligase complex [13,14]. SGT1 binds with SKP1 via its N-terminal TPR domain, and possibly contributes to the function of the SCF complex because the *Arabidopsis sgt1b* mutant exhibits an altered SCF^{TIR1}-mediated auxin response [15].

Interestingly, silencing of *SGT1* in *N. benthamiana* suppresses HR-like cell death induced by $MEK2^{DD}$, which is neither R protein nor effector. Although the induction of HR-like cell death by the constitutive active form of the group C MAPKK, to which MEK2 belongs, is commonly observed across phyla, the molecular role of SGT1 in the HR-like cell death induced by $MEK2^{DD}$ is yet to be elucidated. Herein, we show that silencing of *SGT1* and *HSP90*, but not *SKP1*, suppresses $MEK2^{DD}$-induced HR-like cell death. Silencing *HSP90* resulted in reduced levels of the $MEK2^{DD}$ transcript. In contrast, silencing *SGT1* did not affect $MEK2^{DD}$ transcription, and favorably affected protein accumulation of $MEK2^{DD}$, but not $MEK2^{WT}$. These results suggest that SGT1 plays a positive role in the accumulation of the active form of the MEK2 protein.

2. Material and methods

2.1. Biomaterial and growth condition

Nicotiana benthamiana plants were germinated in a 1:1 mixture of compost and peat, and grown in a controlled-environment chamber with 16 h of light and 8 h of dark at 25 °C.

2.2. Virus-induced gene silencing and Agrobacterium-mediated transient expression (Agroinfiltration) in N. benthamiana

Agrobacterium tumefaciens strains GV3101 and C58C1 were used for VIGS or *Agrobacterium*-mediated transient expression (Agroinfiltration). *A. tumefaciens* was cultured in L Broth (1% Bacto Tryptone, 0.5% Yeast extract, 0.5% NaCl, 0.1% D-Glucose) supplemented with appropriate antibiotics at 28 °C. VIGS of *N. benthamiana* was performed using a tobacco rattle virus (TRV) vector as previously described [11]. Three weeks after inoculation, silenced *N. benthamiana* plants were used for the agroinfiltration experiment. For silencing genes involved in immunity signaling, *EDS1* and *RAR1* [16], *SGT1* [11], *HSP90* (10–186hsp) [17], *SIPK* (TRV-SIPK construct was kindly provided by S.C. Peck), and *SKP1* (this study) were used. *N. benthamiana* cDNA was prepared using by the TRIzol reagent (Thermo Fisher Scientific, Grand Island, NY) and the Superscript III (Thermo Fisher Scientific) as described in semi-quantitative RT-PCR (below). PCR primers (5′-ATG AAG ATG ATC GTG CTA AGG AGT TC-3′) and (5′-AGT GAA GTC ATT TTT AAT GTT AAA TGT CTT ACG-3′) to amplify *SKP1* cDNA for silencing were designed based on the methods of Liu et al. [18]. PCR was performed once at 94 °C for 2 min and 35 cycles at (94 °C for 20 s, 55 °C for 20 s, and 72 °C for 1 min). The PCR product of the expected size (421 bp) was purified in agarose gel and subcloned into a pGEM-T Easy vector (Promega Madison, WI). The inserted cDNA was sequenced and confirmed as an objective SKP1 cDNA. The SKP1 cDNA fragment was subcloned into the TRV vector.

Infection of the VIGS silencing vector by agroinfiltration was performed as previously described [11,17]. Agroinfiltration was also used to express INF1 and constitutive active or wild-type forms of *Solanum tuberosum* MEK2, the original name of which was StMEK1 [8], with $A_{600} = 0.2$, unless otherwise stated.

2.3. Total RNA preparation and semiquantitative RT-PCR analysis

Leaf tissues of *N. benthamiana* were frozen in liquid nitrogen and RNA was extracted using the TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. DNase I-treated RNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in H_2O. Reverse transcriptase reactions using Superscript III (Thermo Fisher Scientific) were performed with 1 μg RNA and 0.5 μg of oligo$(dT)_{12\text{-}18}$ primers, according to the manufacturer's instructions. ExTaq polymerase (Takara Bio, Japan) was used for the RT-PCR reaction according to the manufacturer's instructions. The following primers were used for RT-PCR: *Actin* (*Nb* actin F: 5′-ATG GCA GAC GGT GAG GAT ATT CA-3′; *Nb* actin R: 5′-GCC TTT GCA ATC CAC ATC TGT TG-3′), *MEK2* (MEK2 F: 5′-CAT ATC CCT CTC GAA CAA CCT CTC TC-3′; Nos terminator R: 5′-AAG ACC GGC AAC AGG ATT CAA TC-3′), *SKP1* (*Nb* SKP1 RT-PCR Fw: 5′-CGC TTT GTC TCT GTG TTA GGG TTT-3′; *Nb* SKP1 RT-PCR Rv: 5′-AAG AGG GTA CTC TGG TCA ACT TTG-3′), *RAR1* (*Nb* RAR1 F1: 5′-CGC CAG GGA TTC TTT TGT TCT-3′; *Nb* RAR1 R1: TGT CAG CCT GTT AGG ACG CT-3′), and *EDS1* (*Nb* EDS1 F1: 5′-CAA GAG CAC GGT TGT GTC TTC-3′; *Nb* EDS1 R1: 5′- AAC CTA TAA CGC TTC GGC CTA-3′).

2.4. Protein extraction, immunoblot analysis, and in-gel kinase assay

Leaf tissue samples were frozen in liquid nitrogen and proteins were extracted with a buffer containing 100 mM HEPES-KOH pH 7.4, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 5 mM DTT, and a proteinase inhibitor cocktail (Roche, Basel, Switzerland) as previously described [19]. Equal amounts of proteins were separated by SDS-PAGE [20] and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Immunoblot analysis was performed as described previously [19]. HA antibody (3F10, Roche) was diluted to 1/2000 for immunoblot analysis. The *Arabidopsis* MPK6 antibody, which is raised against the C-terminal peptide (KELIYREALAFNPEYQQ, Sawady Technology, Tokyo, Japan) of MPK6 and is also capable of SIPK recognition, was diluted to 1/5000 for immunoblot analysis. In-gel kinase assay was performed as described previously [19].

3. Results

SGT1 is an evolutionarily conserved and multifunctional protein involved in HSP90 co-chaperon function for R protein stability and activity [21]. Not only for R proteins, SGT1 is required for HR-like cell death induced by INF1 [11]. SGT1 has to have a positive role in non-R protein-induced HR-like cell death; however, it is unclear how SGT contributes to the non-canonical pathway leading to HR-like cell death. We therefore asked whether silencing *SGT1* suppressed HR-like cell death induced by $MEK2^{DD}$, a constitutively active form of MEK2. To separate the SCF and co-chaperon function of SGT1, we silenced *SKP1*, *RAR1,* and *HSP90* genes by VIGS in

N. benthamiana. In addition, *EDS1* and *SIPK*, commonly required for TIR-NBS-LRR type R proteins and downstream MAPK of MEK2, respectively, were also silenced. Three weeks after inoculation, MEK2^{DD} or its empty vector were expressed transiently by agro-infiltration with different concentrations and in symmetrical positions in silenced *N. benthamiana* leaves (Fig. 1a). MEK2^{DD}-induced HR-like cell death was clearly observed in the MEK2^{DD}-infiltrated part of the leaf, with agrobacterium concentrations from A_{600} 0.3 to 0.03 (Fig. 1b). Dot-like HR-like cell death was seen in the area of A_{600} 0.01 (Fig. 1b). HR-like cell death induced by MEK2^{DD} was suppressed by silencing *SGT1*, *HSP90,* and *SIPK* genes. In the *SGT1*-silenced plant, partial HR-like cell death was observed in the A_{600}-0.3 infiltration area, and dot-like HR-like cell death was only visible in infiltration areas at A_{600} 0.1 and 0.03. No HR-like cell death was seen in the case of A_{600} 0.01 (Fig. 1c). In the *HSP90* silenced plant, no HR-like cell death was observed at any concentration of agrobacterium (Fig. 1d). In the case of *SIPK* silencing, the results were similar to that of *SGT1* (Fig. 1e). In contrast, HR-like cell death was observed in the leaves of the VIGS vector control, *SKP1*, *RAR1,* and *EDS1*-silenced plants (Fig. 1f, g, h), even though reduced transcript levels of corresponding target genes were detected in those plants (Fig. S1). In the case of *SKP1* silencing, HR-like cell death was even stronger than that of the vector control. These results suggested that the chaperon complex function of SGT1 was probably required for MEK2^{DD}-induced HR-like cell death, and SGT1's interaction function with SKP1 was not likely to play a role in HR-like cell death induction.

3.1. SGT1 and HSP90 silencing results in low protein accumulation of MEK2^{DD}

To gain insight into the HR-like cell death suppression by *SGT1* silencing, we examined MAPK activity using an in-gel kinase assay and myelin basic protein as a substrate. In the empty-vector control for silencing, SIPK and WIPK were activated by transient expression of MEK2^{DD}. In contrast, the activity of MAPKs was significantly reduced in the *SGT1*- and *HSP90*-silenced plants (Fig. 2a). Silencing of *SKP1*, *RAR1,* and *EDS1* did not markedly affect WIPK and SIPK activity (Fig. 2b). Suppression of MAPKs activity may be caused by a reduction of MAPK protein level. Therefore, we examined SIPK protein levels as a representative using an immunoblot analysis and the anti-*Arabidopsis* MPK6 antibody, which can also recognize SIPK

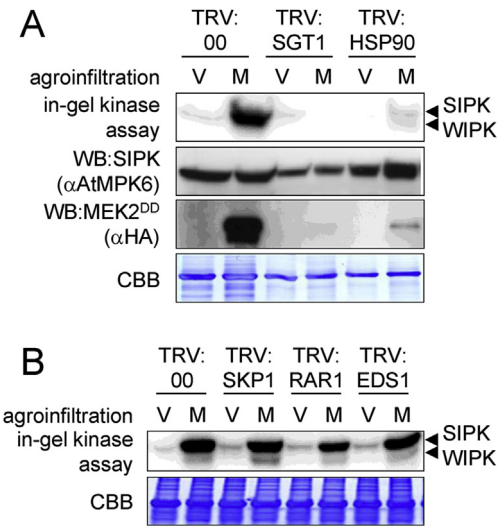

Fig. 2. Reduced WIPK and SIPK activity, as well as low protein accumulation of MEK2^{DD} in *SGT1* and *HSP90* silenced *Nicotiana benthamiana*. The VIGS was performed as described in Peart et al. [11]. Approximately three weeks after virus inoculation, *Agrobacterium* suspensions (A_{600} = 0.2) of either the vector control (V) or MEK2^{DD} (M) were infiltrated into upper leaves. Leaf discs were taken from infiltrated leaf 2 d post-infiltration and subjected to crude protein extraction. (a) Silencing of *SGT1* or *HSP90*. Top panel: autoradiograph of the in-gel kinase assay using crude protein extract prepared from *N. benthamiana* infected with TRV:00 (an empty vector of VIGS), TRV:SGT1, and TRV:HSP90. The second and third panels correspond to western blot analysis of SIPK and MEK2^{DD}, respectively. The bottom panel shows CBB staining to visualize protein loading. (b) Silencing of *SKP1*, *RAR1*, and *EDS1*. Top panel: autoradiograph of the in-gel kinase assay using crude protein extract. The bottom panel shows CBB staining to visualize protein loading.

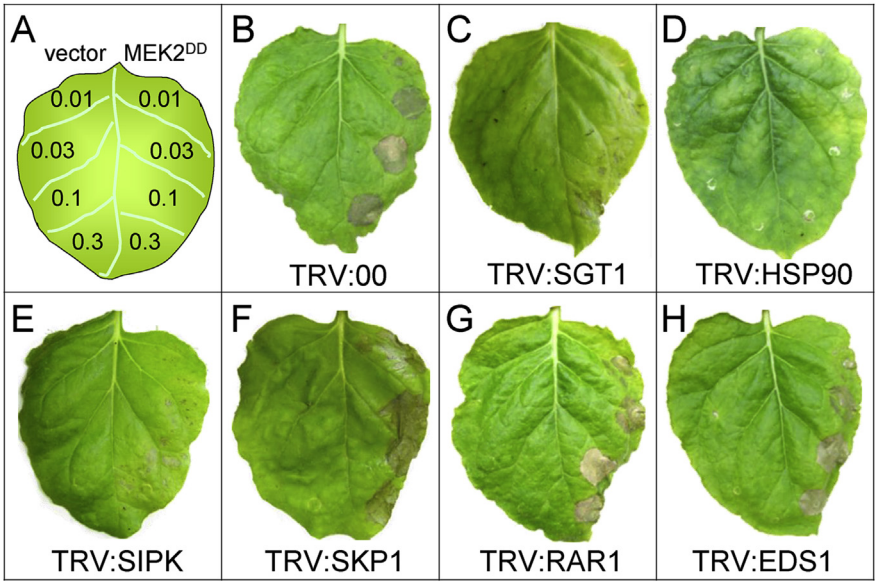

Fig. 1. Silencing of *SGT1*, *HSP90,* or *SIPK* suppresses MEK2^{DD}-induced HR-like cell death. (a) Schematic representation of infiltrated leaf positions and A_{600} for HR-like cell death assay with *Agrobacterium* suspensions of the vector control (left side) and MEK2^{DD} (right side). Using *Nicotiana benthamiana* seedlings, the VIGS was performed as described in Peart et al. [11]. Approximately three weeks after virus inoculation, *Agrobacterium* suspensions with different concentrations of the vector control or MEK2^{DD} were infiltrated into upper leaves. (b) TRV:00, an empty vector for the VIGS, (c) TRV:SGT1, (d) TRV:HSP90, (e) TRV:SIPK, (f) TRV:SKP1, (g) TRV:RAR1, and (h) TRV:EDS1. Photographs were taken 5 days after MEK2^{DD} infiltration.

protein (Fig. S2). However, SIPK protein levels in the control, *SGT1,* and *HSP90* silenced plants were not associated with MAPK activities (Fig. 2a). We then analyzed protein levels of MEK2^{DD} by immunoblotting with the anti-HA antibody because it was tagged with an HA epitope. As a result, MEK2^{DD} protein levels correlated well with MAPK activity (Fig. 2a). These results showed that silencing *SGT1* and *HSP90* resulted in the reduction of WIPK and SIPK activities because of significantly low-level accumulations of MEK2^{DD} protein.

3.2. SGT1 silencing does not affect Agrobacterium-mediated gene expression of MEK2^{DD}

Silencing *SGT1* and *HSP90* exhibited low MEK2^{DD} protein accumulation, resulting in reduced activation of WIPK and SIPK, which lead to HR-like cell death suppression. We next asked whether low MEK2^{DD} protein abundance was likely to be caused by reduced levels of MEK2^{DD} transcripts. We therefore performed a semi-quantitative RT-PCR analysis to determine the MEK2^{DD} mRNA levels in *N. benthamiana* plants with different silencing constructs. We observed that only *HSP90* silencing resulted in almost no transcripts of MEK2^{DD}, whereas transcript levels were comparable to that of the silencing control in all other cases, including *SGT1* (Fig. 3). The *MEK2^{DD}* PCR product in the *HSP90* silenced plant was barely detectable with more PCR cycles, but the PCR product was most likely to have come from coexisting transfer DNA because it was also detected in the sample without reverse transcription (Fig. S3). These results suggested that silencing *HSP90,* but not *SGT1,* affected transcription of MEK2^{DD}. Reduction of MEK2^{DD} protein levels in the *SGT1* silenced plants occurred at the post-transcriptional or posttranslational levels.

3.3. Silencing SGT1 selectively affected accumulation of MEK2^{DD} protein but not MEK2^{WT}

Low protein accumulation of MEK2^{DD} was observed in *SGT1*-silenced plants, although transcription was not affected. MEK2^{DD}, a phospho-mimic form, functions as a constitutive active form when it is expressed in plant cells. To determine whether differences between MEK2^{WT} and MEK2^{DD} corresponded to the inactive and constitutive active from, respectively, we transiently expressed both and analyzed their protein levels in the *SGT1* and *HSP90* silenced plants. Interestingly, protein levels of MEK2^{WT} were comparable in both the control and *SGT1* silenced plants (Fig. 4a). As expected, MEK2^{WT} protein levels were significantly reduced in the HSP90-silenced plant, probably because of the non-detectable transcription, as seen in Fig. 3. Significant reduction of MEK2^{DD} protein in both *SGT1*- and *HSP90*-silenced plants compared to the silencing control was reproducibly observed (Fig. 4a). These results suggest that SGT1 specifically contributes to the protein level of

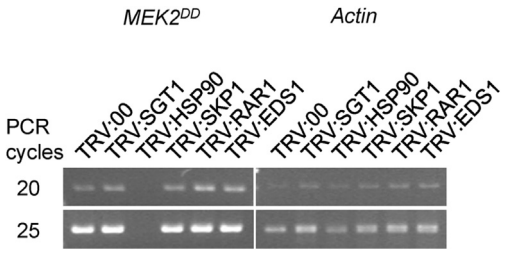

Fig. 3. Silencing of *HSP90* reduces transcription of *MEK2^{DD}*. The VIGS and agroinfiltration was performed as described above. Leaf discs were taken from infiltrated leaf 2 d post-infiltration and subjected to total RNA preparation. Equal loading was verified using *Actin* primers.

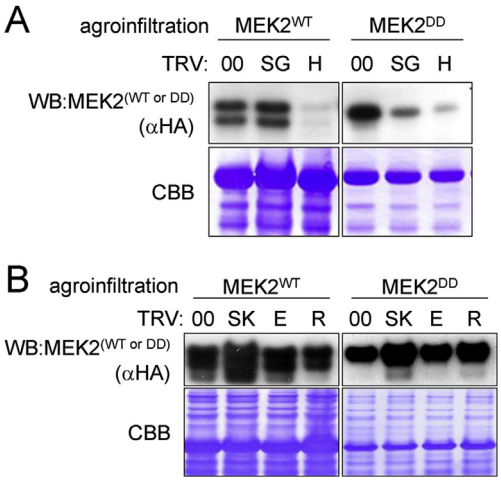

Fig. 4. Silencing *SGT1* resulted in low protein accumulation of MEK2^{DD}, not MEK2^{WT}. The VIGS and agroinfiltration of MEK2^{WT} and MEK2^{DD} followed by western blot analysis were performed as described in Fig. 2. (a) 00, SG, and H correspond to empty vector controls for silencing, silencing of *SGT1* and *HSP90*, respectively. Top panels: western blot analysis with anti-HA antibody to detect MEK2^{WT} or MEK2^{DD} proteins. The bottom panel shows CBB staining to visualize protein loading. (b) 00, SK, E, and R correspond to empty vector controls for silencing, silencing of *SKP1*, *EDS1*, and *RAR1*, respectively. Top and bottom panels: as described above.

MEK2^{DD}. Next, we further asked whether *SGT1* silencing affected MEK2^{WT} protein level after elicitation by INF1, which presumably converts MEK2^{WT} to the active form during induction of HR-like cell death. The MEK2^{WT} was expressed in the leaves of both control (TRV:00) and *SGT1*-silenced plants, 2 days later followed by agroinfiltration of INF1 or GFP as a control. We then compared protein levels of MEK2^{WT} by western blot analysis, and we did not observe apparent difference between control and *SGT1*-silenced plants (Fig. S4).

By contrast to the case of MEK2^{DD} protein in *SGT1* silencing, protein levels of neither MEK2^{WT} nor MEK2^{DD} were reduced by silencing of *SKP1*, *EDS1,* and *RAR1,* which did not suppress MEK2^{DD}-induced HR-like cell death (Fig. 4b). We noticed slight increases in MEK2^{WT} and MEK2^{DD} protein levels with *SKP1* silencing. This may correlate with stronger HR-cell death induction by MEK2^{DD} in the *SKP1* silenced plant (Fig. 1). One possible explanation is that SGT1 preferentially engaged in the chaperon function as *SKP1* was silenced, resulting in a reduced demand for the SKP1 binding function.

4. Discussion

In this study, we performed VIGS of *SGT1*, *HSP90, SIPK, SKP1*, *RAR1,* and *EDS1* to determine whether these genes were required for MEK2^{DD}-induced HR-like cell death. Among of these genes, silencing of *SGT1*, *HSP90,* and *SIPK* suppressed HR-like cell death by MEK2^{DD}. Because SIPK is the substrate of MEK2, silencing of *SIPK* resulted in suppression of HR-like cell death induced by MEK2^{DD}.

The VIGS of *HSP90* showed drastic reduction of agroinfiltrated MEK2^{DD} transcript leading to little or no protein accumulation. We think that the loss of MEK2^{DD} protein caused suppression of HR-like cell death. This result seems not consistent to those from Kanzaki et al. [22] and Takabatake et al. [23]. In our study, the 10–186hsp construct was used for *HSP90* silencing [17]. The construct contains the conserved region of *HSP90* fragment which presumably has a broad specificity to other *HSP90* genes. Because the 10–186hsp construct is very close to that of Takabatake et al. [23], the difference may largely depend on degree of silencing. Recent studies in

mammalian and yeast cells have shown that HSP90 is involved in maturation of transcription factors with chaperon activity and regulation of transcription machinery [24]. Considering that HSP90 is a highly conserved protein, this might be the case in plants when the silencing was severe.

Silencing of *SGT1* reduced protein levels of MEK2^{DD}, but not MEK2^{WT}, without affecting transcription. Although SGT1 was originally isolated in budding yeast, plant SGT1 (actually *Arabidopsis* SGT1a and SGT1b) was first identified as an RAR1 binding protein by a yeast two-hybrid screen [14]. The *Arabidopsis SGT1b* was also simultaneously identified as a gene required for *RPP5* resistance against the *Hyaloperonospora arabidopsidis* isolate Noco2 in *Arabidopsis* [25]. SGT1 consists of the N-terminus a tetratricopeptide repeat (TPR) domain, two variable regions (VR1 and VR2), the CS motif (present in metazoan CHORD and SGT1 proteins), and the C-terminus SGS (SGT1-specific) motif. In plants, SGT1 exerts different functions dependent on its interaction partners.

4.1. The possible role of SGT1 in the co-chaperon function in MEK2^{DD}-induced HR-like cell death

Herein we discuss possible involvement of SGT1 in regulation of MEK2^{DD} protein levels from the point of view that depends on SGT1-interacting proteins. First, SGT1 regulates folding and stability of proteins via formation of a chaperon complex with HSP90 and RAR1. SGT1 and RAR1 play a role as a co-chaperon for the molecular chaperon protein HSP90, which has central function in protein folding and stability. SGT1 binds to HSP90 via a CS motif, which shares structural similarities with small heat-shock proteins, α-crystallin, and the Hsp90 co-chaperone p23/Sba1 [26,27]. SGT1 simultaneously binds to the RAR1 CHORD2 domain using a different side of the CS motif from HSP90. In this case, SGT1 together with RAR1 binds to HSP90 to form a chaperone complex with the R immune receptor as a client [12]. In our research, silencing of RAR1 was not able to suppress HR-like cell death induced by MEK2^{DD}. Although insufficient silencing of RAR was not ruled out, the presence of both SGT1 and RAR1 is not always required. For example, *RPP2*, which confers resistance against the *H. arabidopsidis* isolate Cala2, requires SGT1b but not RAR1. There are more cases of different requirements of SGT1 or RAR1 for individual R proteins [28,29]. In contrast to RAR1, whose co-chaperon function is rather specific to R proteins, SGT1 may serve more diverse roles in the stability control of non-R proteins. SGT1 is important for HR or HR-like cell death by non-R proteins, such as Cf-4, Cf-9, RPW8, INF1, and MEK2^{DD} [11]. Therefore, SGT1 may facilitate different types of proteins to induce cell death. Comparing protein levels of MEK2^{DD} and MEK2^{WT}, SGT1 may specifically support the stability of the activated MEK2 protein, directly or indirectly. However, *SGT1* silencing did not alter protein level of MEK2^{WT} after elicitation by INF1, which induces HR-like cell death and presumably makes MEK2^{WT} activated. A possible explanation unable to observe reduced protein level of MEK2^{WT} after elicitation is that only limited amount of expressed MEK2^{WT} proteins may become active. Therefore, the reduction of activated MEK2^{WT} protein level may be masked by relatively large amount of inactive MEK2^{WT} proteins.

Cytosolic (heat shock cognate 70 kD) HSC70 chaperon is also known to associate with SGT1 [30]. Interaction of HSC70 and SGT1 occurs through the SGS domain of SGT1. A VIGS study revealed the requirement for HSP70 in the cell death response of *N. benthamiana* in the case of INF1, non-host resistance against *Pseudomonas cichorii* and tabtoxinine-β-lactam [22,31]. However, Kanzaki et al. showed that VIGS of HSP70 did not suppress HR-like cell death induced by MEK2^{DD} [22]. It is possible that VIGS was not fully effective because of a functional redundancy among HSP70 isoforms and that HSP70 is dispensable for MEK2^{DD}-induced HR-like cell death.

4.2. SGT1 functions as a component of SCF ubiquitin ligase complex and is not likely involved in MEK2^{DD}-induced HR-like cell death

SGT1 contains the TPR domain at the N-terminus. The function of the TPR domain in SGT1 has already been revealed in budding yeast and plants, and TPR is involved in binding to SKP1 [13,14]. Binding of SGT1 and SKP1 is necessary for assembling the kinetochore complex and SCF-type ubiquitin ligase complex in yeast. In plants, SGT1 is indeed associated with SKP1 and is involved in the function of SCF^{TIR1} and SCF^{COI1} [15]. The *Arabidopsis sgt1b* mutant was also identified as the *eta3* mutant, which confers enhanced auxin insensitivity to the *tir1* mutant. It is well known that *TIR1* encodes the F-box protein, which binds to SKP1 to form the auxin receptor complex SCF^{TIR1} [32]. The *eta3* mutant also showed reduced sensitivity to jasmonate, whose receptor COI1 is also the F-box protein, a substrate determinant for the SCF complex, to degrade JAZ proteins. Therefore, plant SGT1 has a positive role in SCF complex function through its binding to SKP1. In addition, silencing of *SKP1* in *N. benthamiana* with *N* gene, which confers resistance against TMV, suppressed the *N*-gene mediated resistance [18]. Our results showed that silencing of *SKP1* did not suppress HR-like cell death induced by MEK2^{DD}. Consequently, the function of SGT1 through SKP1 binding in the SCF ubiquitin ligase complex is not likely to be involved in MEK2^{DD}-induced HR-like cell death. Even though SKP1 has a positive function in *N*-mediated resistance, it may be independent to HR. Moreover, Azevedo et al. [33] showed that the TPR domain of SGT1, essential for SKP1 binding, was not mandatory for the disease resistance function of SGT1. This is consistent with our results using *SKP1* silencing.

5. Conclusions

The present study showed that silencing of *SGT1* resulted in suppression of HR-like cell death induced by MEK2^{DD} in *N. benthamiana* leaves. This was caused by reduced protein, but not mRNA, levels of MEK2^{DD} when the *SGT1* was silenced, but not the case of *SKP1* silencing. The SKP1 is the essential component of SCF E3 ubiquitin ligase. The SGT1 has a positive role for the SCF E3 ubiquitin ligase function, which was however dispensable for MEK2^{DD}-induced HR like cell death. The comparison of protein levels of MEK2^{DD} and MEK2^{WT} in *SGT1* silencing revealed that only MEK2^{DD} was affected by loss-of-function of *SGT1*. These results suggest that SGT1 has a novel function for maintaining protein levels of MEK2 active form. This possible function of SGT1 may have a role in HR induction upon effector recognition by NLR proteins in *Solanaceae*.

HR cell death is the most intense defense response triggered by various kinds of R proteins. However, the mechanism of HR cell death is still largely unknown because of signaling complexity and redundancy [34]. These features serve as a basis of signaling robustness during ETI; they also made it difficult to elucidate the mechanism of HR cell death. The MEK2-WIPK, SIPK pathway plays an important role in cell death induction triggered by several R proteins. Therefore, a specific MAPK pathway-targeted strategy, such as suppressor mutant screening using constitutive active MAPKK-induced HR-like cell death, could be effective to elucidate a part of the molecular mechanism in HR cell death. In addition, screening HR-suppressing pathogen effectors and using a chemical biology approach to identify HR-inhibiting compounds would provide “molecular probes” to determine important factors that are essential to HR induction, and could be a solution to overcome the

signaling redundancy problem.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2016.04.001.

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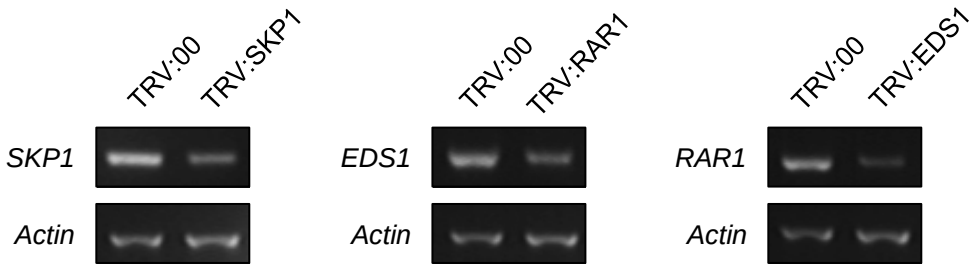

Fig. S1. Reduced transcript levels of *SKP1*, *RAR1*, and *EDS1* by silencing

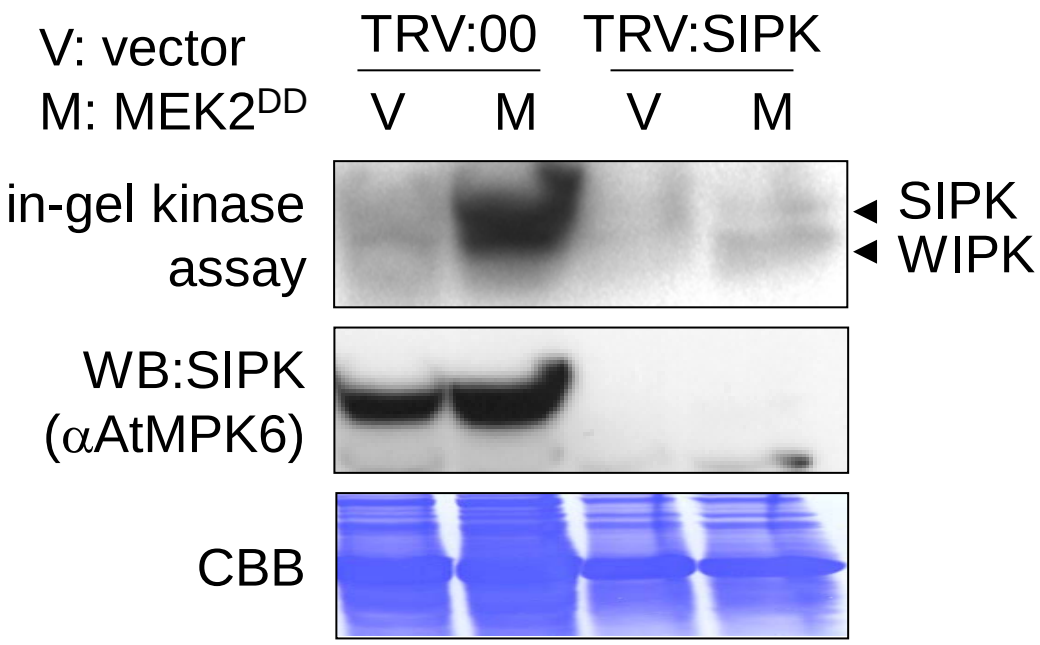

Fig. S2. Silencing of *SIPK* abolished HR-like cell death by MEK2^{DD}

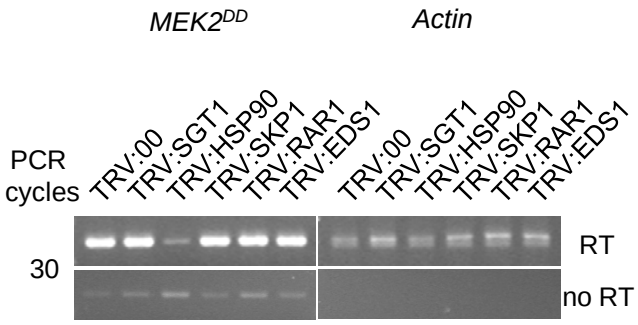

Fig. S3. Silencing of HSP90 abolished transcription of *MEK2^{DD}*

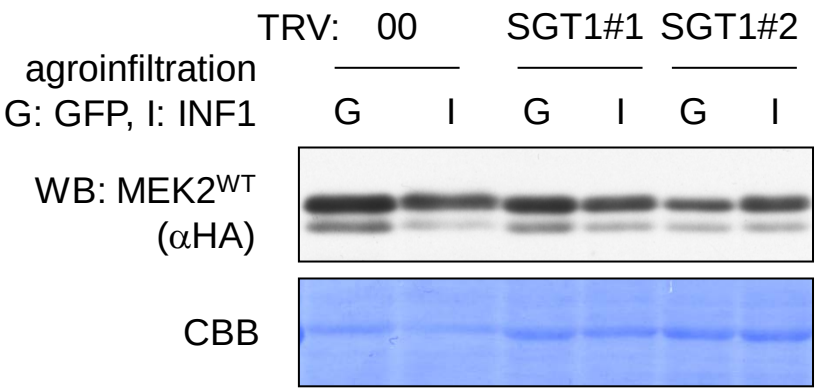

Fig. S4. The *SGT1* silencing did not significantly reduce MEK2^{WT} protein level after elicitation by INF1.