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

Kazuya Ichimura [a, *], Takehito Shinzato [a], Miki Edaki [a], Hirofumi Yoshioka [b], Ken Shirasu [c]

[a] Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa, 761-0795, Japan
[b] Laboratory of Defense in Plant–Pathogen Interactions, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan
[c] Plant Immunity Research Group, RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045, Japan

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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.

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

* Corresponding author.
E-mail address: kichimura@ag.kagawa-u.ac.jp (K. Ichimura).

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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