Protein Phosphatase 5 Serves as a Co-Chaperone to Regulate the Accumulation of Immune Receptor SUMM2

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Article

Protein Phosphatase 5 Serves as a Co-Chaperone to Regulate the Accumulation of Immune Receptor SUMM2

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One Sentence Summary

Arabidopsis PP5 functions as a co-chaperone to regulate the accumulation of NLR receptor SUMM2.

Abstract

Protein Phosphatase 5 (PP5) is an evolutionarily conserved serine/threonine phosphatase with a unique tetratricopeptide domain. It has been implicated in a wide range of cellular processes in mammals, but its function in plants is unknown. Here we uncovered that *Arabidopsis* PP5 is required for immunity mediated by the nucleotide-binding leucine-rich repeat immune receptor protein SUMM2. Loss-of-function mutations in *PP5* suppress the autoimmune phenotypes caused by the activation of SUMM2 due to the disruption of the MEKK1-MKK1/MKK2-MPK4 kinase cascade. Further biochemical analysis revealed that SUMM2 interacts with Heat Shock Protein 90 and PP5, and SUMM2 level is reduced in *pp5* knockout mutant plants, suggesting that PP5 functions as a co-chaperone to regulate the accumulation of SUMM2.

Keywords: protein phosphatase 5 (PP5); SUMM2; HSP90; co-chaperone

Introduction

To defend themselves against infections from various microbial pathogens, plants have evolved sophisticated immune systems [1,2]. Pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) is initiated by recognizing conserved microbial features through cell surface-localized pattern recognition receptors (PRRs). Activation of PTI is believed to be sufficient to stop most pathogen infections. However, adapted pathogens have evolved effector proteins to inhibit PTI and promote colonization. Recognition of pathogen effectors by plant resistance (R) proteins leads to activation of effector-triggered immunity (ETI). Most R proteins identified so far are nucleotide-binding leucine-rich repeat (NLR) proteins with either a Toll/Interleukin-1 receptor (TIR) or a coiled-coil (CC) domain at their N-termini [3]. Recognition of effectors by R proteins can be directly through physical interaction or indirectly through detecting effector-mediated modification of host proteins [4]. PTI and ETI are functionally interdependent, mutually potentiating each other to establish robust plant immunity [5–8].

In *Arabidopsis*, the MEKK1–MKK1/MKK2–MPK4 mitogen-activated protein (MAP) kinase cascade constitutes a key signaling module in plant innate immunity [9,10]. This cascade is essential not only for basal resistance against pathogens but also for maintaining immune homeostasis, as its integrity is actively monitored by the CC-type NLR (CNL) SUMM2 [11]. Consequently, loss of

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MEKK1, MKK1/MKK2, or MPK4 leads to constitutive, SUMM2-dependent activation of defense responses, indicating that SUMM2 detects disruption of this cascade. Mechanistically, SUMM2 physically interacts with CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 3 (CRCK3), a direct substrate of MPK4 [12]. In wild-type plants, MPK4-mediated phosphorylation of CRCK3 keeps the protein in a state that prevents SUMM2 activation. When the MEKK1–MKK1/MKK2–MPK4 cascade is compromised, CRCK3 phosphorylation is reduced, and this hypophosphorylated state is sensed by SUMM2 as a "modified-self" signal, thereby triggering downstream immune responses. Thus, CRCK3 functions as a classical guardee, coupling the integrity of this MAP kinase cascade to NLR-mediated immunity.

The establishment of robust NLR-mediated immune responses hinges on proper accumulation of NLR immune receptors, which are tightly controlled by the molecular chaperone network. Heat Shock Protein 90 (HSP90), a family of highly conserved molecular chaperones, promotes the folding of client proteins and is indispensable for plant immunity. In *Arabidopsis*, cytosolic HSP90s, together with the co-chaperones RAR1 (Required for MLA12 resistance 1) and SGT1 (Suppressor of the G2 allele of Skp1), are required for the proper accumulation and/or folding of multiple NLRs, including MLA, RPM1, RPS2, RPS4, N, and I2 [13–18]. Intriguingly, certain mutations in HSP90.2 and HSP90.3 lead to increased rather than decreased levels of SNC1, RPS2, and RPS4 [14,19], suggesting that HSP90s also assist SGT1 in the assembly of SCF E3 ubiquitin ligase complexes to promote the degradation of specific NLRs. Thus, the HSP90 chaperone machinery can both stabilize and destabilize NLR proteins, and its functional output is likely dictated by distinct sets of co-chaperones. However, whether SUMM2 is regulated by a specific co-chaperone remains unknown.

In this study, we report the identification and characterization of SUMM5, a positive regulator of SUMM2-mediated immunity. SUMM5 was isolated from a modified suppressor screen of the *mkk1 mkk2* double mutant, and loss-of-function mutations in SUMM5 partially suppressed the autoimmune phenotypes of *mekk1*, *mkk1 mkk2*, and *mpk4* plants. Positional cloning revealed that SUMM5 encodes Protein Phosphatase 5 (PP5). Further mechanistic analyses demonstrated that PP5 physically associates with HSP90 and functions as a co-chaperone specifically required for the proper accumulation of SUMM2. Importantly, bacterial growth assays showed that the *pp5* mutant supported wild-type levels of *Pseudomonas syringae pv. tomato* DC3000 carrying AvrRpt2, AvrRPS4, AvrPphB, or the T3SS-deficient *hrcC* mutant strain, indicating that PP5 does not detectably affect RPS2-, RPS4-, RPS5-, or PTI-mediated resistance. Collectively, these findings establish PP5/SUMM5 as an HSP90 co-chaperone that specifically couples the chaperone network to SUMM2-mediated surveillance of the MEKK1–MKK1/MKK2–MPK4 kinase cascade, without broadly influencing other NLR signaling pathways.

Results

Identification and Characterization of summ5 mkk1 mkk2 ndr1

In our previous *mkk1 mkk2* suppressor screen, the *mkk1 mkk2* mutant exhibited strong sterility, which severely limited the size of the M_2 population. Consequently, only SUMM1/MEKK2, SUMM2, SUMM3/CRCK3, and SUMM4/MKK6 were identified from this screen [11,12,20,21]. To identify additional regulators of SUMM2-mediated defense, we extended this screen using a *mkk1-1 mkk2-1 ndr1-1* (*mkk1/2 ndr1*) background, which overcomes the sterility of *mkk1 mkk2* and permits larger-scale screening. In addition to SUMM6/MDS1, which we previously reported from this screen [22], another mutant we characterized is *summ5-1*. As shown in Figure 1A, the *summ5 mkk1 mkk2 ndr1* quadruple mutant is markedly larger than the *mkk1 mkk2 ndr1* triple mutant, although slightly smaller than wild-type. Quantitative RT-PCR analysis revealed that the constitutive expression of *PR1* and *PR2* in *mkk1 mkk2 ndr1* is strongly reduced by *summ5-1* (Figure 1B,C). Moreover, the enhanced resistance of *mkk1 mkk2 ndr1* to the virulent oomycete pathogen *Hyaloperonospora arabidopsidis* (*H. a.*) Noco2 was attenuated by the *summ5-1* mutation (Figure 1D). Together, these data demonstrate that *summ5-1* largely suppresses the autoimmune phenotypes of *mkk1 mkk2 ndr1*.

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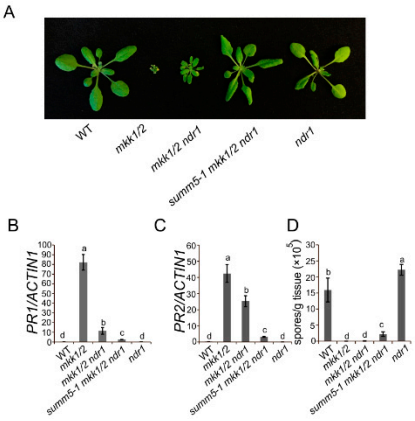

Figure 1. Characterization of the *summ5-1 mkk1-1 mkk2-1 ndr1-1* mutant. (A) Morphology of three-week-old soil-grown wild-type (WT) Col-0, *mkk1-1 mkk2-1* (*mkk1/2*), *mkk1-1 mkk2-1 ndr1-1* (*mkk1/2 ndr1*), *ndr1-1* (*ndr1*) and *summ5-1 mkk1-1 mkk2-1 ndr1-1* (*summ5 mkk1/2 ndr1*) plants. (B, C) Expression levels of *PR1* (B) and *PR2* (C) in WT, *mkk1/2*, *mkk1/2 ndr1*, *ndr1* and *summ5 mkk1/2 ndr1* seedlings. Two-week-old seedlings grown on 1⁄2 MS plates were used. Values were normalized to the expression levels of *ACTIN1*. (D) Growth of *H.a.* Noco2 on WT, *mkk1/2*, *mkk1/2 ndr1*, *ndr1* and *summ5 mkk1/2 ndr1* seedlings. Error bars represent the standard deviations of three measurements. Statistical differences among the samples are labeled with different letters ($P < 0.01$, One-way ANOVA and Tukey's test; n=3).

SUMM5 Encodes Protein Phosphatase 5 (PP5)

To map the *summ5-1* mutation, *summ5-1 mkk1 mkk2 ndr1* (in the Col-0 background) was crossed with Landsberg *erecta* (L*er*) to generate a segregating F2 population. 24 plants that are homozygous for *mkk1 mkk2 ndr1* in the F2 progeny were identified and used for crude mapping. *summ5-1* was initially mapped to chromosome 2 between markers T2N18 (15.9Mb) and F14M4 (19.28Mb). Further fine mapping narrowed down the mutation to a region between marker T24P15 (17.6Mb) and T3F17 (18.7Mb) (Figure 2A). Whole-genome resequencing identified two nonsense mutations in this region, each introducing a premature stop codon in *At2g42750* and *At2g42810*, respectively.

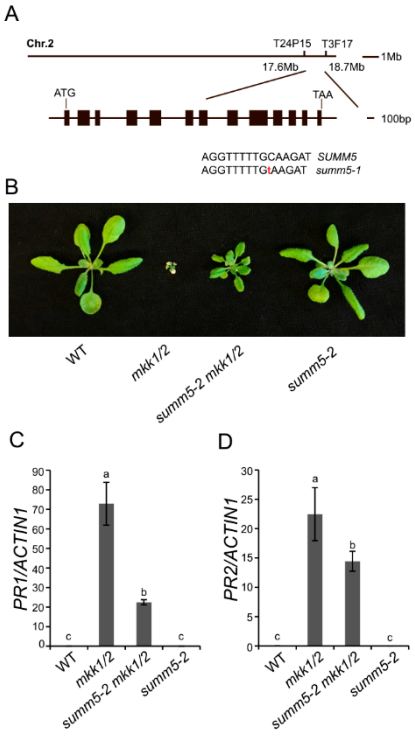

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Figure 2. Positional cloning of *SUMM5*. (A) Map position and location of the *summ5-1* mutations. (B) Morphology of wild-type (WT), *mkk1-1 mkk2-1* (*mkk1/2*), *summ5-2 mkk1-1 mkk2-1* (*summ5 mkk1/2*) and *summ5-2* plants. Plants were grown on soil and photographed three weeks after planting. (C, D) Expression levels of *PR1* (C) and *PR2* (D) in WT, *mkk1/2*, *summ5-2 mkk1/2* and *summ5-2* seedlings as compared to *ACTIN1*. Error bars represent the standard deviations of three measurements. Statistical differences among the samples are labeled with different letters ($P < 0.01$, One-way ANOVA and Tukey's test; n=3).

To determine which mutation causes the *summ5-1* phenotype, we obtained T-DNA insertional mutants of these two candidate genes from the Arabidopsis Biological Resource Center (ABRC). The two T-DNA mutants SALK_051866C (*AT2G42750*) and WiscDsLox368D06/CS864321 (*AT2G42810*) were confirmed to carry insertions in the exons, therefore were crossed to *mkk1 mkk2*. Only the T-DNA mutant CS864321 partially suppresses the autoimmune phenotype of *mkk1 mkk2* (Figure 2B–D), indicating that *SUMM5* is *AT2G42810.* Thus we renamed CS864321 as *summ5*-2.

To further confirm that the mutation found in *AT2G42810* is responsible for the suppression of *mkk1 mkk2 ndr1* mutant phenotypes, we transformed a construct expressing *AT2G42810* under the control of its native promoter with a C-terminal Green Fluorescent Protein (GFP) tag into *summ5-1 mkk1 mkk2 ndr1*. As shown in Figure S1, the transgene restored the dwarf morphology and elevated *PR1* gene expression of *mkk1 mkk2 ndr1*, further confirming that *SUMM5* is *AT2G42810. AT2G42810* encodes PROTEIN PHOSPHATASE 5 (PP5), which is a unique member of phosphoprotein phosphatase (PPP) family of serine/threonine phosphatase [23]. It contains a C-terminal phosphatase catalytic domain and an N-terminal tetratricopeptide (TPR) domain.

Defense Responses in mekk1 and mpk4 Are Partially Suppressed by summ5

As MEKK1, MKK1/MKK2 and MPK4 function in a kinase cascade, we tested whether the autoimmune phenotypes of *mekk1* and *mpk4* can also be suppressed by *summ5*. The *mekk1-1 summ5-2* and *mpk4-3 summ5-2* double mutants were obtained by crossing *summ5-2* with *mekk1-1* and *mpk4-3* respectively. As showed in Figure 3A, *summ5-2 mekk1-1* is still much smaller than wild type but is considerably bigger than *mekk1-1*. qRT-PCR analysis showed that the elevated expression levels of *PR1* and *PR2* in *mekk1-1* were also partially suppressed by *summ5-2* (Figure 3B,C). Similarly, *summ5-2* partially suppresses the dwarf morphology and constitutive *PR* gene expression in *mpk4-3* (Figure 3D–F). Taken together, these genetic data indicate that SUMM5 functions downstream of the MEKK1-MKK1/MKK2-MPK4 kinase cascade.

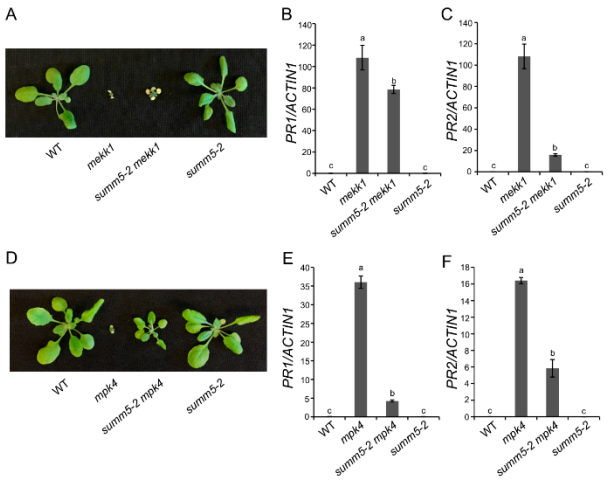

Figure 3. Characterization of the *summ5-1 mekk1-1* and *summ5-1 mpk4-3* double mutants. (A) Morphology of three–week-old wild-type (WT), *mekk1-1*, *summ5-1 mekk1-1* and *summ5-1* plants grown on soil. (B, C) Expression levels of *PR1* (B) and *PR2* (C) in the indicated genotypes as determined by quantitative RT–PCR. Two-week-old seedlings grown on 1⁄2 MS plates were used. Values were normalized to the expression levels of *ACTIN1*. (D) Morphology of three-week-old WT, *mpk4-3*, *summ5-1 mpk4-3*, and *summ5-1* plants grown on soil. (E, F)

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Expression levels of *PR1* (E) and *PR2* (F) in the indicated genotypes. Two-week-old seedlings grown on 1⁄2 MS plates were used. Values were normalized to the expression levels of *ACTIN1*. Error bars represent the standard deviations of three measurements. Statistical differences among the samples are labeled with different letters ($P < 0.01$, One-way ANOVA and Tukey's test; n=3).

PP5 Affects the Accumulation of SUMM2

In mammals, PP5 functions as a co-chaperone of HSP90 [24]. To determine whether *SUMM5* is required for the accumulation of SUMM2, WT and *summ5-2* plants were transformed with a construct expressing the SUMM2-HA fusion protein under the control of 35S promoter. Two representative transgenic lines with similar transcriptional expression levels of *SUMM2-HA* in WT and *summ5-2* mutant background were identified by RT-PCR (Figure 4A). Western blot analysis showed that the SUMM2-HA protein levels in the *summ5-2* background is considerably lower than that in the WT background (Figure 4B), suggesting that PP5 contributes to the accumulation of SUMM2.

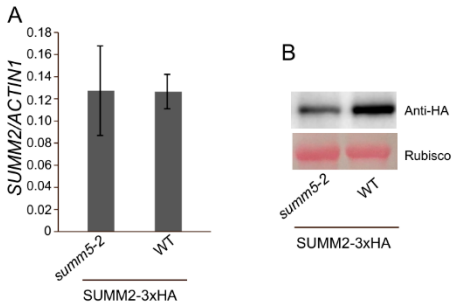

Figure 4. SUMM2 protein level is reduced in *summ5-2*. (A) Expression levels of *SUMM2-HA* in wild-type (WT) and *summ5-2* as determined by quantitative RT–PCR. Two-week-old seedlings grown on 1⁄2 MS plates were used. Values were normalized to the expression levels of *ACTIN1*. (B) Western blot analysis of SUMM2-HA protein levels in WT and the *summ5-2* mutant background. Total protein was extracted from two-week-old seedlings grown on half-strength MS plates. Rubisco was used as the protein loading control.

SUMM2 Interacts with PP5 and HSP90 in Planta

As PP5 affects SUMM2 accumulation, we further tested whether SUMM2 interacts with PP5. We transiently expressed HA-tagged SUMM2 together with FLAG-ZZ-tagged PP5 in *Nicotiana (N.) benthamiana* by *Agrobacteria*-mediated transformation and carried out immunoprecipitation using anti-FLAG beads. As shown in Figure 5A, SUMM2-HA co-immunoprecipitated with PP5-FLAG-ZZ, suggesting that SUMM2 and PP5 associate with each other *in planta*. We further tested interaction between HA-tagged SUMM2 and FLAG-tagged HSP90 in *N. benthamiana*. As shown in Figure 5B, SUMM2-HA co-immunoprecipitated with HSP90-FLAG-ZZ, suggesting that SUMM2 also associates with HSP90 *in vivo*.

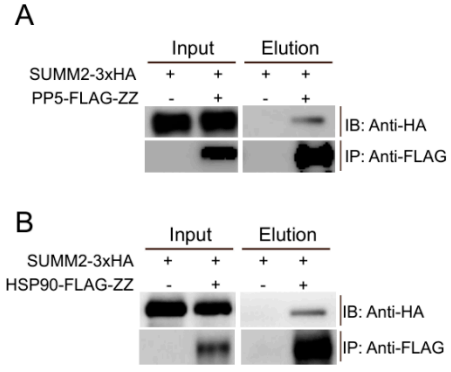

Figure 5. PP5 associates with SUMM2 and HSP90 *in planta*. (A) Co-IP analysis to test the interaction between PP5 and SUMM2. PP5-FLAG-ZZ and SUMM2-3×HA proteins were transiently expressed in *N. bethamiana*. The

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PP5-FLAG-ZZ protein was immunoprecipitated with anti-FLAG conjugated beads and detected by western blot using anti-FLAG antibody. The co-immunoprecipitated SUMM2-3×HA protein was detected by western blot using anti-HA antibody. Similar results were obtained in three independent experiments. (B) Co-IP analysis of interaction between HSP90 and SUMM2. HSP90-FLAG-ZZ and SUMM2-3×HA proteins were transiently expressed in *N. bethamiana*. HSP90-FLAG-ZZ protein was immunoprecipitated with anti-FLAG conjugated beads and detected by western blot using anti-FLAG antibody. SUMM2-3×HA protein co-IPed was detected by western blot using anti-HA antibody. Similar results were obtained in three independent experiments.

Phosphatase Activity of PP5 Is Not Required for SUMM2 Activation

As dephosphorylation of CRCK3 leads to activation of SUMM2-mediated immune responses and PP5 is required for this activation, we initially speculated that PP5 might dephosphorylate CRCK3. However, we were unable to detect a direct interaction between CRCK3 and PP5 by co-immunoprecipitation (Co-IP) in *N. benthamiana*. To further test whether the phosphatase activity of PP5 is involved in the regulation of SUMM2-mediated defense signaling, we mutated the conserved histidine residue (H344) in the catalytic domain of PP5 to alanine and generated transgenic lines expressing PP5^{H344A} in the *summ5 mkk1 mkk2 ndr1* background. H344 is the homologous residue to the histidine 304 in human PP5 which was previously shown to be necessary for its phosphatase activity [25]. To our surprise, the PP5^{H344A} variant restored the dwarf morphology of *mkk1 mkk2 ndr1* (Figure 6A). Analysis of *PR* gene expression showed that the expression levels of *PR1* and *PR2* in the *PP5^{H344A}* transgenic lines are comparable to those in *mkk1 mkk2 ndr1* (Figure 6B,C), indicating that PP5^{H344A} can complement the mutant phenotype of *summ5*. These data suggest that the phosphatase activity of PP5 may not be essential for its function in regulating defense responses mediated by CNL SUMM2.

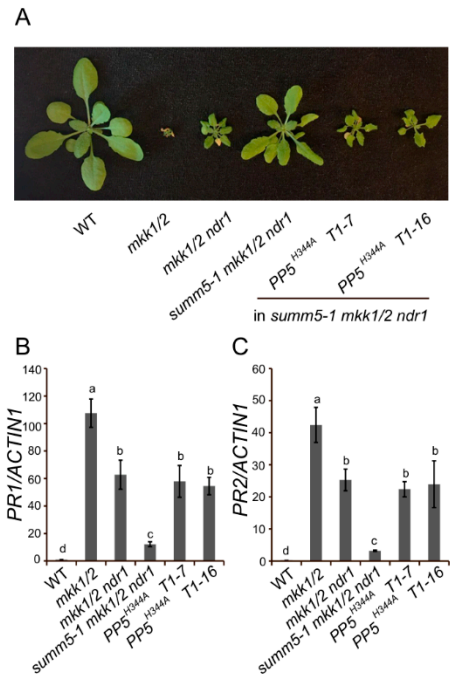

Figure 6. A conserved His (H344) residue in the phosphatase domain is not required for the function of PP5 in the activation of defense responses in *mkk1 mkk2 ndr1*. (A) Morphology of three-week-old wild-type (WT), *mkk1-1 mkk2-1* (*mkk1/2*), *mkk1-1 mkk2-1 ndr1-1* (*mkk1 mkk2 ndr1*), *summ5-2 mkk1 mkk2 ndr1* and *PP5^{H344A}* transgenic lines grown on soil. PP5^{H344A} T1-7 and PP5^{H344A} T1-16 are independent transgenic lines expressing the PP5^{H344A} mutant protein in the *summ5-2 mkk1 mkk2 ndr1* background. (B, C) Expression levels of *PR1* (B) and *PR2* (C) in the indicated genotypes. Total RNA was extracted from two-week-old seedlings grown on 1/2 MS medium. Values were normalized to the expression levels of *ACTIN1*. Error bars represent the standard deviations of

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three measurements. Statistical differences among the samples are labeled with different letters ($P < 0.01$, One-way ANOVA and Tukey's test; n=3).

RPS4, RPS2- and RPS5-Mediated Defense Responses Are Not Affected in summ5 Mutants

To determine whether PP5 is required for resistance conferred by other NLR proteins, we infiltrated WT and *summ5* mutant plants with *Pseudomonas syringae* pv. *tomato* (*P.s.t.*) DC3000 strains carrying *AvrRpt4*, *AvrRpt2* or *AvrPphB*, which are recognized by the TIR-type NLR RPS4 and CNLs RPS2 and RPS5, respectively [26–28]. As shown in Figure S2, growth of these bacterial strains was comparable in WT and *summ5* plants, suggesting that PP5 is not essential for resistance mediated by RPS4, RPS2 or RPS5.

PP5 Is Not Required for PAMP-Triggered Immunity

To determine whether *PP5* is involved in PTI, we infiltrated WT and *summ5* mutant plants with *P.s.t.* DC3000 *hrcC*, a bacterial strain deficient in the type III secretion system often used to test PTI response [29,30]. As shown in Figure S3, the growth of *P.s.t.* DC3000 *hrcC* in *summ5-1* and *summ5-2* leaves was comparable to that in WT plants, suggest that PP5 is not required for PTI.

Discussion

We previously established a model in which activation of the CNL SUMM2 depends on the integrity of the MEKK1–MKK1/MKK2–MPK4 kinase cascade, with the phosphorylation status of CRCK3 serving as the key signal [12]. To further understand how SUMM2-mediated immunity is regulated, we performed a suppressor screen in the *mkk1 mkk2 ndr1* background and identified *SUMM5*, which encodes the conserved serine/threonine phosphatase PP5 (Figures 1 and 2). Loss-of-function mutations in *SUMM5*/*PP5* partially suppress the autoimmune phenotypes of *mekk1*, *mkk1 mkk2*, and *mpk4* mutants (Figure 3), suggesting that SUMM5/PP5 functions as a positive regulator of this immune signaling branch. Loss of *PP5* in *Arabidopsis* reduces SUMM2 protein levels, indicating that PP5 positively regulates SUMM2-mediated immunity by promoting the accumulation of SUMM2.

In animals, PP5 participates in diverse cellular processes, whereas plant PP5 has been linked to light signaling, thermotolerance, and interaction with the HSP90–SGT1–RAR1 chaperone complex. Previous work showed that PP5 can associate with the tomato NLR I-2, but its functional relevance remained unclear [18]. Here, we uncover a specific requirement for PP5 in the accumulation and activation of SUMM2. We showed that PP5 interacted with SUMM2 and is required for the accumulation of SUMM2 (Figures 4 and 5). As its phosphatase activity is not required for its function (Figure 6), PP5 most likely serves as a molecular chaperone to stabilize SUMM2 *in vivo*.

Unlike other protein phosphatases, PP5 contains a unique N-terminal TPR domain, which is involved in the interaction with other proteins. The TPR region was also shown to interact with its C-terminal phosphatase domain to inhibit its phosphatase activity [31]. Interestingly, mutating a conserved His residue in the catalytic domain of PP5 does not affect its function in SUMM2-mediated defense (Figure 6), suggesting that the phosphatase activity of PP5 is either not required for its function in promoting the folding and accumulation of SUMM2, or a very low level of phosphatase activity is sufficient in this process. During the preparation of this manuscript, an independent study reported that PP5 directly interacts with CRCK3 and that its phosphatase activity is required for regulating SUMM2-mediated immunity [32]. However, we were unable to detect a physical interaction between PP5 and CRCK3 in our hands using co-immunoprecipitation assays. It is possible that PP5 may engage distinct protein complexes under different experimental conditions or genetic backgrounds, and that its phosphatase activity becomes essential only in certain scenarios. In our model, the primary requirement for PP5 in SUMM2-mediated immunity is to act as a scaffold that facilitates the accumulation of SUMM2.

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Loss of PP5 only partially suppress the autoimmune phenotypes of *mekk1*, *mkk1 mkk2* and *mpk4*, indicating that it functions as a fine-tuning co-chaperone rather than an absolute determinant of SUMM2 accumulation. Furthermore, *pp5* mutations do not detectably affect resistance mediated by RPS2, RPS4, or RPS5, implying that PP5 is either not required for the biogenesis of these NLRs or its contribution falls below the detection limit of our assays. The contribution of PP5 to immunity mediated by different R proteins may vary depending on the nature of specific NLRs. This also raises the interesting possibility that distinct co-chaperones exhibit NLR-client specificity, thereby adding a layer of regulatory precision to immune receptor homeostasis.

Material and Methods

Plant Materials and Growth Conditions

Plants were grown under 16-h light at 23°C and 8-h dark at 19°C unless specified. *mekk1-1*, *mkk1-1 mkk2-1*, *mpk4-3* and *ndr1-1* mutants were described previously [9,33]. *mkk1-1 mkk2-1 ndr1-1* was obtained by crossing *mkk1-1 mkk2-1* with *ndr1-1*. The *summ5-1* single mutant was obtained by crossing *summ5-1 mkk1-1 mkk2-1 ndr1-1* with WT plants. *summ5-2* (WiscDsLox368D06) was obtained from ABRC. The *summ5-2 mkk1-1 mkk2-1, summ5-2 mekk1-1* and *summ5-2 mpk4-3* double mutants were generated by crossing *summ5-2* with *mkk1-1 mkk2-1*, *mekk1-1* and *mpk4-3* respectively.

Mutant Characterization and Genetic Mapping

For gene expression analysis, total RNA was extracted from 12-day-old seedlings grown on 1/2 MS medium using EZ-10 Spin Column Plant RNA Mini-Preps Kit (Bio Basic, Inc.). Reverse transcription was conducted with M-MuLV reverse transcriptase (ABM, Inc.). qPCR was carried out using the Takara SYBR Premix Ex TaqII kit. The primers used to amplify *PR1*, *PR2*, and *ACTIN1* have been described previously [34] The primers used to amplify *SUMM2-HA* are SUMM2-RT-F and HA-R (Table S1).

To map the *summ5-1* mutation, the *summ5-1 mkk1-1 mkk2-1 ndr1-1* mutant in Col-0 background was crossed with L*er* plants to obtain a segregating F2 population. F2 plants with *mkk1 mkk2*-like morphology were used for crude and fine mapping. To identify the *summ5-1* mutation, genomic DNA of *summ5-1 mkk1-1 mkk2-1 ndr1-1* was extracted and sequenced using an Illumina Genome Analyzer. Candidate mutations in this region between markers T24P15 and T3F17 were identified by comparing the whole genome sequencing data with the Arabidopsis Col-0 reference genome sequence.

Plasmid Construction

For transgene complementation, a genomic DNA fragment containing *PP5* was amplified from WT genomic DNA using primers 1305-PP5-Kpn1-F and PP5-Xba1-R (Table S1). The PCR fragment was digested with Kpn1/Xba1 and inserted into a modified pCambia1305 vector with an in-frame GFP tag at the C-terminus. For constructing the $PP5^{H344A}$ mutant plasmid, two overlapping *PP5* genomic DNA fragments were amplified using primers 1305-PP5-Kpn1-F and PP5-H344A-R or primers PP5-H344A-R and PP5-Xba1-R, and the $PP5^{H344A}$ mutant gene was subsequently obtained by overlapping PCR using primers 1305-PP5-Kpn1-F and PP5-Xba1-R and the two above DNA fragments as template (Table S1). After digestion with Kpn1/Xba1, the $PP5^{H344A}$ DNA fragment was cloned into pCAMBIA1305-FLAGZZ.

For Co-IP analysis in *N. benthamiana*, the genomic DNA of *PP5* was amplified using the primers PP5-Kpn1-F and PP5-Xba1-R and cloned into pCambia1300-35S-FALGZZ. The genomic DNA of HSP90 was amplified using the primers HSP90.3-Kpn1-F and HSP90.3-Pst1-R and cloned into pCambia1300-35S-FALGZZ. The 1300-35S-SUMM2-3xHA plasmids were described previously [11].

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Co-IP Analysis

For co-IP experiments in *N. benthamiana*, fresh colonies of *Agrobacterium tumefaciens* GV3101 strains containing pCambia1300-35S-SUMM2-3xHA, pCambia1300-35S-PP5-FLAGZZ, pCambia1300-35S-HSP90-FLAGZZ or an empty vector were inoculated to Luria broth (LB) media supplemented with appropriate antibiotics. The bacteria were grown at 28°C for 16 h before collection and resuspension in buffer containing 10 mM MES (pH 5.6), 10 mM $MgCl_2$ and 150μM acetosyringone. *Agrobacteria* suspensions carrying different constructs were mixed in 1:1 ratio with a final concentration of OD600 = 0.6 and kept on the bench for three hours at room temperature. The bacterial mixtures were then infiltrated into leaves of four-week-old *N. benthamiana* plants. For each sample, about 1 g of tissue was harvested from the infiltrated area two days later infiltration.

For co-IP analysis, the tissue was ground in liquid nitrogen and resuspended in 2 volumes of grinding buffer (10% glycerol, 25 mM Tris–HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10 mM DTT, 2% PVPP, 1× protease inhibitor cock-tail from Roche). After the samples were fully lysed at 4°C, they were centrifuged twice at 15,000×*g* for 10 min. The supernatants were collected and transferred to a new tube containing anti-FLAG-conjugated beads. After incubation for 2 h, the beads were collected by centrifugation and washed four times with the extraction buffer plus 1.5% NP-40. Proteins bound to the beads were subsequently eluted with 100 μl of 0.1 mg/ml 3xFLAG peptide and were subjected to SDS–PAGE electrophoresis and western blot analysis.

Pathogens Infection

For *H.a.* Noco2 infection, two-week-old soil-grown plants were sprayed with spores of *H.a.* Noco2 (5×104 spores ml–1). Afterwards the plants were covered with a transparent plastic lid and kept in a growth chamber (18°C, 12h light/12h dark cycles, and ~80% humidity) for seven days. The growth of *H.a.* Noco2 was then quantified as described previously [35].

For infection assays of *P.s.t.* DC3000 *hrc*C and *P.s.t.* DC3000 with different effectors, plants were grown at 22°C under 12h light/12h dark cycles. Two leaves of four-week-old plants were infiltrated with bacterial suspensions in 10 mM MgCl2. Leaf discs were collected for 3 days post inoculation and ground in 10 mM MgCl2. The samples were diluted and plated on LB medium. Bacterial colonies were counted 2 days later to determine the bacterial titer.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. **Figure S1. Complementation of *summ5-1* by *PP5-GFP*.** (A) Morphology of wild-type (WT), *mkk1-1 mkk2-1* (*mkk1/2*), *mkk1-1 mkk2-1 ndr1-1* (*mkk1/2 ndr1*), *summ5-1 mkk1-1 mkk2-1 ndr1-1* (*summ5 mkk1/2 ndr1*) and two *PP5-GFP* transgenic lines in *summ5-1 mkk1-1 mkk2-1 ndr1-1* background. The photo was taken with three-week-old soil-grown plants. (B, C) Expression levels of *PR1* (B) and *PR2* (C) in the indicated genotypes as determined by quantitative RT–PCR. Two-week-old seedlings grown on 1⁄2 MS plates were used. Values were normalized to the expression levels of *ACTIN1*. **Figure S2. RPS4-, RPS2- and RPS5-mediated immunity is not affected in *summ5* mutants.** (A, B, C). Growth of bacteria in wild type (WT) and *summ5* mutant plants. Four-week-old soil-grown plants were inoculated with *P.s.t.* DC3000 AvrRpt2 (A), *P.s.t.* DC3000 AvrPphB (B) and *P.s.t.* DC3000 AvrRps4 (C). Bacterial titers at day 0 and day 3 were determined by taking leaf disks within the inoculated area. The data are shown as mean ± SD (n = 6), which was analyzed with one-way ANOVA and Tukey's test ($P < 0.01$). Labels with the same letters indicate no significant differences. The experiments were repeated twice with similar results. **Figure S3. Growth of *P.s.t.* DC3000 *hrcC* in wild type (WT) and *summ5* mutants.** Four-week-old soil-grown plants were inoculated with *P.s.t.* DC3000 *hrcC*. Bacterial titers at day 0 and day 3 were determined by taking leaf disks within the inoculated area. The data are shown as mean ± SD (n = 6), which was analyzed with one-way ANOVA and Tukey's test ($P < 0.01$). Labels with the same letter indicate no significant differences. The experiments were repeated twice times with similar results. **Table S1. Primers used in this study.**

Author Contributions: YL and HX conducted the experiments; YL, HX, and YZ designed the experiments and analyzed the data; YL, HX, and YZ wrote the paper.

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