Simultaneous mutations in SMN1 and SUMM2 fully suppress the dwarf and autoimmune phenotypes of Arabidopsis mpk4 mutant

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\textbf{ABSTRACT}

Disruption of the Arabidopsis mitogen-activated protein kinase pathway, MEKK1–MKK1/MKK2–MPK4 (hereafter designated as MEKK1 pathway), leads to the activation of distinct NLRs (nucleotide-binding and leucine-rich repeat receptors), TNL (TIR-type NLR) SMN1, and CNL (CC-type NLR) SUMM2, resulting in dwarf and autoimmune phenotypes. Unlike mekk1 and mkk1mkk2 mutants, the dwarf and autoimmune phenotypes of mpk4 are only partially suppressed by the summ2 mutation, suggesting a significant contribution of SMN1 to the mpk4 phenotypes. However, full suppression of mpk4 by the smn1summ2 double mutation remains to be elucidated. To address this key question, we generated a mpk4smn1summ2 triple mutant and analyzed the dwarf and constitutive cell death phenotypes. The mpk4smn1summ2 triple mutant showed restoration of plant size with no detectable cell death, indicating full suppression of the dwarf and autoimmune phenotypes. These results suggest that SMN1 and SUMM2 constitute a robust surveillance system for the MEKK1 pathway against pathogen infection.

The Arabidopsis thaliana MEKK1 pathway functions downstream of the pattern-recognition receptors and has a positive role in resistance against virulent oomycetes and bacterial pathogens.\textsuperscript{1,2} Disruption of the MEKK1 pathway leads to dwarf and autoimmune phenotypes due to constitutive defense responses such as spontaneous cell death and accumulation of reactive oxygen species.\textsuperscript{1,3–6} SUMM2, a CNL protein-encoding gene involved in this phenotype, was identified via a suppressor screen of the mkk1 mkk2 background.\textsuperscript{7} The summ2 mutation suppressed the dwarf and autoimmune phenotypes of mkk1 mkk2 and mekk1 mutants but did not fully suppress these phenotypes in the mpk4 mutant.\textsuperscript{2} In addition, the mutation in EDS1 required for TNL function also partially does, suggesting that additional component(s) such as TNL protein are likely involved in the case of mpk4.\textsuperscript{7} In our recent study, we performed novel suppressor screening to identify the components involved in dwarf and autoimmune phenotypes of the mekk1 mutants by using a dwarf autoimmune line that overexpressed the N-terminal regulatory domain of MEKK1, and we identified SMN1, also known as RPS6, encoding a TNL protein that detects the HopA1 effector.\textsuperscript{8} This suggests that TNL SMN1 and CNL SUMM2 proteins monitor the integrity of the MEKK1 pathway. In the present study, we hypothesized that SMN1 is the additional TNL protein involved in the dwarf and autoimmune phenotypes of mpk4. Along this line, we focused on testing the key hypothesis concerning the suppression of mpk4 phenotypes by simultaneous mutations of smn1 and summ2. MPK4 is also the component of the ANPs–MKK6–MPK4 pathway. It is to be noted that disruption of MPK4 results in defects in cytokinesis leading to developmental dwarfism.\textsuperscript{9} However, this is the case with Col-0 mpk4-2 mutant, which is reported to be a null allele.\textsuperscript{10} In this study, we used Ler (Landsberg erecta) background mpk4-1 mutant, which is reported to be a weak allele with no defect in cytokinesis.\textsuperscript{9} The dwarf phenotype of mpk4-1 is temperature dependent. At 28°C, morphology of mpk4-1 plant seems to be normal (Figure 1 Suppl.).

We generated a mpk4smn1summ2 triple mutant and compared the phenotypes of mpk4, mpk4smn1, and mpk4summ2. Experiments were performed as described by Takagi et al. (2019, 2020)\textsuperscript{8,11} except for that shown in Figure 2. We omitted the smn1 and summ2 single mutant controls because the effects of the single mutations to mpk4 phenotypes with the controls were already analyzed in Takagi et al. (2019) and Zhang et al. (2012), respectively.\textsuperscript{2,8} First, we analyzed the shoot length. At 24°C, the mpk4smn1summ2 triple mutants showed almost the same length as Ler (Figure 1), suggesting full suppression of the dwarf and autoimmune phenotypes of mpk4. We further observed suppression of the dwarf phenotype in the mpk4smn1 double mutant, compared to the mpk4 single mutant.\textsuperscript{8} The mpk4summ2 showed more significant suppression of the mpk4 dwarf phenotype than mpk4smn1. Consistent with previous results,\textsuperscript{2} mpk4summ2 was slightly shorter than Ler. We also analyzed the rosette sizes of mpk4, mpk4smn1, mpk4smn1summ2, and mpk4summ2. Unexpectedly, mpk4smn1summ2 was larger than Ler, suggesting full suppression of mpk4 dwarfism (Figure 2). The larger rosette size of mpk4smn1summ2 than Ler may be ascribed to a decreased cost of resistance, which is often associated with a reduction in growth,\textsuperscript{12} due to simultaneous mutations in two NLR genes.
Figure 1. Shoot morphology of Ler, mpk4-1, mpk4-1smn1-98, mpk4-1summ2-8, and mpk4-1smn1-98summ2-8 mutants. Plants were germinated and grown on GM for 10 d, and then grown in soil at 24°C until 1 month of age. Bar = 5 cm. Data is representative of three biological replicates.

Figure 2. (a) Rosette morphology of Ler, mpk4-1, mpk4-1smn1-98, mpk4-1summ2-8, and mpk4-1smn1-98summ2-8 mutants. The plants were germinated and grown in soil at 22°C for 3 weeks. Bars = 2 cm. (b) Rosette size of the plants. Values represent averages from the following replicates (Ler n = 15; mpk4-1 n = 32; mpk4-1smn1-98 n = 4; mpk4-1summ2-8 n = 17; mpk4-1smn1-98summ2-8 n = 29), and the error bars denote standard deviation. Asterisks indicate significant differences (*p < .05, **p < .001) compared to the indicated two genotypes (in Welch’s t-test).
SUMM2 and SMN1. Although mpk4summ1 and mpk4summ2 were larger than mpk4, these plants were smaller than both Ler and mpk4smn1summ2. These results are consistent with the findings of our previous study and those of Zhang et al. (2012).2,8,11

To analyze the autoimmune phenotype, we performed trypan blue staining to observe the cell death of mpk4, mpk4smn1, mpk4summ2, and mpk4smn1summ2 grown at 22°C. Consistent with the previous results, we observed a scattering pattern of stained cells in both the mesophyll and vasculature.8,11 The number of stained cells was significantly lower in mpk4summ2. Stained cells were negligible in mpk4summ2, mpk4smn1summ2, and Ler (Figure 3). Considering the results of these phenotypic analyses, both SMN1 and SUMM2 contributed to the dwarf autoimmune phenotype of mpk4. The contribution of SUMM2 to the mpk4 phenotype was more significant than that of SMN1. To analyze the suppression of autoimmune phenotypes of mpk4 via gene expression, we also compared the expression levels of defense gene markers PR1 and PR2 with those reported in our previous study8 in mpk4, mpk4smn1, mpk4summ2, and mpk4smn1summ2 grown at 22°C in soil. Compared to mpk4, PR1 and PR2 expression decreased in a graded manner in mpk4smn1, mpk4summ2, and mpk4smn1summ2 (Figure 4). The PR1 expression level was 37% lower in mpk4smn1summ2 than Ler. The PR2 expression levels were almost similar in Ler and mpk4smn1summ2. Collectively, our data suggested that mpk4smn1summ2 is comparable to Ler with respect to defense marker gene expression.

In summary, we generated a triple mutant and showed that simultaneous mutations of smn1 and summ2 fully suppress the dwarf and autoimmune phenotype of mpk4. This supports our hypothesis that the MEKK1 pathway is under the surveillance of distinct types of NLR proteins, SMN1 and SUMM2.8 In addition, disruption of the ANP2/ANP3–MKK6–MPK4 pathway also results in dwarfism and an autoimmune phenotype partially dependent on PAD4 and EDS1, which are required for TNL function.13 This may explain the partial suppression of the mpk4 phenotype by summ2 because MPK4 is shared downstream of both MEKK1–MKK1–MKK2 and ANP2/ANP3–MKK6 pathways. Therefore, it may be hypothesized that SMN1 is activated by the disruption of not only the MEKK1 pathway but also the ANP2/ANP3–
MKK6–MPK4 pathway. Generating multiple mutants of *anp2anp3* or *mkk6* with *smn1* would provide further clues to elucidate the intricate functional crosstalk of the distinct MAP kinase pathways.

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No potential conflict of interest was reported by the authors.

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