Addendum

A Novel MAPKKK Involved in Cell Death and Defense Signaling

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ABSTRACT

A high-throughput in planta overexpression screen of a Nicotiana benthamiana cDNA library identified a mitogen activated protein kinase kinase (MAPKKK), NbMKK1, as a potent inducer of hypersensitive response (HR)-like cell death. NbMKK1-mediated cell death was attenuated in plants whereby expression of NbSIPK, an ortholog of tobacco SIPK and Arabidopsis AIMPK6, was knocked down by virus-induced gene silencing (VIGS), suggesting that NbMKK1 functions upstream of NbSIPK. In accordance with this result, NbMKK1 phosphorylated NbSIPK in vitro, and furthermore NbMKK1 and NbSIPK physically interacted in yeast two-hybrid assay. VIGS of NbMKK1 in N. benthamiana resulted in a delay of Phytophthora infestans INF1 elicitin-mediated HR as well as in the reduction of resistance against a non-host pathogen Pseudomonas cichorii. Our data of NbMKK1, together with that of LeMKK4,1 demonstrate the presence of a novel defense signaling pathway involving NbMKK1/LeMKK4 and SIPK.

Mitogen activated protein kinase (MAPK) cascades are highly conserved signaling pathways in eukaryotes, comprising three tiers of protein kinase, MAPKKK (MAPKKK kinase), MAPKK and MAPK, that sequentially relay phosphorylation signals.2 The Arabidopsis genome carries genes for 20 MAPKs, 10 MAPKKs and more than 25 MAPKKKs.3 In plants, MAPK signaling is known to function in various biotic4,5 and abiotic6 stress responses and cytokinins.7 In defense signaling, extensive research has been carried out for two tobacco MAPKs, SIPK8 (salicylic-acid-induced protein kinase; hereafter designated as NtSIPK) and WIPK9 (wound-induced protein kinase = NtWIPK), and their orthologs in Arabidopsis10 (AtMPK6 and AtMPK3, respectively), partly because kinase activities of these two MAPKs are easy to detect by an in gel kinase assay using myeline basic protein (MBP) as substrate.11 Both NtSIPK and NtWIPK are activated by the interaction between host resistance (R)-gene and cognate avirulence gene of pathogen12,13 and elicitor perception by host cells.13,14 Shuqun Zhang and his group showed that an upstream kinase of both NtSIPK and NtWIPK is NtMEK2.15 Transient overexpression of constitutively active NtMEK2 caused phosphorylation of NtSIPK and NtWIPK, resulting in rapid HR-like cell death in tobacco leaves.15 Later, the same lab showed that overexpression of NtSIPK alone also caused HR-like cell death.16 The downstream target proteins of NtSIPK and AtMPK6 are being identified and include 1-aminoacyclopropane-1-carboxylic acid synthase-6 (ACS-6).17,18 Although recent studies identified another MAPK cascade (NtMEK1→Ntf6) involved in defense responses19,20 we can still say that the current research focus of MAPK defense signaling centers around the cascade comprising [NtMEK2→NtSIPK/NtWIPK→target proteins] of tobacco and its orthologous pathways in other plant species.

In an effort to search for plant genes involved in HR-like cell death, we have been employing a high-throughput in planta expression screen of N. benthamiana cDNA libraries. In this experimental system, a cDNA library was made in a binary potato virus X (PVX)-based expression vector pSfTnX.21 The cDNA library was transferred to Agrobacterium tumefaciens, and 40,000 of the bacterial colonies were individually inoculated by toothpicks onto leaf blades of N. benthamiana leaves. The phenotype around the inoculated site was observed 1-2 weeks following the inoculation. This rapid screen identified 30 cDNAs that caused cell death after overexpression, including genes coding for ubiquitin proteins, RNA recognition motif (RRM) containing proteins, a class II ethylene-responsive element binding factor (EREBP)-like protein22 and a MAPKK protein (this work). Such an in planta screening technique has been used before for the isolation of fungal23 and oomycete24,25 elicitors and necrosis inducing genes, but not for isolation of plant genes. Overexpression screening of cDNA libraries is a common practice in prokaryotes, yeast and animal cells,26 so it is a surprise that this approach...
MAPK cascade

Pathogens

MAPKKK

? ?

MAPKK

MEK2

NbMKK1 /LeMKK4

MEK1

WIPK

SIPK

NTF6

Defense responses

HR-cell death

Figure 1. Defense signaling through NbMKK1/LeMKK4. Two defense signal pathways involving NbMEK2 (indicated as MEK2) - WIPK/SIPK and NbMEK1 (indicated as MEK1) - NTF6 are well documented. By our and Pedley and Martin’s work, another novel MAPKK, NbMKK1/LeMKK4 was demonstrated to participate in defense signaling by phosphorylation of SIPK.

has not been systematically applied in plants. Given its throughput, we propose that this virus-based transient overexpression system is a highly efficient way to isolate novel plant genes by functional screen. Since overexpression frequently causes non-specific perturbation of signaling, genes identified by overexpression should be further validated by loss-of-function assays, for instance, VIGS.

Overexpression of the identified MAPKK gene, NbMKK1, triggered a rapid generation of H₂O₂, followed by HR-like cell death in N. benthamiana leaves (this work). NbMKK1-GFP fusion protein overexpression also caused cell death, and curiously NbMKK1-GFP was shown to localize consistently in the nucleus. Sequence comparison classified NbMKK1 to the Group D of MAPKs about which little information is available. So far, a MAPK, LeMKK4, from tomato belonging to the Group D MAPKs, was shown to cause cell death after overexpression. Based on amino acid sequence similarity and phylogenetic analyses, LeMKK4 and NbMKK1 seem to be orthologs. To see whether NbMKK1 transduces signals through SIPK and WIPK, we performed NbMKK1 overexpression in N. benthamiana plants whereby the expression of either NbSIPK or NbWIPK (WIPK ortholog in N. benthamiana) was silenced by VIGS. NbMKK1 did not induce cell death in NbSIPK-silenced plants, suggesting that the NbMKK1 cell death signal is transmitted through NbSIPK. Indeed, NbMKK1 phosphorylated NbSIPK in vitro, and NbMKK1 and NbSIPK physically interacted in yeast two-hybrid assay. These results suggest that NbMKK1 interacts with NbSIPK, most probably with its N-terminal docking domain, and phosphorlates NbSIPK in vivo to transduce the cell death signal downstream.

NbMKK1 exhibits constitutive expression in leaves. To determine the function of NbMKK1 in defense, we silenced NbMKK1 by VIGS, and such plants were challenged with Phytophthora infestans INF1 elicitor and Pseudomonas cichorii, a non-host pathogen. INF1-mediated HR cell death was remarkably delayed in NbMKK1-silenced plants. Likewise, plant defense against P. cichorii was compromised in NbMKK1-silenced plants. These results indicate that NbMKK1 is an important component of signaling of INF1-mediated HR and non-host resistance to P. cichorii.

Together, our analyses of NbMKK1 and independent work from Greg Martin’s lab on LeMKK4 suggest that a Group D MAPKK, NbMKK1/LeMKK4, functions upstream of SIPK and transduces defense signals in these solanaceous plants (Fig. 1). In plants as well as in other eukaryotes, it is common that kinases have multiple partners. The work on these kinases fits this concept. A single MAPK (e.g., SIPK) is phosphorylated by multiple MAPKs (e.g., NtMEK2 and NbMKK1), and a single MAPK (e.g., NbMEK2) can phosphorylate multiple MAPKs (e.g., NbSIPK and NtWIPK).

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