Plant Cell Physiol. 46(12): 1902–1914 (2005)
doi:10.1093/pcp/pci211, available online at www.pcp.oupjournals.org
JSPP © 2005

Rapid Paper

A Mitogen-activated Protein Kinase NtMPK4 Activated by SIPKK is Required for Jasmonic Acid Signaling and Involved in Ozone Tolerance via Stomatal Movement in Tobacco

Kenji Gomi [1, 5], Daisuke Ogawa [2, 3], Shinpei Katou [1], Hiroshi Kamada [2], Nobuyoshi Nakajima [3], Hikaru Saji [3], Takashi Soyano [4], Michiko Sasabe [4], Yasunori Machida [4], Ichiro Mitsuhara [1], Yuko Ohashi [1, 5] and Shigemi Seo [1, 5, *]

[1] *Plant Physiology Department, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, 305-8602 Japan*
[2] *Graduate School of Life and Environmental Sciences, Tsukuba University, Tsukuba, Ibaraki, 305-0033 Japan*
[3] *Environmental Biology Division, National Institute for Environmental Studies, Tsukuba, Ibaraki, 305-8506 Japan*
[4] *Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya, 464-8602 Japan*
[5] *Program for Promotion of Basic Research Activities for Innovative Bioscience, Minato-ku, Tokyo, 105-0001 Japan*

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

The mitogen-activated protein kinase (MAPK) cascade is involved in responses to biotic and abiotic stress in plants. In this study, we isolated a new MAPK, NtMPK4, which is a tobacco homolog of *Arabidopsis* MPK4 (AtMPK4). NtMPK4 was activated by wounding along with two other wound-responsive tobacco MAPKs, WIPK and SIPK. We found that NtMPK4 was activated by salicylic acid-induced protein kinase kinase (SIPKK), which has been isolated as an SIPK-interacting MAPK kinase. In NtMPK4 activity-suppressed tobacco, wound-induced expression of jasmonic acid (JA)-responsive genes was inhibited. NtMPK4-silenced plants showed enhanced sensitivity to ozone. Inversely, transgenic tobacco plants, in which SIPKK or the constitutively active type SIPKK^{EE} was overexpressed, exhibited greater responsiveness to wounding with enhanced resistance to ozone. We further found that *NtMPK4* was expressed preferentially in epidermis, and the enhanced sensitivity to ozone in NtMPK4-silenced plants was caused by an abnormal regulation of stomatal closure in an ABA-independent manner. These results suggest that NtMPK4 is involved in JA signaling and in stomatal movement.

Keywords: Jasmonic acid — MAP kinase — Ozone — Stomata — Wound response.

Abbreviations: ACS, 1-aminocyclopropane-1-carboxylate synthase; AOS, allene oxide synthase; GST, glutathione *S*-transferase; JA, jasmonic acid; MAPK/MPK, mitogen-activated protein kinase; MAPKK/MEK/MKK, MAPK kinase; MBP, myelin basic protein; ORF, open reading frame; PI-II, proteinase inhibitor-II; PPFD, photosynthetic photon flux density; PR, pathogenesis-related; RACE, rapid amplification of cDNA ends; RT–PCR; reverse transcription–PCR; SA, salicylic acid; SIPK, salicylic acid-induced protein kinase; SIPKK, salicylic acid-induced protein kinase kinase; WIPK, wound-induced protein kinase

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number AB212070.

Introduction

Plants are sessile organisms lacking any possible means of avoiding environmental challenges, and thus have developed a network of signaling events leading to defensive responses by producing defensive compounds. Jasmonic acid (JA) or its methyl ester (MeJA) is a plant signaling compound involved in the regulation of many stress responses and development (Turner et al. 2002). It is induced by a wide range of biotic and abiotic stresses, such as wounding, ozone exposure, water deficit and pathogen attack (Creelman and Mullet 1997, Pieterse et al. 1998, Staswick et al. 1998, Overmyer et al. 2000, Rao et al. 2000, Turner et al. 2002, Farmer et al. 2003, Rojo et al. 2003). The JA biosynthetic pathway has been well studied, and much information about the type and subcellular localization of its enzymes is available (Mueller 1997, Berger 2002, Turner et al. 2002, Li et al. 2005). In contrast, information about the JA signaling pathway is limited. The few signaling components described have mostly been identified by mutant screens for plants displaying either a reduced sensitivity to JA or a constitutive or enhanced response to JA (Weber 2002). Recent studies have revealed that some mitogen-activated protein kinase (MAPK/MPK) cascades are involved in the wound/JA signaling pathway (Zhang and Klessig 1998, Seo et al. 1999, Ichimura et al. 2000, Petersen et al. 2000, Matsuoka et al. 2002). MAPKs constitute a familiy of serine/threonine protein kinases that have been well conserved in all eukaryotes throughout evolution (Nishida and Gotoh 1993, Jonak et al. 1994, Nishihama et al. 1995). They are enzymatically activated when threonine and tyrosine residues within the TXY motif in subdomain VIII are phosphorylated (Anderson et al. 1990) by MAPK kinase (MAPKK/MEK/MKK). In the sequenced *Arabidopsis* genome, 20 genes have been identified as encoding putative MAPKs (MAPK Group 2002). In *Arabidopsis*, two MAPKs, AtMPK3 and AtMPK6, are respon-

* Corresponding author: E-mail, sseo71@affrc.go.jp; Fax, +81-29-838-7469.

sive to various forms of stress (Ichimura et al. 2000, Yuasa et al. 2001, Droillard et al. 2002). In tobacco, it has been revealed that WIPK (wound-induced protein kinase) and SIPK (salicylic acid-induced protein kinase), which are the orthologs of AtMPK3 and AtMPK6, respectively, are involved in the response to wounding and ozone (Seo et al. 1995, Zhang and Klessig 1998, Samuel and Ellis 2002). In addition, the *Arabidopsis mpk4* mutant was reported to exhibit constitutive systemic acquired resistance with elevated salicylic acid (SA) levels and AtMPK4 is required for JA-responsive gene expression (Petersen et al. 2000). Furthermore, AtMPK4 is activated by various stresses (Ichimura et al. 2000, Droillard et al. 2004), indicating that MPK4 has an important role in the plant defense system as well as AtMPK3/WIPK and AtMPK6/SIPK.

However, to our knowledge, there is no evidence about the function of MPK4 in other plant species. Recently, it has been revealed that JA signaling is similar but not the same among plant species. For example, a homolog of coronatine-insensitive1 (COI1), which is required for the JA signaling pathway, has been isolated from tomato (Li et al. 2004). The sterility of the mutant was caused by a defect in maternal control of seed maturation rather than male sterility (Li et al. 2004), whereas the *Arabidopsis coi1* mutant exhibits a male sterile phenotype (Feys et al. 1994). Thus, characterization and functional analyses of MPK4 from other plant species would be interesting and important.

In order to study the role of MPK4 in other plant species, we isolated a tobacco MPK4 gene (*NtMPK4*) and identified SIPK kinase (SIPKK) as an upstream MAPKK of NtMPK4. To elucidate the biological function of NtMPK4, we generated NtMPK4-silenced tobacco plants by introducing the inverted repeat sequence of *NtMPK4* and *SIPKK*-overexpressing tobacco plants using either wild-type SIPKK or constitutively active SIPKK^{EE} genes. Using transgenic plants, we show that NtMPK4 has an essential role in the response to wounding, and is also involved in ozone tolerance by regulating stomatal closure in an ABA-independent manner.

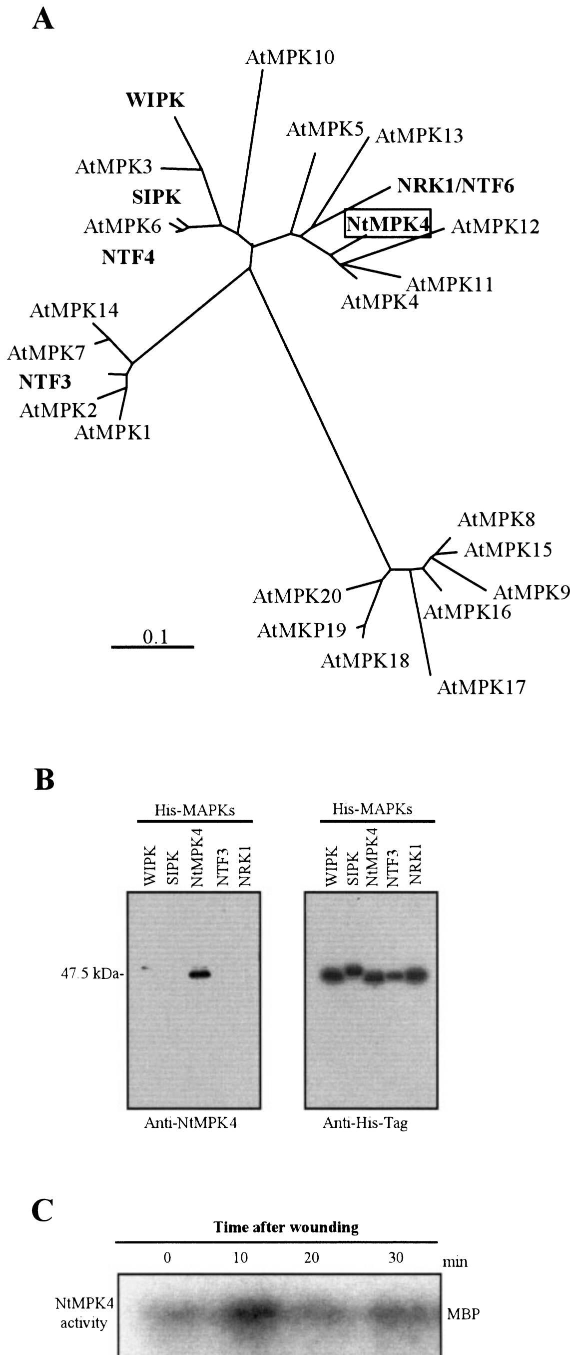


Fig. 1 NtMPK4 is a homolog of AtMPK4 and is activated by wounding. (A) Phylogenic relationship of MAPK proteins from *Arabidopsis* and tobacco. The sequence information of *Arabidopsis* and tobacco MAPKs was obtained from the Ahlfors 2004. The alignment for this analysis was created using the ClustalW program (http// www.ddbj.nig.ac.jp/E-mail/clustalw-j.html). Tobacco MAPKs are represented in bold type and NtMPK4 is represented in boxed type. (B) The specificity of NtMPK4 antibody. His-tagged MAPKs (25 ng) were loaded onto duplicate blots and subjected to immunoblot analysis with antibodies raised against NtMPK4 (anti-NtMPK4) and His-tag (anti-His-Tag). (C) Immune complex kinase assay of NtMPK4 after wounding. Leaves of 2-month-old wild-type tobacco plants were harvested at the indicated time after wounding. Crude extracts containing 50 μg of total protein were immunoprecipitated with anti-NtMPK4 antibody, and the immunoprecipitates were subjected to an immune complex kinase assay with myelin basic protein (MBP) as a substrate. Phosphorylation of MBP was detected by autoradiography after SDS–PAGE.

Results

Isolation of NtMPK4 cDNA from tobacco

Using a partial sequence of *NtMPK4* obtained by degenerate PCR amplification from RNA of tobacco leaves, we synthesized gene-specific primers for 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE. All overlapping sequences among these RACE products were identical, and the full sequence of the tobacco MPK4 cDNA, designated *NtMPK4*, was identified

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

(GenBank accession number AB212070). This gene consisted of 1,760 bp and the deduced amino acid sequence consisting of 373 amino acids contained 11 conserved kinase domains and the amino acids TEY (residues 198–200) that are required for the activation of MAPK (data not shown). To compare the amino acid sequence of NtMPK4 with that of MAPKs already isolated from tobacco and *Arabidopsis*, we produced a phylogenetic tree using the CLUSTALW algorithm and TREEVIEW program (Fig. 1A). NtMPK4 was classified into the same clade as AtMPK4 and could therefore be a homolog of AtMPK4.

Activation of NtMPK4 by wounding

The *Arabidopsis mpk4* mutant exhibits a JA-insensitive phenotype and suppresses wound-responsive gene expression, indicating that MPK4 is required for wound/JA signaling (Petersen et al. 2000). Thus, to clarify whether our newly isolated gene is also involved in these signaling pathways, we analyzed the kinase activity of NtMPK4 after wounding by immune complex kinase assay with myelin basic protein (MBP) as an artificial substrate. Before performing the experiment, we generated a specific antibody against NtMPK4 and confirmed that the antibody recognizes NtMPK4 but not WIPK, SIPK, or two other tobacco MAPKs (i.e. NTF3 and NRK1) (Fig. 1B). The kinase activity of NtMPK4 transiently increased at 10 min and declined to the basal level at 30 min in wounded tobacco leaves (Fig. 1C).

SIPKK activates NtMPK4 and NRK1 in vitro

To determine which MAPKK activates NtMPK4, we selected SIPKK as a candidate because SIPKK is classified into the same group as AtMKK1 and AtMKK2 (MAPK Group 2002), which activate AtMPK4 in vitro and in vivo (Huang et al. 2000, Matsuoka et al. 2002, Teige et al. 2004). SIPKK was originally isolated as an SIPK-interacting MAPKK in tobacco by yeast two-hybrid screening, but was reported to have no activity for the activation of SIPK (Liu et al. 2000). To verify whether SIPKK can activate NtMPK4 in vitro, we generated glutathione *S*-transferase (GST)–SIPKK and GST–SIPKK^{EE}; the latter is a constitutively active form produced by replacing both Thr220 and Thr226 with glutamic acid. On conducting in vitro kinase assays with MBP, we found that SIPKK^{EE} but not SIPKK could activate NtMPK4 (Fig. 2A). Next, we tested the activation of different tobacco MAPKs (WIPK, SIPK, NTF3 and NRK1) by SIPKK^{EE}. SIPKK^{EE} could activate NtMPK4 strongly and NRK1 slightly, indicating that SIPKK is the potential upstream MAPKK of NtMPK4 and NRK1, but not of WIPK, SIPK and NTF3 (Fig. 2B).

Generation of NtMPK4-silenced tobacco

To obtain more information on the biological function of NtMPK4 in tobacco, we attempted to generate NtMPK4-silenced plants using RNA interference technology. A 762 bp

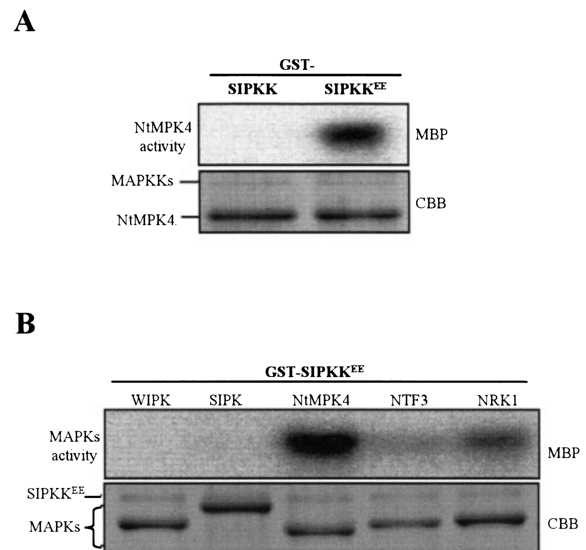


Fig. 2 Constitutively active SIPKK, SIPKK^{EE}, activates NtMPK4 and NRK1 in vitro. Tobacco MAPKs were incubated with wild-type SIPKK or SIPKK^{EE} in the kinase reaction buffer containing MBP, and the samples were separated by SDS–PAGE and subjected to autoradiography, and protein staining with Coomassie brilliant blue (CBB). (A) In vitro activation of NtMPK4 by wild-type SIPKK or constitutively active SIPKK, SIPKK^{EE}. (B) In vitro activation of different MAPKs of tobacco by SIPKK^{EE}.

region of *NtMPK4* (covering a portion of the 5′ coding region) was inserted, in inverse orientation, into the binary vector pBE2113 (Mitsuhara et al. 1996). The inverted repeat construct was introduced into tobacco plants by the *Agrobacterium*-mediated method. The second generation of nine independently transformed lines was selected and checked for the expression of the endogeneous *NtMPK4* by reverse transcription–PCR (RT–PCR) (Fig. 3A). Seven *NtMPK4*-silenced lines exhibited substantially low levels of the *NtMPK4* transcript (Fig. 3A). The two lines of NtMPK4 (NtMPK4IR-11 and NtMPK4IR-6) were used for further experiments. Both NtMPK4-silenced plants exhibited a dwarf phenotype but the NtMPK4IR-6 line was a more severe dwarf phenotype than that of the NtMPK4IR-11 line (Fig. 3B). To confirm that NtMPK4 kinase activity was suppressed in NtMPK4-silenced plants, we analyzed the activity of NtMPK4 after wounding. As shown in Fig. 3C, NtMPK4 kinase activity was clearly suppressed in both NtMPK4-silenced plants.

Generation of SIPKK- and SIPKK^{EE}-overexpressing tobacco

To strengthen our analysis of the biological function of NtMPK4, we generated *SIPKK*- and *SIPKK^{EE}*-overexpressing lines. Thirty-two (SIPKK) and 38 (SIPKK^{EE}) independent lines were resistant to kanamycin. RNA gel blot analysis revealed that nine *SIPKK*- and seven *SIPKK^{EE}*-overexpressing lines exhibited substantially elevated levels of the *SIPKK* or *SIPKK^{EE}* transcript (Fig. 4A). The second generations of each of two

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

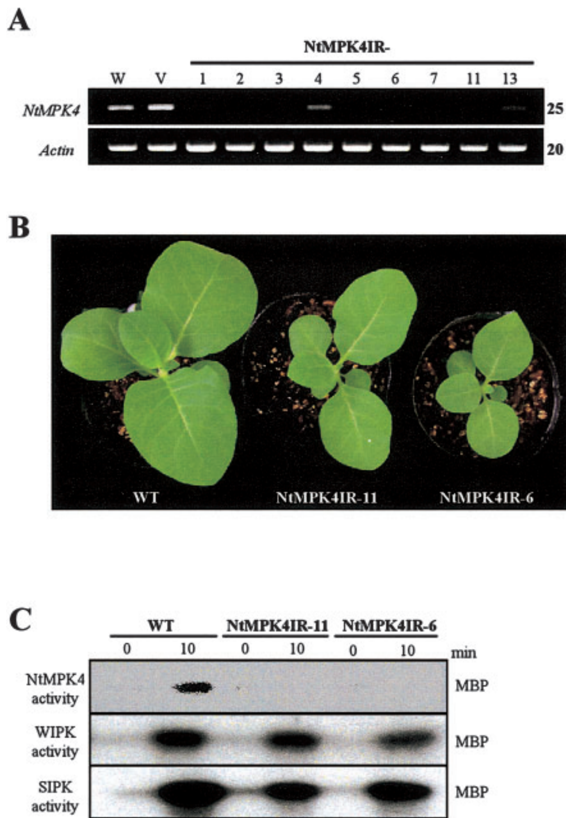


Fig. 3 Generation of NtMPK4-silenced tobacco. (A) RT–PCR analysis of leaves from wild-type and NtMPK4-silenced (NtMPK4IR) plants for expression of *NtMPK4* and *actin*. Wild-type and vector control lines are indicated by W and V, respectively. The cycle number of the RT–PCR is shown at the right. (B) Morphology of 1-month-old wild-type (WT) and NtMPK4-silenced tobacco plants. (C) MAP kinase activity in response to wounding. Leaves of 2-month-old wild-type and NtMPK4-silenced tobacco plants were harvested at the indicated time after wounding. Immune complex kinase assays were performed by using anti-NtMPK4, anti-WIPK and anti-SIPK antibodies.

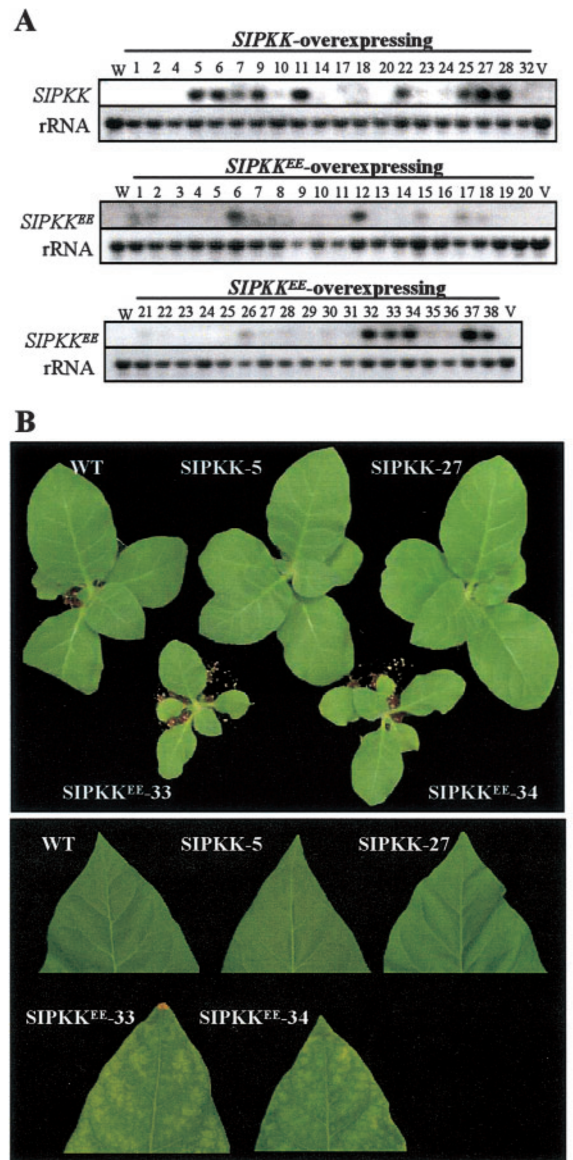


Fig. 4 Generation of *SIPKK*- and *SIPKK^{EE}*-overexpressing tobacco plants. (A) Screening of *SIPKK*- or *SIPKK^{EE}*-overexpressing transgenic plants. RNA gel blots showing *SIPKK* (upper panel) or *SIPKK^{EE}* (middle and lower panels) expression in untreated leaves. Wild-type and vector control lines are indicated by W and V, respectively. The methylene blue-stained rRNA is shown as a loading control. (B) Morphology of wild-type (WT), *SIPKK*-overexpressing and *SIPKK^{EE}*-overexpressing lines. The upper panel shows the plants of 2-month-old wild-type (WT), *SIPKK*-overexpressing, and *SIPKK^{EE}*-overexpressing lines. The lower panel shows leaves of the same age at 3 months after germination. (C) Activity of each MAPK in untreated leaves. Leaves of 2-month-old wild-type (W), *SIPKK*-overexpressing and *SIPKK^{EE}*-overexpressing plants were harvested. Immune complex kinase assays were performed by using anti-WIPK, anti-NtMPK4 and anti-SIPK antibodies. (D) Activities of NtMPK4 and NRK1 in untreated leaves. The result after 1 h (upper panel) or 24 h (lower panel) exposure is shown.

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

lines of SIPKK (SIPKK-5 and SIPKK-27) or $SIPKK^{EE}$ ($SIPKK^{EE}$-33 and $SIPKK^{EE}$-34) were used for further experiments. As shown in Fig. 4B, *$SIPKK^{EE}$*-overexpressing lines exhibited a dwarf phenotype, whereas *SIPKK*-overexpressing lines had no obvious phenotype under normal growth conditions. In addition, leaves of *$SIPKK^{EE}$*-overexpressing lines exhibited some lesion-like chlorotic spots, whereas leaves of wild-type and *SIPKK*-overexpressing lines of the same age did not (Fig. 4B). To confirm that SIPKK activates both NtMPK4 and NRK1 in planta as well as in vitro (Fig. 2B), we analyzed the activity of each MAPK under non-treatment conditions in these transgenic plants. NtMPK4 was activated in both *SIPKK*- and *$SIPKK^{EE}$*-overexpressing lines, but was more strongly activated in *$SIPKK^{EE}$*-overexpressing lines (Fig. 4C), indicating that SIPKK has the potential to activate NtMPK4 in vivo. NRK1 activity could be detected after 24 h but not 1 h exposure when considerable NtMPK4 activity was detectable (Fig. 4D). Activation of NRK1 was found in only *$SIPKK^{EE}$*-overexpressing lines (Fig. 4D). Interestingly, SIPK activity, which was detectable at 1 h exposure under non-treatment conditions, was significantly suppressed in both *SIPKK*- and *$SIPKK^{EE}$*-overexpressing lines compared with the wild type (Fig. 4C).

NtMPK4 activity alters the expression of some defense-related genes

To investigate the role of NtMPK4 in the regulation of wound-responsive genes, RNA gel blot analysis was performed on *allene oxide synthase* (*AOS*) and *proteinase inhibitor-II* (*PI-II*) in NtMPK4-silenced and wild-type plants after wounding. AOS is a key enzyme for the biosynthesis of JA and it was reported that *AOS* gene expression was increased by wounding in *Nicotiana attenuata* (Ziegler et al. 2001). *PI-II* has been used as markers of wound/JA-responsive genes (Seo et al. 1995). After wounding, the expression of *AOS* gene was not altered in NtMPK4-silenced plants compared with wild-type plants (Fig. 5A). The expression of *PI-II* was suppressed in both NtMPK4-silenced plants after wounding but was strongly suppressed in the NtMPK4IR-6 line (Fig. 5A). To clarify whether NtMPK4 is involved in JA signaling as well as AtMPK4 in *Arabidopsis*, we next performed an RT–PCR analysis of *acidic PR-1* and *NtWRKY3*, which were SA-inducible genes (Sasaki et al. 2002, Chen and Chen 2000) in NtMPK4-silenced plants, because the antagonistic effect of SA on JA signaling was well known (Sasaki et al. 2002). As shown in Fig. 5, expression of *acidic PR-1* and *NtWRKY3* was up-regulated in NtMPK4-silenced plants under non-treatment conditions, indicating that NtMPK4 was involved in the JA signaling pathway.

To confirm the above results, we performed similar experiments using *SIPKK*- and *$SIPKK^{EE}$*-overexpressing lines. As shown in Fig. 5C, the expression of *NtWRKY3* was suppressed in *$SIPKK^{EE}$*-overexpressing lines under non-treatment conditions. After wounding, expression of the *PI-II* gene was induced more strongly in *SIPKK*-overexpressing lines, or more

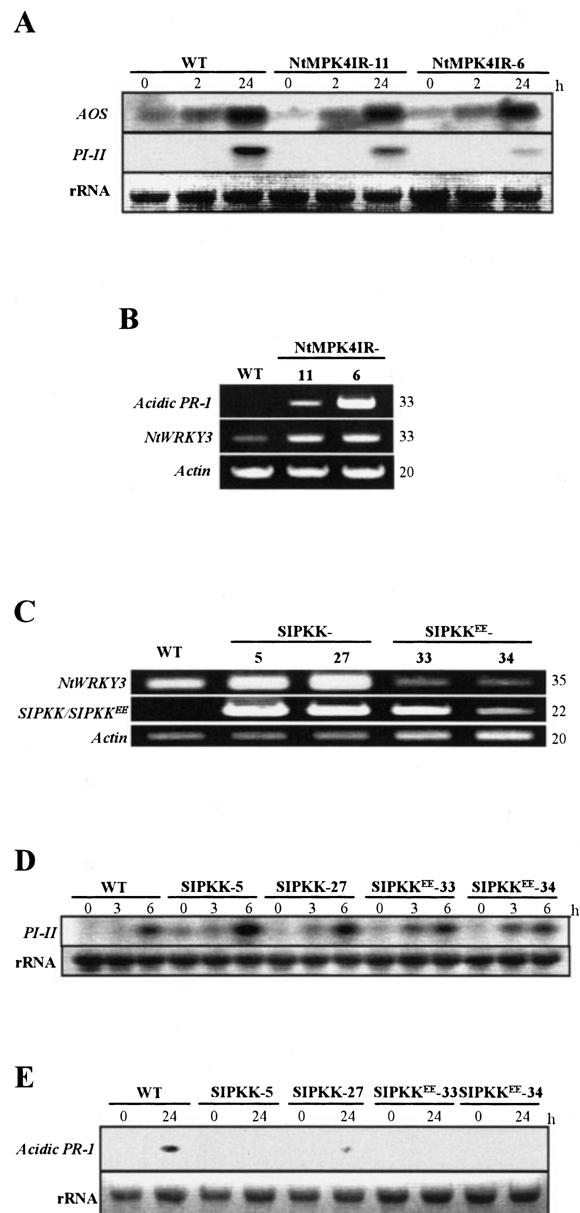


Fig. 5 Differential expression of wound- and SA-responsive genes in NtMPK4-silenced, *SIPKK*-overexpressing and *$SIPKK^{EE}$*-overexpressing tobacco plants. (A) Effect of *NtMPK4* silencing on induction of wound-responsive genes at 0, 2 and 24 h after wounding. (B) RT–PCR analysis of SA-responsive genes in untreated leaves of wild-type (WT) and NtMPK4-silenced plants. The level of actin was assayed as a control. The cycle number of the RT–PCR is shown at the right. (C) RT–PCR analysis of the *NtWRKY3* gene in untreated leaves of wild-type (WT), *SIPKK*-overexpressing and *$SIPKK^{EE}$*-overexpressing plants. The level of actin was assayed as a control. The cycle number of the RT–PCR is shown at the right. (D) Effect of *SIPKK* or *$SIPKK^{EE}$* on induction of the wound-responsive gene at 0, 3 and 6 h after wounding. (E) Effect of *SIPKK* or *$SIPKK^{EE}$* on induction of *acidic PR-1* at 0 and 24 h after SA treatment.

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

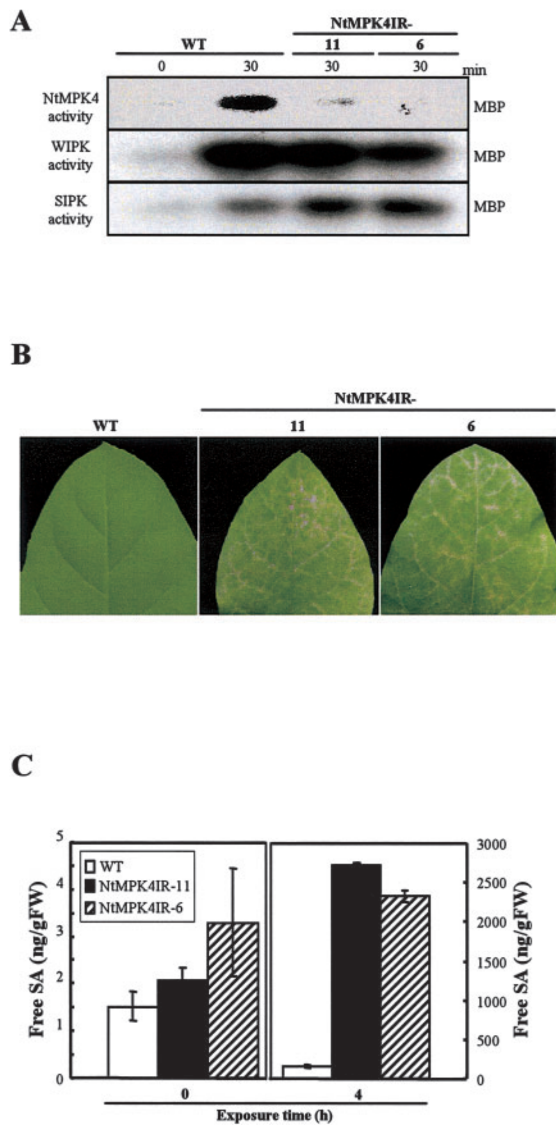
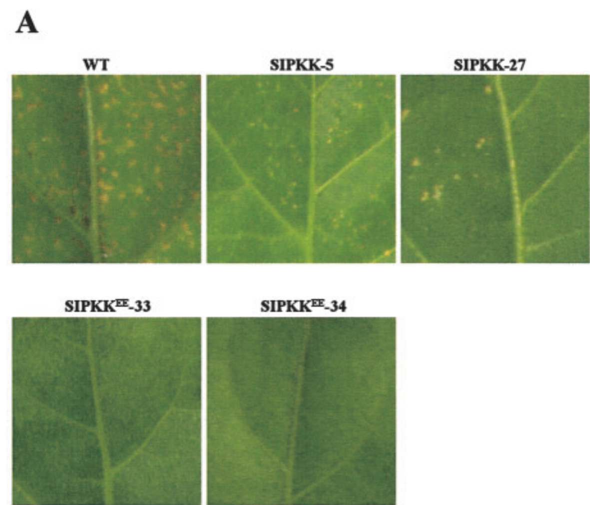


Fig. 6 Activity of NtMPK4 is involved in ozone tolerance. (A) NtMPK4 activity after exposure to ozone. Leaves of 1-month-old wild-type (WT) and NtMPK4-silenced plants were harvested at the indicated time after ozone exposure (0.2 ppm), and immune complex kinase assays were performed using anti-NtMPK4, anti-WIPK and anti-SIPK antibodies. (B) Morphology of wild-type (WT) and NtMPK4-silenced leaves of the same age at 3 d after the onset of a 4 h exposure to ozone (0.2 ppm). (C) Free SA content at 4 h after ozone exposure (0.2 ppm) in wild-type (WT) and NtMPK4-silenced leaves of the same age. Values are means ± SD of six samples.



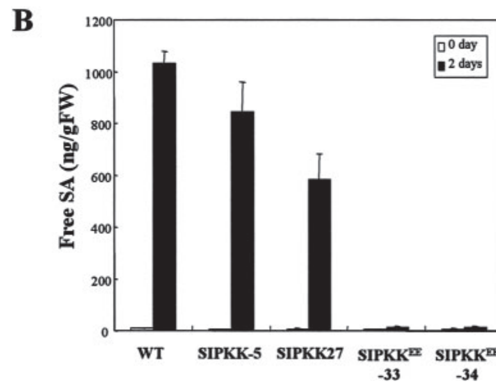


Fig. 7 *SIPKK*- and *SIPKK^{EE}*-overexpressing lines are resistant to ozone. (A) Morphology of wild-type (WT), *SIPKK*-overexpressing and *SIPKK^{EE}*-overexpressing leaves of the same age after the onset of a 2 d exposure to ozone (0.2 ppm). The same region of the leaf was photographed. The experiments were repeated three times with similar results. (B) Free SA content after a 2 d exposure to ozone (0.2 ppm) in wild-type (WT), *SIPKK*-overexpressing and *SIPKK^{EE}*-overexpressing leaves of the same age. Values are means ± SD of six samples.

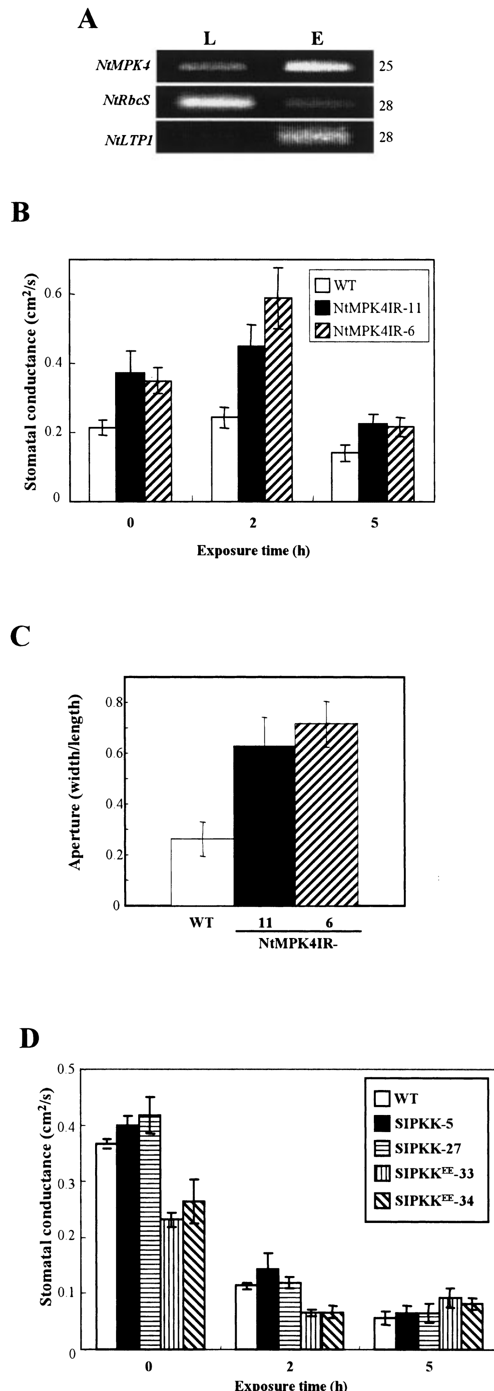
rapidly in *SIPKK^{EE}*-overexpressing lines, compared with the wild type (Fig. 5D). Furthermore, expression of *acidic PR-1* after treatment with SA was suppressed in both transgenic lines (Fig. 5E). These results strongly indicate that NtMPK4 regulates part of the wound-based JA signaling pathway positively and the SA signaling pathway negatively.

NtMPK4-silenced plants are sensitive to ozone

The ethylene and JA signaling pathways act synergistically in induced systemic resistance (Pieterse et al. 1998) and in regulating the expression of pathogenesis-related (PR) and wound-inducible genes (O'Donnell et al. 1996, Norman-Setterblad et al. 2000). Thus we asked the question whether NtMPK4 is required for ethylene signaling because expression of *PI-II* was also induced by ethylene (Sasaki et al. 2002). To clarify whether NtMPK4 acts as a regulator of JA signaling, we analyzed the sensitivity of NtMPK4-silenced plants to ozone, because it has been revealed that ethylene and JA have opposite effects on the response to ozone. Ethylene stimulates cell

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

death, requiring for the spreading cell death, whereas JA protects tissues from cell death (Mehlhorn and Wellbum 1987, Overmyer et al. 2000, Rao et al. 2000, Nakajima et al. 2002, Tuominen et al. 2004). Furthermore, it has been reported that SIPK/AtMPK6 and WIPK/AtMPK3 are activated by ozone in tobacco and *Arabidopsis* (Samuel and Ellis 2002, Ahlfors et al. 2004). To determine whether NtMPK4 is involved in the response to ozone, first we analyzed the NtMPK4 kinase activity after ozone exposure. As shown in Fig. 6A, when wild-type plants were exposed to 0.2 ppm of ozone for 30 min, NtMPK4 activity increased as well as WIPK and SIPK activity. SIPK activity was significantly stronger in NtMPK4-silenced plants than wild-type plants, while WIPK activity was little changed (Fig. 6A). Next, when we observed a visible change of NtMPK4-silenced plants, we found no visible change after exposure to 0.2 ppm of ozone for 4 h (data not shown). However, obvious necrotic lesions were found in NtMPK4-silenced leaves at 3 d after the onset of a 4 h exposure to ozone, while no necrosis was visible in wild-type leaves under the same conditions (Fig. 6B). This result strongly indicates that NtMPK4 functions in the JA signaling pathway but not the ethylene signaling pathway and its activity is required for ozone tolerance. The suppression of the NtMPK4 kinase activity in these plants was confirmed by immune complex kinase assay (Fig. 6A).



Next, we measured the amount of SA in NtMPK4-silenced plants exposed to ozone, because it has been reported that SA signaling is involved in the induction of cell death upon exposure to ozone (Örvar et al. 1997, Rao et al. 2000). At a concentration of ozone that caused no visible symptoms in wild-type plants (0.2 ppm for 4 h), NtMPK4-silenced plants accumulated free SA at 14.0- to 16.4-fold compared with that in the wild-type plants (Fig. 6C).

To confirm the above results, *SIPKK*- and *SIPKK^{EE}*-overexpressing lines were exposed to 0.2 ppm of ozone for 2 d. Under these conditions, many necrotic lesions formed on wild-type leaves, while fewer lesions developed in the *SIPKK*-overexpressing lines and no necrotic lesions in the *SIPKK^{EE}*-overexpressing lines (Fig. 7A). Notably, significantly less SA accumulated in *SIPKK^{EE}*-overexpressing lines exposed to ozone for 2 d than in the wild type (Fig. 7B). In the *SIPKK*-overexpressing lines, SA accumulation was slightly reduced compared with that in the wild type (Fig. 7B) with a slight reduction of necrotic lesions (Fig. 7A). The overexpression of

Fig. 8 NtMPK4 is involved in stomatal regulation. (A) RT–PCR analysis of the *NtMPK4* gene in leaves (L) and abaxial epidermis (E). *NtLTP1* as a highly expressed gene in epidermal tissue (Canevascini et al. 1996), and *NtRbcS* as a gene expressed to only a small extent (Kopka et al. 1997) were used as controls. The cycle number of the RT–PCR is shown at the right. (B) Changes in stomatal conductance of ozone-exposed wild-type (WT) and NtMPK4-silenced plants. One-month-old plants were exposed to ozone (0.2 ppm). Diffusion resistance was measured in the light (PPFD, 300 μE m^{-2} s^{-1}) by using a diffusion porometer. The data were then processed and expressed as stomatal conductance. Values are means ± SD of five independent points per leaf in two replicate measurements. The experiments were repeated twice with similar results. (C) Stomatal apertures of wild-type (WT) and NtMPK4-silenced plants. The width and length of at least 70 stomatal pores were measured and used to calculate the average stomatal aperture (width/length). Values are means ± SD. (D) Changes in stomatal conductance of ozone-exposed wild-type (WT), *SIPKK*-overexpressing and *SIPKK^{EE}*-overexpressing plants. Two-month-old plants were exposed to ozone (0.2 ppm). Values are means ± SD of five independent points per leaf in two replicate measurements. The experiments were repeated three times with similar results.

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

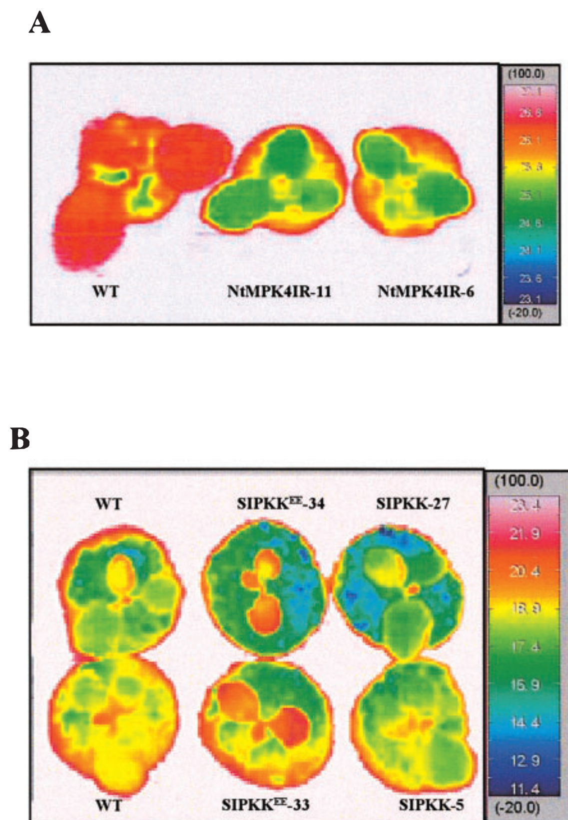


Fig. 9 NtMPK4 activity affects transpiration. (A) Pseudo-color infrared image of 1-month-old wild-type (WT) and NtMPK4-silenced plants under normal growth condition. (B) Pseudo-color infrared image of 1-month-old wild-type (WT), *SIPKK*-overexpressing and *SIPKK*^{EE}-overexpressing plants under normal growth conditions.

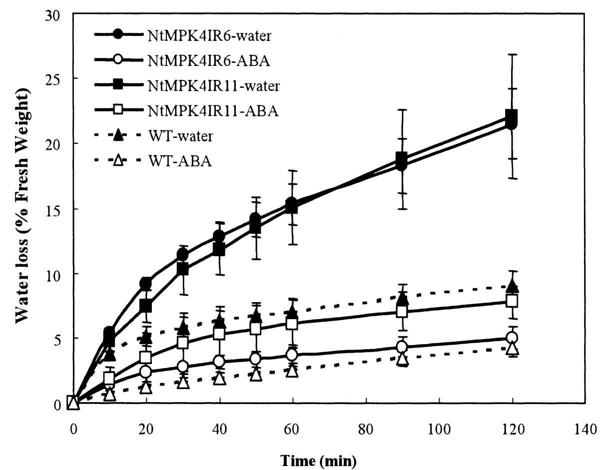


Fig. 10 NtMPK4 does not affect ABA signaling for stomatal closure. Kinetics of water loss from ABA-treated leaves of the wild-type (WT) and NtMPK4-silenced plants. Detached leaves were incubated in water containing ABA (20 μM) for 12 h. Then, the water loss from four detached leaves was measured. Water loss is expressed as a percentage of the initial fresh weight. Values are means ± SD of four leaves each. The experiments were repeated twice with similar results.

the *SIPKK* and *SIPKK*^{EE} genes in these plants was confirmed by RT–PCR (data not shown).

NtMPK4 is involved in stomatal regulation

How is NtMPK4 involved in the response to ozone through the JA signaling pathway? Ozone enters the plant mesophyll tissue through the stomata and diffuses through inner air spaces. It has been reported that the *AtMPK4* gene is strongly expressed in guard cells (Petersen et al. 2000) and that JA signaling is required for stomatal closure (Suhita et al. 2004). Thus, we performed RT–PCR for *NtMPK4* using total RNA extracted from abaxial epidermal peels. As control genes, we used *NtLTP1*, whose expression was higher in epidermal tissue and the guard cells (Canevascini et al. 1996), and *RbcS*, whose expression was lower in the epidermis (Kopka et al. 1997). As shown in Fig. 8A, the *NtMPK4* gene was highly expressed in abaxial epidermis. Thus, we predicted that the highly sensitive phenotype of NtMPK4-silenced plants was caused by abnormal regulation of stomatal closure. To evaluate whether NtMPK4 activity is required for stomatal regulation, the diffusion resistance of NtMPK4-silenced and wild-type plants during ozone exposure was measured and used to calculate the stomatal conductance. Surprisingly, NtMPK4-silenced plants showed approximately 1.5-fold higher stomatal conductance than wild-type plants at 0 time (Fig. 8B). Furthermore, the stomatal conductance of NtMPK4-silenced plants was approximately 1.5-fold greater than that of wild-type plants after 5 h of ozone exposure (Fig. 8B). Consistent with the above result, stomatal aperture was significantly greater in NtMPK4-silenced plants than wild-type plants under normal growth conditions (Fig. 8C). The density of stomata was similar in both wild-type and NtMPK4-silenced plants (data not shown). On the other hand, the *SIPKK*^{EE}-overexpressing line showed significantly lower stomatal conductance than the wild type until 2 h after the onset of ozone exposure, while the *SIPKK*-overexpressing line was similar to the wild type (Fig. 8D). These results indicate that NtMPK4 activity is required for the regulation leading to stomatal closure.

The greater stomatal conductance in NtMPK4-silenced plants also had an effect on transpiration. Leaf temperature in NtMPK4-silenced plants measured by thermal imaging was significantly lower than that of the wild type (Fig. 9A). When the temperature of *SIPKK*^{EE}-overexpressing lines was monitored, quite the opposite was found, confirming that NtMPK4 activity is needed for stomatal closure (Fig. 9B).

NtMPK4 regulates stomatal closure in an ABA-independent manner

The abnormal regulation of stomatal closure prompted us to determine whether ABA has a role in NtMPK4-silenced plants. ABA is well known as a regulator of stomatal closure (Suhita et al. 2004). To study the effect of ABA, we measured

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

transpirational water loss after ABA treatment in NtMPK4-silenced leaves. Transpirational water loss was 9 and 4% in untreated and ABA-treated wild-type leaves at 120 min, respectively (Fig. 10). In NtMPK4-silenced leaves, it was approximately 22 and 6% in untreated and ABA-treated leaves, respectively (Fig. 10). This result indicates that the response to ABA in stomata is almost normal in NtMPK4-silenced plants, and NtMPK4 might regulate stomatal closure in an ABA-independent manner.

Discussion

In this study, we generated NtMPK4-silenced or -activated transgenic plants, and demonstrated that, in tobacco, activation of NtMPK4 is required for the wound-based JA signaling pathway and has an essential role in the response to ozone. We also identified SIPKK as a possible upstream MAPKK of NtMPK4 and NRK1. In *Arabidopsis*, AtMKK2 activates both AtMPK4 and AtMPK6, which are a homolog of NtMPK4 and an ortholog of SIPK respectively, and AtMKK1 activates only AtMPK4 (Teige et al. 2004). On the other hand, SIPKK could activate NRK1, which was from the same clade as AtMPK5 and AtMPK13, but not SIPK. Neither AtMKK1 nor AtMKK2 interacts with AtMPK5 or AtMPK13 (Teige et al. 2004). These results suggest that SIPKK might not be a tobacco ortholog of AtMKK1/AtMKK2 and its function is different from that of these MAPKKs. This idea is supported by the phenotype of each transgenic plant. AtMKK2^{EE}-overexpressing plants had no obvious phenotype, but the *SIPKK^{EE}*-overexpressing tobacco plants showed a dwarf phenotype (Teige et al. 2004, Fig. 4B).

NRK1 was isolated by yeast two-hybrid screening using NQK1 (NtMEK1) as bait and is involved in cytokinesis (Soyano et al. 2003). It has been reported that NQK1 activates NRK1 in vitro and in vivo. In this study, we found that SIPKK also activates NRK1, suggesting the possible involvement of SIPKK as well as NQK1 in cytokinesis. These results are consistent with the dwarf phenotype of *SIPKK^{EE}*-overexpressing lines, which may be caused by acceleration of the cell cycle due to hyperactivation of NRK1. Although we do not have any direct evidence at this point, it would be interesting to study this transgenic plant from the viewpoint of cytokinesis.

In the *Arabidopsis mpk4* mutant, the induction of JA- and wound-responsive genes was blocked (Petersen et al. 2000), suggesting that AtMPK4 is a positive regulator in the wound/JA signaling pathway. In this study, we demonstrated that wound-induced expression of the *AOS* gene, encoding a key enzyme for JA biosynthesis, was not altered, but that of *PI-II* was altered in NtMPK4-silenced plants. These results indicate that NtMPK4 regulates a part of wound signaling in tobacco. Furthermore, we demonstrated that NtMPK4-silenced plants exhibited the antagonistic effect of SA on JA signaling, indicating that NtMPK4 was involved in JA signaling. In addition, *SIPKK^{EE}*-overexpressing lines exhibited spontaneous chlorotic spots with leaf senescence (Fig. 4B). As JA signaling is involved in leaf senescence in *Arabidopsis* (He et al. 2002, Xiao et al. 2004), this phenomenon may be explained by hyper-acceleration of NtMPK4-mediated JA signaling.

Recently, plant MAPK cascades have been suggested to be involved in the response to ozone. SIPK/AtMPK6 and WIPK/AtMPK3 are activated on exposure to ozone in tobacco and *Arabidopsis* (Samuel and Ellis 2002, Ahlfors et al. 2004). In this study, we revealed that NtMPK4 was activated by ozone, as were WIPK and SIPK (Fig. 6A), suggesting that NtMPK4-mediated signaling has an important role in the modulation of the cellular response to ozone. Generally, ethylene and JA seem to have opposite effects on this response. For example, JA protects tobacco plants from ozone-induced cell death when applied before ozone treatment (Örvar et al. 1997). The *Arabidopsis* JA-insensitive mutant *jar1* and the JA-defective *fad3 fad7 fad8* triple mutant are highly ozone sensitive (Overmyer et al. 2000, Rao et al. 2000, Tuominen et al. 2004). On the other hand, the ethylene-insensitive mutant *ein2* is ozone tolerant (Tuominen et al. 2004). Tobacco transgenic plants, in which antisense DNA for an ozone-inducible 1-aminocyclopropane-1-carboxylate synthase (ACS) from tomato was introduced, showed an ozone-tolerant phenotype (Nakajima et al. 2002). In this study, we found that NtMPK4-silenced plants were highly sensitive to ozone (Fig. 6B), indicating that NtMPK4 functions in the JA signaling but not the ethylene signaling pathway. This is consistent with the finding that *Arabidopsis* AtMPK4 does not act in the ethylene signaling pathway (Petersen et al. 2000). More recently, it has been reported that the *ACS* gene family except *ACS1* and *ACS9* in *Arabidopsis* is highly expressed in the epidermal cell layer and guard cells (Tsuchisaka and Theologis 2004). To our knowledge, there is no evidence of antagonistic or synergetic interaction between the ethylene and JA signaling pathways in epidermal cells or guard cells. Studies using transgenic plants or mutants for each hormone signaling pahway may provide new findings regarding the interactions between the ethylene and JA signaling pathways in the epidermis.

Interestingly, SIPK activity was significantly stronger in NtMPK4-silenced plants than wild-type plants after exposure to ozone (Fig. 6A). Recently, it has been reported that AtMPK6/SIPK was involved in the biosynthesis of ethylene through the phosphorylation of ACS2 and ACS6, which is the rate-limiting enzyme of the biosynthesis (Liu and Zhang 2004). Thus, the marked activation of SIPK may be caused by an antagonistic effect of ethylene on NtMPK4-mediated JA signaling. However, surprisingly, both the overexpression and the suppression of SIPK caused great sensitivity to ozone in tobacco (Samuel and Ellis 2002). In the above scenario, SIPK-silenced plants should be resistant to ozone. Although it is not clear why the suppression of SIPK caused such sensitivity, one possible explanation of this phenotype is the putative abnormal hyperactivation of WIPK in SIPK-silenced tobacco, in

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

which the hyperactivation continued after exposure to ozone (Samuel and Ellis 2002).

It has been reported that SA was accumulated in ozone-exposed plants and its signaling was involved in promoting cell death (Örvar et al. 1997, Rao et al. 2000). Under normal growth conditions, the level of SA in NtMPK4-silenced plants was similar to that in wild-type plants. In the *Arabidopsis mpk4* mutant, the SA level is higher than in the wild type (Petersen et al. 2000). This may be a result of the difference between the 'knock-out mutant' and 'knock-down transgenic plant', because expression of the *acidic PR-1* and *NtWRKY3* genes in NtMPK4-silenced plants could be detected by RT–PCR analysis but not RNA gel blot analysis (Fig. 5B, data not shown). After exposure to ozone, more SA was accumulated in NtMPK4-silenced plants than in wild-type plants (Fig. 6C), suggesting that the sensitivity of NtMPK4-silenced plants depends on the accumulation of SA.

Our data shown in Fig. 5E, 6C and 7B revealed that NtMPK4 activity influences the SA biosynthesis and signaling pathways by regulating JA signaling, although precisely how is not clear. One possible explanation is a positive feedback of SA signaling via NtMPK4. Indeed, the *Arabidopsis mpk4* mutant exhibits high-level expression of the *PR1* gene independent of NPR1, which is a key regulatory component of SA signaling and thought to regulate a negative feedback pathway in the signaling (Shah 2003, Dong 2004).

We demonstrated that NtMPK4-silenced plants had high stomatal conductance, and reduced stomatal closure on exposure to ozone (Fig. 8B), indicating that the NtMPK4-silenced plants could not restrict the influx of ozone into leaf tissue. Thus, the sensitivity to ozone in NtMPK4-silenced plants is probably caused by an acceleration of cell death following the accumulation of SA due to an excessive influx of ozone. The *Arabidopsis jar1* mutant exhibits similar stomatal conductance compared with wild type (Tuominen et al. 2004), suggesting that the NtMPK4-mediated JA pathway is distinct from the JAR1-mediated JA pathway. Interestingly, the stomatal aperture was significantly greater in NtMPK4-silenced plants than in wild-type plants under normal growth conditions (Fig. 8C). *Arabidopsis ost1*, which is an ABA-insensitive mutant in terms of stomatal closure, and *jar1* mutants exhibit a similar stomatal aperture to the wild type under normal growth conditions (Mustilli et al. 2002, Suhita et al. 2004). This suggests that NtMPK4 is involved not only in the response to stress, but also in the developmental regulation of stomata. Recently, it has been reported that some MAPKKKs control stomatal development in tobacco and *Arabidopsis* (Nishihama et al. 2001, Bergmann et al. 2004), indicating that the MAPK cascade has an important role in stomatal development. However, we found that the density of stomata was similar in both wild-type and NtMPK4-silenced plants. Furthermore, the nuclear division of guard cells in NtMPK4-silenced plants was normal (data not shown). These results suggest that NtMPK4-mediated stomatal regulation is distinct from that of other known MAPK cascades.

The abnormal regulation of stomatal closure prompted us to examine the altered sensitivity to ABA in NtMPK4-silenced plants. We demonstrated that the stomatal response to ABA in NtMPK4-silenced plants was almost normal (Fig. 10). Consistent with our results, AtMPK4 was activated by low humidity but not by ABA in *Arabidopsis* (Ichimura et al. 2000). These results suggest that NtMPK4 is not involved in the ABA signaling pathway for stomatal regulation. In comparison with ABA signaling, very few studies have focused on the JA signaling pathway leading to stomatal closure. Suhita et al. (2004) conducted a detailed examination of the role of JA signaling in stomatal closure, finding that protein phosphorylation is an important factor by using protein kinase inhibitors.

Interestingly, SIPK activity was suppressed in both *SIPKK*- and *SIPKK^{EE}*-overexpressing lines (Fig. 4C). This phenomenon would be explained by the putative formation of an SIPKK–SIPK complex which inhibits SIPK activity, because SIPKK can interact with SIPK in vitro but was reported to have no activity for the activation of SIPK (Liu et al. 2000). Further study, such as an immunoprecipitation assay, on the SIPKK complex is necessary.

Taken together, our findings strongly indicate that the activation of NtMPK4 has an important role in transmitting a JA-mediated signal and ozone sensitivity. A number of issues, however, still remain to be addressed such as the relationship among NtMPK4, WIPK and SIPK in the response to stress, and identification of downstream targets of NtMPK4.

Materials and Methods

Plant materials, wounding and salicylic acid treatment

Tobacco (*Nicotiana tabacum* cv. Samsun NN) plants were grown in a temperature-controlled greenhouse maintained at 25°C with a 16 h light/8 h dark cycle. The wounding of leaves was performed by cutting them into small pieces with a razor blade. For SA treatment, pieces of tobacco leaf were floated on water containing 50 µM SA.

cDNA cloning

Total RNA from leaves was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For the isolation of *NtMPK4*, degenerated oligonucleotides were made from the sequence conserved between *Arabidopsis* AtMPK4 (Mizoguchi et al. 1993) and *Medicago* MMK2 (Jonak et al. 1995), which fell into the same group (MAPK Group 2002), and used as primers in PCR amplification. A full-length cDNA of NtMPK4 was isolated by using a SMART RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Palo Alto, CA, USA). Partial cDNA of *NtAOS* was amplified from the SMART cDNA library by using degenerated primers made from the sequence of *Nicotiana attenuate* (Ziegler et al. 2001). DNA sequencing was performed with a DNA sequencing kit and ABI DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Production of NtMPK4 antibody and immunoblot analysis

A polyclonal antiserum was raised against the synthetic peptide SGDQGVQSNFK at the N-terminus of NtMPK4. Immunoblot analysis using anti-NtMPK4 and anti-His-Tag antibodies (Novagen,

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

Darmstadt, Germany) was performed as described previously (Seo et al. 1999).

Expression and purification of fusion proteins

The open reading frames (ORFs) of *NtMPK4*, *WIPK* (D61377), *SIPK* (U94192), *NTF3* (X69971) and *NRK1* (AB055515) were amplified by PCR using gene-specific primers. These amplicons were cloned in-frame into the expression vector pET32a (Novagen, Madison, WI, USA). These fusion proteins produced in *Escherichia coli* were purified according to the manufacturer's instructions. The ORFs of *SIPKK* and $SIPKK^{EE}$ were cloned into pGEX4T-1 (Amersham Pharmacia Biotech, Buckinghamshire, UK), and the GST-fused proteins produced in *E. coli* were purified according to the manufacturer's directions. Site-directed mutagenesis of *SIPKK* was performed by using a Site-directed Mutagenesis System (TAKARA SHUZO CO. LTD, Tokyo, Japan) following the instructions provided. The amino acids replaced and the sites of replacements are described in the Results.

In vitro kinase assay and immune complex kinase assay

Each MAPK (500 ng) and MBP (5 μg, Sigma, St Louis, MO, USA) were incubated with GST–SIPKK or GST–$SIPKK^{EE}$ (50 ng) at 30°C for 20 min in a kinase reaction buffer containing 40 mM HEPES-KOH, pH 7.5, 20 mM $MgCl_2$, 2 mM dithiothreitol, 100 μM ATP and 50 nM [γ-^{32}P]ATP (Amersham; ~4,000 Ci/mmol). The immune complex kinase assay was performed by using anti-NtMPK4, anti-WIPK, anti-SIPK and anti-NRK1 antibodies according to Seo et al. (1999). Reaction products were separated by SDS–PAGE, and phosphorylation of MBP was analyzed by autoradiography.

Construction of NtMPK4-silencing, SIPKK-overexpressing and SIPKKEE-overexpressing vectors, and tobacco transformation

The double-stranded RNA interference construct was tailored through a PCR-mediated approach using a 762 bp region of *NtMPK4* (corresponding to positions 141–902). The amplified DNA fragments were inserted, in inverse orientation and separated by a β-glucuronidase sequence linker, into the binary vector pBE2113 vector (Mitsuhara et al. 1996). cDNAs of *SIPKK* and $SIPKK^{EE}$ were also subcloned into the same vector. These constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation (Wen-jun and Forde 1989). Transformation of Samsun NN tobacco was performed by using the leaf disc co-cultivation method (Horsch et al. 1985). Transformed plants were selected by growth on kanamycin-containing media. Plants of the second generation were used for the experiments.

RT–PCR and RNA gel blot analysis

Total RNA from leaves and abaxial epidermis was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. RT–PCR was performed with RT–PCR High-plus- (TOYOBO, Osaka, Japan) using gene-specific primers designed to target *NtMPK4*, *actin* (X63603), *RbcS* (AY220079), *NtLTP1* (X62395), *acidic PR-1* (X06361), *NtWRKY3* (AF193770) and *SIPKK* (AF165186). The number of cycles was adjusted so that the amplification was within the linear range. Sequences used for RT–PCR were as follows: *NtMPK4*, forward, 5′-GATCACCTGATGATGCCAGT-3′ and reverse, 5′-GAGCTGTGAACTCACCTCCA-3′; *actin*, forward, 5′-CAGGGTTTGCTGGAGATGATGCTC-3′ and reverse, 5′-TGAATGCCTGCAGCTTCCATTCC-3′; *RbcS*, forward, 5′-CCTCTGCAGCAGTTGCCACT-3′ and reverse, 5′-GTAGCCTTCAGGCTTGTAGGC-3′; *NtLTP1*, forward, 5′-TTGTGCATGGTGGTAGCTGC-3′ and reverse, 5′-CTTCCAAATTGTGCGGGG-3′; *acidic PR-1*, forward, 5′-GTAATATCCCACTCTTGCCGTGCC-3′ and reverse, 5′-CGTGAAATGGACGTAGGTCG-3′; *NtWRKY3*, forward, 5′-CCAACACGCTTGCAACTC-3′ and reverse, 5′-AGTCATAAGGATTAACAATC-3′; *SIPKK/SIPKK*^{EE}, forward 5′-CGCTCTCAAGGCTATTCAG-3′ and reverse, 5′-TTATAACTCAGTGAGTGTTGCC-3′. The identity of these RT–PCR products was confirmed by sequencing. Total RNA blotting, hybridization, labeling of cDNA probes and RNA gel blot analysis were performed as described previously (Seo et al. 1999).

Ozone exposure, stomatal conductance, stomatal aperture, thermal imaging and water loss

Ozone treatment was performed at 0.2 ppm. The ozone chamber was maintained at 25°C and a relative humidity of 70% under a photosynthetic photon flux density (PPFD) of 300 μE m^{-2} s^{-1}. Ozone was generated with an ozone generator (Sumitomo Seika Chemicals, Tokyo, Japan). After exposure, the same aged leaf of each plant was photographed. Diffusion resistance at five independent points per leaf was measured in the light (PPFD, 300 μE m^{-2} s^{-1}) by using a diffusion porometer (Super porometer, LI-1600; LiCor, Lincoln, NE, USA). The data were then processed and indicated as stomatal conductance. For the measurement of stomatal aperture, abaxial epidermal peels were fixed with cold ethanol at –20°C for 15 min. The width and length of at least 70 stomatal pores were measured with an optical microscope and used to calculate the average stomatal aperture (width/length). Water loss from four detached leaves was measured as described by Leung et al. (1997). For ABA treatment, detached leaves were incubated in water containing ABA (20 μM) for 12 h. Then, the water loss from four detached leaves was measured as described above. Thermal images of plants were obtained using a Thermotracer TH7102MV (NEC san-ei, Japan).

Quantification of SA

Quantification of free SA was performed as described previously (Seo et al. 1995).

Acknowledgments

We thank Hidemi Wakikado (National Agricultural Research Center) for lending us the Thermotracer TH7102MV. This work was supported by the Program for the Promotion of Basic Research Activities for Innovative Biosciences, by a Grant-in-Aid for Scientific Research on Priority Areas (Molecular Mechanisms of Plant–Pathogenic Microbe Interaction) and by a grant from the Japan Society for the Promotion of Science Research Fellowship for Young Scientists.

References

Ahlfors, R., Macioszek, V., Rudd, J., Brosché, M., Schlichting, R., Scheel, D. and Kangasjärvi, J. (2004) Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thaliana* during ozone exposure. *Plant J.* 40: 512–522.

Anderson, N.G., Maller, J.L., Tonks, N.K. and Sturgill, T.W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 343: 651–653.

Berger, S. (2002) Jasmonate-related mutants of *Arabidopsis* as tools for studying stress signaling. *Planta* 214: 497–504.

Bergmann, D.C., Lukowitz, W. and Somerville, C.R. (2004) Stomatal development and pattern controlled by a MAPKK kinase. *Science* 304: 1494–1497.

Canevascini, S., Caderas, D., Mandel, T., Fleming, A.J., Dupuis, I. and Kuhlemeier, C. (1996) Tissue-specific expression and promoter analysis of the tobacco *Ltp1* gene. *Plant Physiol.* 112: 513–524.

Chen, C. and Chen, Z. (2000) Isolation and characterization of two pathogen- and salicylic acid-induced genes encoding WRKY DNA-binding proteins from tobacco. *Plant Mol. Biol.* 42: 387–396.

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

Creelman, R.A. and Mullet, J.E. (1997) Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. *Plant Cell* 9: 1211–1223.

Dong, X. (2004) NPR1, all things considered. *Curr. Opin. Plant Biol.* 7: 547–552.

Droillard, M.J., Boudsocq, M., Barbier-Bygoo, H. and Lauriére, C. (2002) Different protein kinase families are activated by osmotic stresses in *Arabidopsis thaliana* cell suspensions. *FEBS Lett.* 527: 43–50.

Droillard, M.J., Boudsocq, M., Barbier-Bygoo, H. and Lauriére, C. (2004) Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of *Arabidopsis thaliana*: activation by hypoosmolarity and negative role in hyperosmolarity tolerance. *FEBS Lett.* 574: 42–48.

Farmer, E.E., Almeras, E. and Krishnamurthy, V. (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. *Curr. Opin. Plant Biol.* 6: 372–378.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male-sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6: 751–759.

He, Y., Fukushige, H., Hildebrand, D.F. and Gan, S. (2002) Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. *Plant Physiol.* 128: 876–884.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231.

Huang, Y., Li, H., Gupta, R., Morris, P.C., Luan, S. and Kieber, J.J. (2000) ATMPK4, an *Arabidopsis* homolog of mitogen-activated protein kinase, is activated in vitro by AtMEK1 through threonine phosphorylation. *Plant Physiol.* 122: 1301–1310.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases AtMPK4 and AtMPK6. *Plant J.* 24: 655–666.

Jonak, C., Heberle-Bors, E. and Hirt, H. (1994) MAP kinases: universal multi-purpose signaling tools. *Plant Mol. Biol.* 24: 407–416.

Jonak, C., Kiegerl, S., Lloyd, C., Chan, J. and Hirt, H. (1995) MMK2, a novel alfalfa MAP kinase, specifically complements the yeast MPK1 function. *Mol. Gen. Genet.* 248: 686–694.

Kopka, J., Provart, N.J. and Muller-Rober, B. (1997) Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. *Plant J.* 11: 871–882.

Leung, J., Merlot, S. and Giraudat, J. (1997) The *Arabidopsis ABSCISIC ACID-INSENSITIVE 2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. *Plant Cell* 9: 759–771.

Li, C., Schilmiller, A.L., Liu, G., Lee, G.I., Jayanty, S., Sageman, C., Vrebalov, J., Giovannoni, J.J., Yagi, K., Kobayashi, Y. and Howe, G.A. (2005) Role of β-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. *Plant Cell* 17: 971–986.

Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E. and Howe, G.A. (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses and glandular trichome development. *Plant Cell* 16: 126–143.

Liu, Y. and Zhang, S. (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. *Plant Cell* 16: 3386–3399.

Liu, Y., Zhang, S. and Klessig, D.F. (2000) Molecular cloning and characterization of a tobacco MAP kinase kinase that interacts with SIPK. *Mol. Plant-Microbe Interact.* 13: 118–124.

MAPK Group (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. *Trend Plant Sci.* 7: 301–308.

Matsuoka, D., Nanmori, T., Sato, K., Fukami, Y., Kikkawa, U. and Yasuda, T. (2002) Activation of AtMEK1, an *Arabidopsis* mitogen-activated protein kinase, in vitro and in vivo: analysis of active mutants expressed in *E. coli* and generation of the active form in stress response in seedlings. *Plant J.* 29: 637–647.

Mehlhorn, H. and Wellbum, A.R. (1987) Stress ethylene formation determines plant sensitivity to ozone. *Nature* 327: 417–418.

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* 37: 49–59.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H. and Shinozaki, K. (1993) ATMPKs: a gene family of plant MAP kinases in *Arabidopsis thaliana*. *FEBS Lett.* 336: 440–444.

Mueller, M.J. (1997) Enzymes involved in jasmonic acid biosynthesis. *Physiol. Plant.* 100: 653–663.

Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. (2002) *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* 14: 3089–3099.

Nakajima, N., Itoh, T., Takikawa, S., Asai, N., Tamaoki, M., Aono, M., Kubo, A., Azumi, Y., Kamada, H. and Saji, H. (2002) Improvement in ozone tolerance of tobacco plants with an antisense DNA for 1-aminocyclopropane-1-carboxylate synthase. *Plant Cell Environ.* 25: 727–735.

Nishida, E. and Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.* 18: 128–131.

Nishihama, R., Banno, H., Shibata, W., Hirano, K., Nakashima, M., Usami, S. and Machida, Y. (1995) Plant homologues of components of MAPK (mitogen-activated protein kinase) signal pathways in yeast and animal cells. *Plant Cell Physiol.* 36: 749–757.

Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T. and Machida, Y. (2001) The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. *Genes Dev.* 15: 352–363.

Norman-Setterblad, C., Vidal, S. and Palva, E.T. (2000) Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Mol. Plant-Microbe Interact.* 13: 430–438.

O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a signal mediating the wound response of tomato plants. *Science* 274: 1914–1917.

Örvar, B.L., McPherson, J. and Ellis, B.E. (1997) Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. *Plant J.* 11: 203–212.

Overmyer, K., Tuominen, H., Kettunen, R., Betz, C., Langebartels, C., Sandermann, H., Jr and Kangasjarvi, J. (2000) Ozone-sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. *Plant Cell* 12: 1849–1862.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., et al. (2000) *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* 103: 1111–1120.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10: 1571–1580.

Rao, M.V., Lee, H.I., Creelman, R.A., Mullet, J.A. and Davis, K.R. (2000) Jasmonic acid signalling modulates ozone-induced hypersensitive cell death. *Plant Cell* 12: 1633–1646.

Rojo, E., Solano, R. and Sanchez-Serrano, J.J. (2003) Interactions between signaling compounds involved in plant defense. *J. Plant Growth Regul.* 22: 82–98.

Samuel, M.A. and Ellis, B.E. (2002) Double jeopardy: both overexpression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. *Plant Cell* 14: 2059–2069.

Sasaki, K., Hiraga, S., Ito, H., Seo, S., Matsui, H. and Ohashi, Y. (2002) A wound-inducible tobacco peroxidase gene expresses preferentially in the vascular system. *Plant Cell Physiol.* 43: 108–117.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H. and Ohashi, Y. (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathway. *Science* 270: 1988–1992.

Seo, S., Sano, H. and Ohashi, Y. (1999) Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. *Plant Cell* 11: 289–298.

Shah, J. (2003) The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* 6: 365–371.

Soyano, T., Nishihama, R., Morikiyo, K., Ishikawa, M. and Machida, Y. (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024

MAPK cascade and is required for plant cytokinesis. *Genes Dev.* 17: 1055–1067.

Staswick, P.E., Yuen, G.Y. and Lehman, C.C. (1998) Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. *Plant J.* 15: 747–754.

Suhita, D., Raghavendra, A.S., Kwak, J.M. and Vavasseur, A. (2004) Cytoplasmic alkalization precedes reactive oxygen species production during methyl jasmonate- and abscisic acid-induced stomatal closure. *Plant Physiol.* 134: 1536–1545.

Teige, M., Scheikl, E., Eulgem, T., Doczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L. and Hirt, H. (2004) The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. *Mol. Cell* 15: 141–152.

Tsuchisaka, A. and Theologis, A. (2004) Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiol.* 136: 2982–3000.

Tuominen, H., Overmyer, K., Keinänen, M., Kollist, H. and Kangasjärvi, J. (2004) Mutual antagonism of ethylene and jasmonic acid regulates ozone-induced spreading cell death in *Arabidopsis*. *Plant J.* 39: 59–69.

Turner, J.G., Ellis, C. and Devoto, A. (2002) The jasmonate signal pathway. *Plant Cell* 14 (suppl.), S153–S164.

Weber, H. (2002) Fatty acid-derived signals in plants. *Trends Plant Sci.* 7: 217–224.

Wen-jun, S. and Forde, B.G. (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. *Nucleic Acids Res.* 17: 8385.

Xiao, S., Dai, L., Liu, F., Wang, Z., Peng, W. and Xie, D. (2004) COS1: an *Arabidopsis* coronatine insensitive1 suppressor essential for regulation of jasmonate-mediated plant defense and senescence. *Plant Cell* 16: 1132–1142.

Yuasa, T., Ichimura, K., Mizoguchi, T. and Shinozaki, K. (2001) Oxidative stress activates ATMPK6, an *Arabidopsis* homologue of MAP kinase. *Plant Cell Physiol.* 42: 1012–1016.

Zhang, S. and Klessig, D.F. (1998) The tobacco wounding-activated mitogen-activated protein kinase is encoded by SIPK. *Proc. Natl Acad. Sci. USA* 95: 7225–7230.

Ziegler, J., Keinanen, M. and Baldwin, I.T. (2001) Herbivore-induced allene oxide synthase transcripts and jasmonic acid in *Nicotiana attenuata*. *Phytochemistry* 58: 729–738.

(Received September 26, 2005; Accepted September 30, 2005)

Downloaded from https://academic.oup.com/pcp/article/46/12/1902/1827370 by guest on 24 April 2024