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OMTK1, a Novel MAPKKK, Channels Oxidative Stress Signaling through Direct MAPK Interaction*

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In common with other eukaryotes, plants utilize mitogen-activated protein kinase (MAPK) cascades to mediate responses to a wide variety of stimuli. In contrast to other eukaryotes, plants have an unusually large number of MAPK components, such as more than 20 MAPKs, 10 MAPK kinases (MAPKKs), and 60 MAPKK kinases (MAPKKKs) in *Arabidopsis* (MAPK Group (2002) *Trends Plant Sci.* 7, 301–308). Presently it is mostly unknown how MAPK signaling specificity is generated in plants. Here we have isolated OMTK1 (oxidative stress-activated MAP triple-kinase 1), a novel MAPKKK from alfalfa (*Medicago sativa*). In plant protoplasts, OMTK1 showed basal kinase activity and was found to induce cell death. Among a panel of hormones and stresses tested, only H_2O_2 was found to activate OMTK1. Out of four MAPKs, OMTK1 specifically activated MMK3 resulting in an increased cell death rate. Pull-down analysis between recombinant proteins indicated that OMTK1 directly interacts with MMK3 and that OMTK1 and MMK3 are part of a protein complex *in vivo*. These results indicate that OMTK1 plays a MAPK scaffolding role and functions in activation of H_2O_2-induced cell death in plants.

Mitogen-activated protein kinases (MAPKs),[1] a specific class of serine/threonine protein kinases, are involved in controlling many cellular functions in all eukaryotes. A general feature of MAPK cascades is their composition of three functionally linked protein kinases. A MAPK is phosphorylated and thereby activated by a MAPK kinase (MAPKK), which itself becomes activated by another serine/threonine protein kinase, a MAPKK kinase (MAPKKK). Targets of MAPKs can be various transcription factors, protein kinases, or cytoskeletal proteins (1).

In plants, MAPK cascades are associated with various physiological, developmental, and hormonal responses, and it became clear that the same MAPK pathway components are involved in responses to different stimuli (2, 3). For instance, SIMKK encoding an alfalfa MAPKK mediates hyperosmotic stress, elicitor, and ethylene signals (4–6). Besides, alfalfa MAPK, MMK3, was found to play a role in elicitor and ethylene signaling (5–7) but also in cytokinesis (8).

A similar situation also exists in other eukaryotes and is particularly apparent in *Saccharomyces cerevisiae*, in which four of the six MAPK cascades use subsets of the same protein kinases (9). This situation put forward the major question, what ensures the fidelity of signaling, particularly in instances in which the same protein kinase functions in more than one pathway. The studies in yeast and mammals revealed that scaffold proteins, which tether MAPK modules in one complex, play important roles in defining the specificity. The first MAPK scaffold discovered was yeast Ste5 protein, which assembles MAPKKK, MAPKK, and MAPK of the pheromone mating pathway (10–13). Since its discovery, a number of MAPK scaffolding molecules were identified in yeast and mammals (14–20). MAPK components themselves were also found to function as scaffolds, such as yeast Pbs2 protein, the MAPKK of the high osmolarity/glycerol pathway (21). Similarly, human MEKK2 shows synergistic interaction with MKK7 and JNK1 on its kinase domain and activates JNK1 most efficiently through MKK7 activation in the tripartite complex (22).

In contrast, nothing is presently known about plant MAPK scaffolds. However, the observation that *Arabidopsis* MAPKKK, MEKK1, interacts with *Arabidopsis* MAPKK, MKK1 and MKK2, and *Arabidopsis* MAPK, MPK4, in yeast (23), implies that plant MAPKKKs might function as scaffold molecules.

In this report, we show that OMTK1 encodes a functional novel alfalfa MAPKKK that is activated by H_2O_2 and induces cell death. OMTK1 possesses scaffolding function to bind and selectively activate the alfalfa MAPK, MMK3. Although MMK3 can be activated by several stimuli, including H_2O_2 (5–7), H_2O_2-induced MMK3 activation completely depends on the presence of OMTK1.

EXPERIMENTAL PROCEDURES

Isolation, Sequence Analysis, and Cloning of OMTK1—Primers corresponding to *Arabidopsis* MEKK1 were used to isolate OMTK1 from alfalfa by PCR. The PCR fragment obtained was cloned into pBluescript SK+ (Stratagene). Sequence analysis revealed the fragment to encode an alfalfa MAPKKK, denoted as OMTK1, which subsequently was used to screen an alfalfa (*Medicago sativa*) cDNA library prepared from somatic embryos. An isolated clone was fully sequenced and found to contain an open reading frame of 592 amino acids. The nucleotide and predicted protein sequences are available in the EMBL data base under accession number AJ575100.

In Vitro Binding Assays—MMK3 was expressed as GST fusion proteins as described previously (4). The protein concentrations of the recombinant proteins were determined with the Bio-Rad detection system (Bio-Rad) using bovine serum albumin as a standard, and the purity of the protein fractions was determined by Coomassie staining

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ575100.

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; OMTK1, oxidative stress-activated mitogen-activated protein triple-kinase 1; SIMK, salt-induced MAPK; SAMK, stress-activated MAPK; MMK2 and -3, *Medicago* MAPK2 and -3, respectively; YE, yeast elicitor; ACC, aminocyclopropane-1-carboxylic acid; MBP, myelin basic protein; PI, propidium iodide; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MEKK, MAPK/ERK kinase kinase; GST, glutathione *S*-transferase; HA, hemagglutinin; FDA, fluorescein diacetate.

A

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MEKK1       1   ------------------------------------------------------------
MEKK2       1   MKKSSDKSPVRQHDTATQINSDAVSSSTSFTDSDSTCSFLTPSMEFPDRISFRRIDFSEA
MEKK3       1   ------------------------------------------------------------
OMTK1       1   ------------------------------------------------------------
consensus   1

MEKK1       1   ----------------------------MDRILARMKKSTGRRGDKNITPVRRLERRDA
MEKK2      61   APTGVVLPSTSSELTRSNSSENKIPNEDISVSTSSRYLVFDKILALMKKSPGRRGDKTSP
MEKK3       1   --------------------------MDVTAIFAGDILVQSREYLIPNDVVDVDGGIKAV
OMTK1       1   -------------------------MNHLPRIFGYRNKSTTEMNSKKKQPKLQRRNAVK
consensus  61

MEKK1      33   ARNINYAAASCSSSSAEDLSVSTSSLMTRSLEFPEPTSFRIGGG-VGEMDRIYRSLGVSG
MEKK2     121   ARRLDRSDAVRRNIDYDAGEDSSSLLITRSLDFENRTSFRVDGVDDGEEDRIYQYIGVSG
MEKK3      35   RPPIIQPEPGRKLPLIDFPGSSWDEITYFAPSKTVKRQSSSSDNTSDKEEVETEETRGM
OMTK1      36   NFNYDAQPSSSSPESSESASSSASILCTRSMDFHDRKSFR-VEGVDGEFDIICRSLGLNG
consensus 121   ..... .....  .  . ...*...*............ .. ...............

MEKK1      92   PDDLAISFDAWEACKKR--SSSDVVNRFKSFDLDKVRDQDLSEEGPSGVVVGSDSMNHKV
MEKK2     181   PEDFAISSDAWKARMEHERSSSDVVNKLKSLDLDSR------EAGPSGGVVASSSMNHKF
MEKK3      95   FVQLGDTAHEACPFATNEADSSSTVSIIS-------------PSYASRGSIVPSWLKRKF
OMTK1      95   PEDFSIPAAAWEAMKFRSSSDVLPRLNVS-----------EFDETKTSGEIDQVGVVDCD
consensus 181   ... .........  . .. ......... . ...         .................

MEKK1     150   QGQDLSEAGPSGGIVTELSEIG--------NLITPVDRLVADGVVENRRVMERTPTIVKS
MEKK2     235   QGHDLSEAGSIGVVVASNFTLSESNKIENLNSLRDKEIVDGDMVENRCGIERKPTILVKS
MEKK3     142   LGRVSLGFVYEGSSGSSVGSESTCSLMTPSLEFPDRISFRKKDFSEKGPSRHVWEKRKLT
OMTK1     144   DRVLVRDSPAESSGGEVNVSIRDSPAKSSGCCTGGEIRVPVRGFDETSNCSTGSGGIKGI
consensus 241   ......... .... ... ..... .. . . ..... ... ...   . ... . ..

MEKK1     202   KGYLVPNNVVAVGVG---------------------------------------------
MEKK2     295   RGYLVHNDDVGVGGGIKGVRPPVLNVPRADKEVVDGGTVESKSGIEWKPTILVKSKGYLV
MEKK3     202   RAKLIEN-----------------------------------------------------
OMTK1     204   R-----------------------------------------------------------
consensus 301   ..... . ..... ..

MEKK1     219   ---------------------------------GGIKGIRPPVLKPPPAMKRPPIDHRGSS
MEKK2     355   SNDGGIKGVTSPVLNLRPTDKEVVDSGTVENRRGIKGVRPSVLKPPPVMKLPPVDLPGSS
MEKK3     209   -----------------------------------------------FCNPEDIEPVTS
OMTK1     205   ----------------------------------------PPMLKPPPGVRVSVVDDTCST
consensus 361                                      ...........................

MEKK1     247   WDFLTHFAPSETVKRPSSSSSSSEDGCDEEE--------GKEEEAEAEEMGARFIQLGDT
MEKK2     415   WDILTHFAPDSEIVRRPSSSSSSENGCDEEE--------AEDDKVEKEETCDMFIQLEDT
MEKK3     221   WLKGQLLGEGSFASVYEAISDSSVGSESTCS--------LMTPSMEFPDR----ISFRKR
OMTK1     226   WDILRDFAPEGEGKEREEQEEEEEKVDEVEEKEEVGELELKVEEEENGDIIGEESRSCSF
consensus 421   *........... .......... ......        ......* ..... .... ..

                                                                    I
MEKK1     299   ADETCSFTTNEGDSSSTVSNTSPIYPDGGAIITSWQKGQLLGRGSFGSVYEGISGDGDFF
MEKK2     467   TDEACSFTTNEGDSSSTVSNTSPICVSGGSINTSWQKGQLLRQGSFGSVYEAISEDGDFF
MEKK3     269   DFSEEGPSGRVKEKRKLMRNKLIENFRKPEDITSWLKGQLLGRGSYASVYEAISEDGDFF
OMTK1     286   TTSQEDDSSSTTTDTRSNSISPNVRLKPVITPGSWQKGELLGRGSFGTVYEGISEDGFFF
consensus 481   ..  ................... ↑... ...**.**.**..**...***.**.**.**

           II                   III        IV                V
MEKK1     359   AVKEVSLLDQGSQAQECIQQLEGEIKLLSQLQHQNIVRYRGTAKDGSNLYIFLELVTQGS
MEKK2     527   AVKEVSLLDQGSQAQECIQQLEGEIALLSQLEHQNILRYRGTDKDGSNLYIFLELVTQGS
MEKK3     329   AVKEVSLLDKGIQAQECIQQLEGEIALLSQLQHQNIVRYRGTAKDVSKLYIFLELVTQGS
OMTK1     346   AVKQVSLLDQGSQGKQSVVQLEHEIALLSQFEHENIVRYIGTEMDESNLYIFIEFVTKGS
consensus 541   ***.*****.*.*......***.**.****. *.**.**.**...*.*.****.*.**.**

                                                    VI               VII
MEKK1     419   LLKLYQRYQLRDSVVSLYTRQILDGLKYLHDKGFIHRDIKCANILVDANGAVKLADFGLA
MEKK2     587   LLELYRRYQIRDSLISLYTKQILDGLKYLHHKGFIHRDIKCATILVDANGTVKLADFGLA
MEKK3     389   VQKLYERYQLSYTVVSLYTRQILAGLNYLHDKGFVHRDIKCANMLVDANGTVKLADFGLA
OMTK1     406   LLSLYRRYKLRDSQVSAYTRQILHGLKYLHDRNIVHRDIKCANILVDANGSVKVADFGLA
consensus 601   ...**.**.......*.**.****.**.***.....*******.******.**.******

                          VIII                       IX
MEKK1     479   KVSKFNDIKSCKGTPFWMAPEVINRKDSDGYGSPADIWSLGCTVLEMCTGQIPYSDLEPV
MEKK2     647   KVSKLNDIKSRKETLFWMAPEVINRKDNDGYRSPADIWSLGCTVLEMCTGQIPYSDLEPV
MEKK3     449   EASKFNDIMSCKGTLFWMAPEVINRKDSDGNGSPADIWSLGCTVLEMCTGQIPYSDLKPI
OMTK1     466   KAIKLNDVKSCQGTAFWMAPEVVRG-KVKGYGLPADIWSLGCTVLEMLTGKIPYSPMECI
consensus 661   . .* **..*...*.*******.......*...***************.**.****.....

                 X                                XI
MEKK1     539   QALFRIGRGTLPEVPDTLSLDARLFILKCLKVNPEERPTAAELLNHPFVRRPLPSVGSGG
MEKK2     707   EALFRIRRGTLPEVPDTLSLDARHFILKCLKLNPEERPTATELLNHPFVRRPLP---SSG
MEKK3     509   QAAFKIGRGTLPDVPDTLSLDARHFILTCLKVNPEERPTAAELLHHPFVINL--------
OMTK1     525   SAMFRIGKGELPPVPDTLSRDARDFILQCLKVNPDDRPTAAQLLDHKFVQRSFSQSSGSA
consensus 721   .*.*.*..*.**.******.***.***.***.**..****..**.*.**.....    ...

MEKK1     599   SGSASPLLRR
MEKK2     764   SGSTSPLIRR
MEKK3           ----------
OMTK1     585   SPHIPRRG--
consensus 781   ... ......
```

B

MEKK4
MEKK1
MEKK2
MEKK3
OMTK1
ANP1
CTR1

FIG. 1. **Primary structure of OMTK1.** *A*, alignment of the predicted protein sequence of alfalfa OMTK1, *Arabidopsis* MEKK1, MEKK2, and MEKK3 proteins. Identical amino acid residues are indicated by *black boxes*; similar amino acids are indicated with *gray boxes*. *Stars* indicate the amino acid residues conserved among these four proteins. *Dots* indicate the amino acid residues, which show similarity among more than two proteins. *Roman numerals* indicate the 11 major conserved subdomains of protein kinases. An *arrow* indicates the position of the ΔOMTK1 construct. *B*, phylogenetic tree showing the evolutionary relationship between alfalfa OMTK1, *Arabidopsis* MEKK1, MEKK2, MEKK3, MEKK4, ANP1, and CTR1 proteins. The putative kinase domains of the MAPKKKs were used for alignment.

after 10% SDS-PAGE. OMTK1 was *in vitro* transcribed/translated in the presence of [^{35}S]methionine using the TNT T7/T3-coupled reticulocyte lysate systems kit (Promega) according to the manufacturer's instructions. 10 μl of *in vitro* transcribed/translated OMTK1 or ΔOMTK1 was incubated overnight at 4 °C in 100 μl of wash buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40) in the presence of 5 μg of GST or GST-MMK3, respectively. Subsequently, 25 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) was added, and the mixture was incubated for 4 h at 4 °C. Protein complexes were washed four times with wash buffer and denatured with SDS loading buffer. The proteins were analyzed by autoradiography after SDS-PAGE.

Transient Expression Assays—Transient expression assays were performed with isolated protoplasts from cultured *Arabidopsis* cell suspension. The isolation, transformation, and cultivation of protoplasts were described earlier (6). HA-tagged SIMK and MMK3 were prepared as described previously (4). The open reading frames of MMK2 and SAMK were fused at their C termini to a triple hemagglutinin (HA) epitope and cloned into the plant expression vector pRT101, and the open reading frames of OMTK1 and ΔOMTK1 were fused at their C termini to a double Myc epitope and cloned into the vector pRT101. *Arabidopsis* protoplasts were transiently transformed via polyethylene glycol with 4 μg each of the plasmids pSH9-SIMK-HA, pRT101-MMK2-HA, pRT100-MMK3-HA, or pRT101-SAMK-HA and the indicated concentrations of pRT101-OMTK1-Myc or pRT101-ΔOMTK1-Myc. Then they were subjected to different treatments 12–16 h after transformation. After treatment, extracts were prepared from the protoplasts and used for immunocomplex kinase assay with antibodies raised against HA or Myc.

Immunocomplex Kinase Assays—Protoplast extracts containing equal protein amounts were subjected to a 2-h preincubation in the presence of 20 μl of mixed protein A- and G-Sepharose beads (1:1). The supernatant was then immunoprecipitated with 5 μl of anti-HA monoclonal antibody and 20 μl of protein G-Sepharose beads overnight at 4 °C. The beads were washed three times with wash buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% Tween 20) and once with kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 5 mM EGTA, and 1 mM dithiothreitol). Kinase reactions of the immunoprecipitated proteins were performed in 15 μl of kinase buffer containing 5 μg of MBP, 0.1 mM ATP, and 2 μCi of [γ-^{32}P]ATP. The protein kinase reactions were performed at room temperature for 30 min, and the reactions were stopped by adding 4× SDS loading buffer. The phosphorylation of MBP was analyzed by autoradiography after separation on 15% SDS-PAGE.

Immunoblots—For Western analysis, equal amounts of protein extracts from protoplasts were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting. Membranes were probed with either anti-HA monoclonal antibody or with anti-Myc polyclonal antibody (A-14, Santa Cruz Biotechnology). Membranes were developed by enhanced chemiluminescence as recommended by the manufacturer (Gene Image, Amersham Biosciences).

Treatment of Transiently Transformed Protoplasts—Transiently transformed *Arabidopsis* protoplasts were treated with 250 mM NaCl, 1 mM aminocyclopropane-1-carboxylic acid (ACC), 2 mM H_2O_2, 500 μg/ml yeast elicitor (YE), or 100 μM proteasome inhibitor MG115. Samples were harvested at 10 min.

Cell Death Assessment—Transiently transformed *Arabidopsis* protoplasts were treated with or without 2 mM H_2O_2 for 6 h, and then the number of live and dead cells was determined by double staining with the fluorescent probes fluorescein diacetate (FDA) and propidium iodide (PI). Protoplasts were incubated with 2 μg/ml FDA and 6 μg/ml PI in culture medium for 30 min and were observed with an inverted fluorescent microscope (Zeiss). Images of the fluorescent signal were captured using a digital camera. Over 1000 cells from three different randomly selected fields were counted to determine the percentage of dead cells. The percentage of induced cell death was calculated by subtracting the percentage of dead cells of empty vector transformed non-treated protoplasts divided by the transformation efficiency of the protoplasts, which was determined by transformation with a vector expressing green fluorescent protein.

RESULTS

Primary Structure of OMTK1—While screening for new members of the plant MAPKKK family from alfalfa, we isolated a novel MAPKKK, OMTK1. The OMTK1 C-terminal putative kinase domain showed 68.1%, 65%, 64.9%, 60.6%, 40.9%, and 23.6% identity to the kinase domains of *Arabidopsis* MEKK1, MEKK2, MEKK3, MEKK4, ANP1, and CTR1, respectively. Identity of the kinase domains and the position of the kinase domains in whole proteins suggest that OMTK1 is closely related to the A1 subgroup of the plant MEKK family of MAPKKKs (24) (Fig. 1, *A* and *B*). The N-terminal non-catalytic region of OMTK1 showed no significant similarity to any known functional sequence motifs.

FIG. 2. **OMTK1 selectively activates MMK3 in protoplasts.** MBP kinase activities of immunoprecipitated SIMK-HA, MMK2-HA, MMK3-HA, and SAMK-HA when expressed transiently in *Arabidopsis* protoplasts alone (*lanes 1*, *4*, *7*, and *10*), with 0.2 μg of plasmid of OMTK1-Myc (*lanes 2*, *5*, *8*, and *11*), or with 0.4 μg of plasmid of OMTK1-Myc (*lanes 3*, *6*, *9*, and *12*). After SDS-PAGE, MAPK activities on MBP were determined by autoradiography. Expression levels of MAPKs-HA and OMTK1-Myc were assessed by immunoblotting with anti-HA and anti-Myc antibody, respectively.

OMTK1 Selectively Activates the MMK3 Pathway in Plant Protoplasts—To investigate which MAPK pathway is regulated by OMTK1, we transiently coexpressed OMTK1 and four different alfalfa MAPKs in plant protoplasts. Because we found that OMTK1 is autoactive to a certain extent in *Arabidopsis* protoplasts (see Fig. 3*D*)[2] we transiently expressed HA-tagged MAPKs alone or with different amounts of Myc-tagged OMTK1 in *Arabidopsis* protoplasts and determined the kinase activity of the HA-immunoprecipitated MAPKs using MBP as a substrate. As shown in Fig. 2, upon coexpression with OMTK1-Myc only MMK3-HA was activated in the protoplasts. As can be seen by immunoblotting, the differences of MAPKs-HA activation by OMTK1-Myc coexpression were not due to differences in MAPK protein amounts. These results indicate that OMTK1 selectively activates MMK3.

OMTK1 Is Activated by H_2O_2—To define the stimuli that can regulate OMTK1 activity, OMTK1-Myc and MMK3-HA were coexpressed in *Arabidopsis* protoplasts, and the protoplasts were treated with different stimuli. As shown in Fig. 3*A*, only H_2O_2 strongly activated MMK3-HA in combination with OMTK1-Myc. Interestingly, MMK3-HA alone was not notably activated by H_2O_2, but coexpression of OMTK1-Myc resulted in severalfold higher activation of MMK3-HA (Fig. 3, *B* and *C*). In addition, OMTK1-induced activation of MMK3 by H_2O_2 occurred in a dose-dependent manner above concentrations of 200 μM H_2O_2 (Fig. 3*B*), and H_2O_2-induced MMK3 activation occurred in 5 min and stayed at nearly the same level more than 90 min (Fig. 3*C*). In contrast, the addition of OMTK1-Myc did not affect the activities of SIMK, MMK2, and SAMK under H_2O_2 treatment (data not shown). Furthermore, we tested other stimuli, methyl jasmonate, salicylic acid, abscisic acid, benzyl alcohol, α-naphthaleneacetic acid, kinetin, and cold stress, but none of them activated MMK3-HA in combination with OMTK1-Myc (data not shown). These data suggest that H_2O_2 activates an OMTK1-dependent MAPK pathway. Interestingly, as can be seen by immunoblotting, protein amounts of OMTK1-Myc were affected by NaCl and H_2O_2 treatment (Fig. 3*A*). This phenomenon is reproducible and was also seen in other experiments. Because of the basal activity of OMTK1, activation of MMK3 by H_2O_2 might be explained by increased

[2] H. Nakagami and H. Hirt, unpublished results.

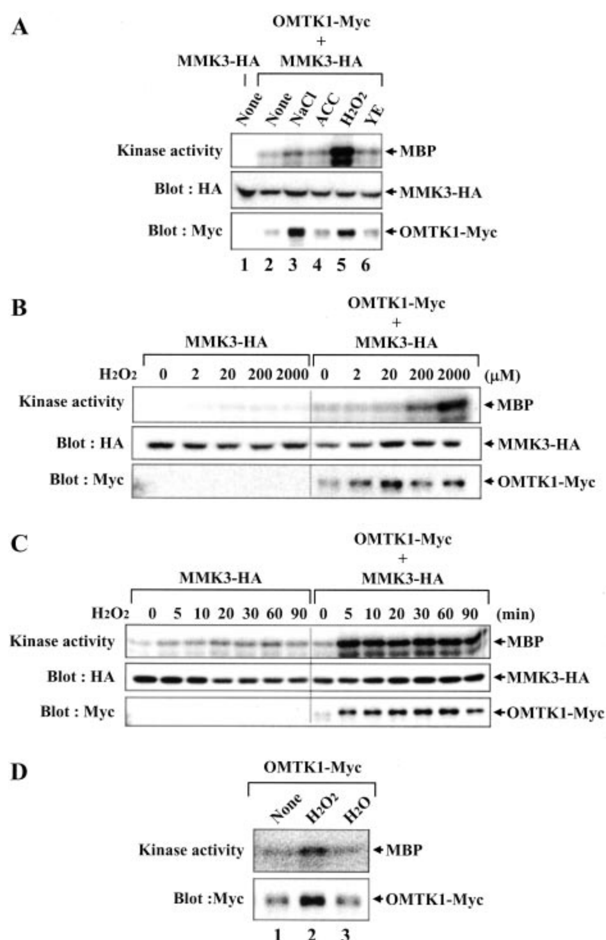

FIG. 3. **OMTK1 is activated by H_2O_2.** *A*, H_2O_2 activates MMK3 in combination with OMTK1 in protoplasts. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA alone (*lane 1*) or MMK3-HA in combination with OMTK1-Myc (0.2 μg of plasmid) (*lanes 2–6*). Protoplasts were treated for 10 min with 250 mM NaCl (*lane 3*), 1 mM ACC (*lane 4*), 2 mM H_2O_2 (*lane 5*), 500 μg/ml YE (*lane 6*), or an equivalent amount of distilled water (*lanes 1* and *2*). MMK3-HA activity was determined from protein extracts by immunoprecipitation with an anti-HA antibody followed by an *in vitro* kinase assay with MBP as a substrate. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies. *B*, MMK3 is activated by H_2O_2 in a dose-dependent manner in the presence of OMTK1. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA alone or MMK3-HA in combination with OMTK1-Myc (0.2 μg of plasmid), and protoplasts were treated for 10 min with different concentrations of H_2O_2. MMK3-HA activity was determined from protein extracts by immunoprecipitation with an anti-HA antibody followed by *in vitro* kinase assays with MBP as a substrate. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies. *C*, kinetics of MMK3 activation by H_2O_2. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA alone or MMK3-HA in combination with OMTK1-Myc (0.2 μg of plasmid). Protoplasts were treated with 2 mM H_2O_2 for the indicated times. MMK3-HA activity was determined from protein extracts by immunoprecipitation with an anti-HA antibody followed by *in vitro* kinase assays with MBP as a substrate. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies. *D*, OMTK1 phosphorylates MBP. *Arabidopsis* protoplasts were transiently transformed with OMTK1-Myc alone (4 μg of plasmid), and protoplasts were treated for 10 min with 2 mM H_2O_2 (*lane 2*), distilled water (*lane 3*), or before treatment (*lane 1*). OMTK1-Myc activity was determined from protein extracts by immunoprecipitation with an anti-Myc antibody followed by *in vitro* kinase assays with MBP as a substrate. The same extracts were immunoblotted with anti-Myc antibody.

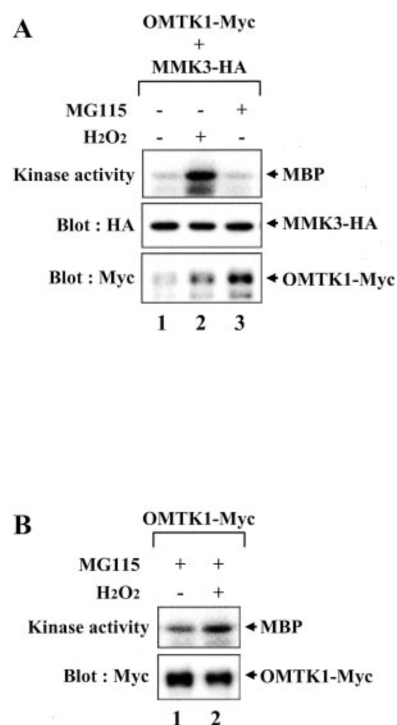

FIG. 4. **Accumulation of OMTK1 is not sufficient to activate MMK3 in protoplasts.** *A*, proteasome inhibitor MG115 treatment stabilizes OMTK1 but does not activate MMK3 in protoplasts. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA in combination with OMTK1-Myc (0.2 μg of plasmid). Protoplasts were treated for 10 min with 2 mM H_2O_2 (*lane 2*), 100 μM MG115 (*lane 3*), or an equivalent amount of distilled water (*lane 1*). MMK3-HA activity was determined from protein extracts by immunoprecipitation with an anti-HA antibody followed by an *in vitro* kinase assay with MBP as a substrate. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies. *B*, stabilized OMTK1 can be further activated by H_2O_2. *Arabidopsis* protoplasts were transiently transformed with OMTK1-Myc (4 μg of plasmid). Protoplasts were preliminary treated with 100 μM MG115 (*lane 3*) for 10 min, then further treated with 2 mM H_2O_2 (*lane 2*) or an equivalent amount of distilled water (*lane 1*) for 10 min. OMTK1-Myc activity was determined from protein extracts by immunoprecipitation with an anti-Myc antibody followed by *in vitro* kinase assays with MBP as a substrate. The same extracts were immunoblotted with anti-Myc antibody.

OMTK1 protein levels, but this mechanism seems not to be sufficient for MMK3 activation in response to NaCl treatment (Fig. 3*A*). To clarify whether OMTK1 is functional as a kinase, we tried to detect its kinase activity. Immunoprecipitated OMTK1-Myc from untreated protoplasts could phosphorylate MBP (Fig. 3*D*, *lane 1*), indicating that OMTK1 possesses low constitutive kinase activity. However, total kinase activity of OMTK1 markedly increased in parallel with OMTK1 protein levels upon treatment of protoplasts with H_2O_2 (Fig. 3*D*, *lane 2*). These results show that OMTK1 activity and protein levels are regulated by H_2O_2.

Accumulation of OMTK1 Protein Is Not Sufficient to Activate the MMK3 Pathway—Activation of OMTK1 by H_2O_2 was generally accompanied with OMTK1 protein accumulation (Fig. 3, *A–D*). Because OMTK1 was expressed in protoplasts under the 35S promoter of Cauliflower mosaic virus, the observed differences in OMTK1 protein amounts cannot be explained by changes of transcription. To investigate the possibility that OMTK1 protein levels are regulated by changes in protein stability, protoplasts expressing OMTK1-Myc and MMK3-HA were treated with the proteasome inhibitor MG115. As shown in Fig. 4*A*, MG115 treatment resulted in rapid and higher accumulation of OMTK1 than after H_2O_2 treatment. These results indicate that OMTK1 is regulated by a proteasome-dependent pathway. Interestingly, in contrast to H_2O_2 treatment, accumulation of OMTK1 by MG115 treatment did not lead to MMK3 activation (Fig. 4*A*, *lane 2* and *3*, respectively). In addition, we found that, under MG115 treatment, OMTK1 could be further activated by H_2O_2 treatment (Fig. 4*B*). These results

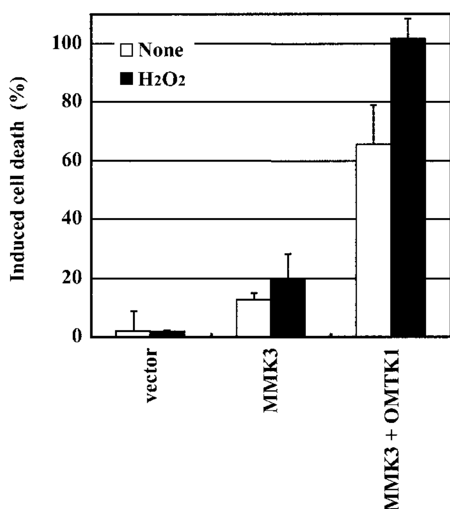

FIG. 5. **OMTK1 induces cell death.** *Arabidopsis* protoplasts were transiently transformed with MMK3-HA alone or MMK3-HA in combination with OMTK1-Myc (4 μg of plasmid), and protoplasts were treated with or without 2 mM H_2O_2 for 6 h. Protoplasts were stained with FDA for living cells and PI for dead cells. To determine the percentage of dead protoplasts, over 1000 protoplasts from three different randomly selected fields were counted under each condition. *Bars* represent the standard deviation between samples.

strongly suggest that besides protein stability other post-translational mechanisms are involved in regulating OMTK1.

OMTK1 Induces Cell Death—H_2O_2 is known to induce programmed cell death. To investigate if the OMTK1 signaling pathway regulates cell death, we analyzed cell viability of transiently transformed *Arabidopsis* protoplasts with or without H_2O_2 treatment. After 6 h of 2 mM H_2O_2 treatment, no significant increase of cell death was observed in protoplasts transformed with empty vector (Fig. 5). Expression of MMK3 slightly induced cell death in the absence and upon H_2O_2 treatment. In contrast, coexpression of OMTK1 and MMK3 highly induced the cell death rate in the absence and presence of H_2O_2. These results indicate that the OMTK1 pathway triggers cell death.

The Kinase Domain of OMTK1 Is Sufficient to Mediate H_2O_2 Signaling to the MMK3 Pathway—In an attempt to define which part of OMTK1 is important to receive H_2O_2 signaling and to activate MMK3, we constructed truncated OMTK1 (ΔOMTK1) which lacks the N-terminal putative regulatory domain (amino acids 1–310, Fig. 1*A*). To compare ΔOMTK1-Myc with full-length OMTK1-Myc, different amounts of ΔOMTK1-Myc were expressed in protoplasts. As observed with full-length OMTK1, ΔOMTK1-Myc poorly activated MMK3-HA and showed much lower protein amounts in the absence of H_2O_2 treatment (Fig. 6*A*).

N-terminal Truncated OMTK1 Shows Reduced Activity to Trigger Cell Death—To clarify the function of the N-terminal non-catalytic domain, we analyzed whether ΔOMTK1 retains the activity to induce cell death. As shown in Fig. 6*B*, coexpression of ΔOMTK1 and MMK3 also induced cell death, however, the rate of ΔOMTK1/MMK3-induced cell death was far lower in comparison with that induced by full-length OMTK1/MMK3. Because induction of cell death showed strong correlation with MMK3 activity (Figs. 3 and 5), these results indicate that the OMTK1 N-terminal domain is necessary to fully activate MMK3. Interestingly, expression of OMTK1 or ΔOMTK1 alone also induced cell death. These observations could be due to the activation of endogenous MAPKs by OMTK1. Reduced activity of ΔOMTK1 is compatible with the idea that the OMTK1 N-terminal domain is needed to fully activate the downstream MAPK.

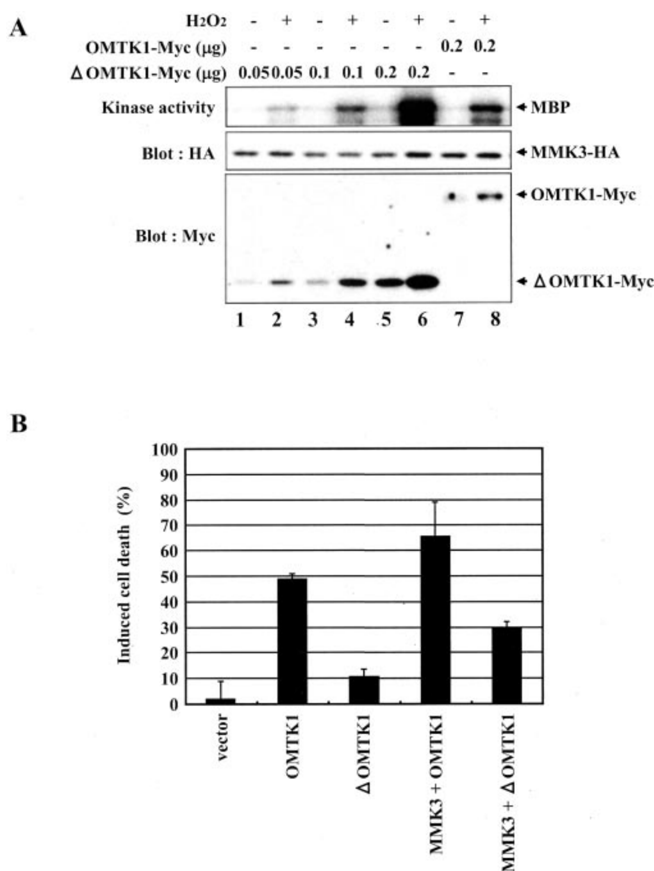

FIG. 6. **Property of ΔOMTK1.** *A*, ΔOMTK1 activates MMK3 under H_2O_2 treatment in protoplasts. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA in combination with 0.05 μg (*lanes 1* and *2*), 0.1 μg (*lanes 3* and *4*), or 0.2 μg (*lanes 5* and *6*) ΔOMTK1-Myc plasmid, or 0.2 μg of OMTK1-Myc plasmid (*lanes 7* and *8*). Protoplasts were treated with (*lanes 2*, *4*, *6*, and *8*) or without (*lanes 1*, *3*, *5*, and *7*) 2 mM H_2O_2 for 10 min. MMK3-HA activities were determined from protein extracts by immunoprecipitation with an anti-HA antibody followed by *in vitro* kinase assays with MBP as a substrate. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies. *B*, ΔOMTK1 shows reduced activity to trigger cell death. *Arabidopsis* protoplasts were transiently transformed with MMK3-HA alone or MMK3-HA in combination with ΔOMTK1-Myc (4 μg of plasmid) or OMTK1-Myc (4 μg of plasmid). Protoplasts were stained with FDA for living cells and PI for dead cells. To determine the percentage of dead protoplasts, over 1000 protoplasts from three different randomly selected fields were counted under each condition. *Bars* represent the standard deviation between samples.

OMTK1 and MMK3 Exist in a Complex in Vivo—So far, our analysis revealed that OMTK1 is an upstream activator of MMK3. In principle, activation of MAPKs by upstream factors can occur in a transient manner or by stable complex formation. Although this mechanism was intensively studied in yeast and mammals, no such investigations have yet been performed in plants. To test whether OMTK1 can form a complex with MMK3 *in vivo*, OMTK1-Myc was coexpressed with MMK3-HA in protoplasts, before immunoprecipitation of OMTK1 with Myc antibody and immunoblotting with HA antibody for detecting MMK3. As shown in Fig. 7, MMK3-HA coimmunoprecipitated with OMTK1-Myc (*lane 2*). To investigate whether the N-terminal non-catalytic region of OMTK1 was involved in this interaction, ΔOMTK1-Myc was also tested for coimmunoprecipitation with MMK3-HA. As shown in Fig. 7, *lane 3*, ΔOMTK1 was also able to form a complex with MMK3. These data indicate that OMTK1 and MMK3 are part of one protein complex *in vivo*.

OMTK1 Directly Interacts with MMK3—Because OMTK1 and MMK3 were found in the same protein complex in proto-

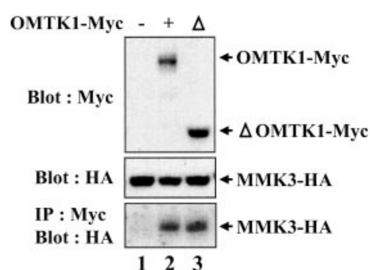

FIG. 7. **OMTK1 forms a complex with MMK3 in protoplasts.** MMK3-HA was coexpressed with OMTK1-Myc (4 μg of plasmid) (*lane 2*) or ΔOMTK1-Myc (4 μg of plasmid) (*lane 3*) in *Arabidopsis* protoplasts. As a negative control, MMK3-HA was expressed alone (*lane 1*). The extracts were immunoprecipitated (*IP*) with anti-Myc antibody, and the immunoprecipitates were immunoblotted (*Blot*) for MMK3-HA with anti-HA antibody. The same extracts were immunoblotted with anti-HA and anti-Myc antibodies.

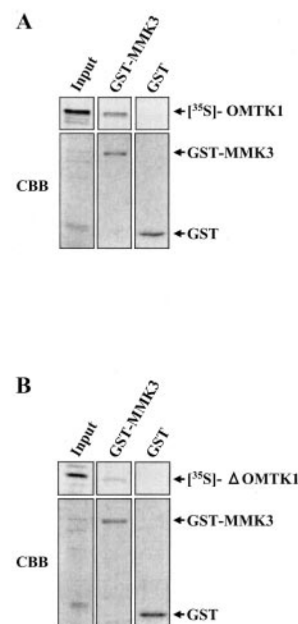

FIG. 8. **OMTK1 directly interacts with MMK3 *in vitro*.** GST or GST-MMK3, immobilized on glutathione beads, was incubated with *in vitro* translated ^{35}S-labeled OMTK1 or ΔOMTK1 protein. Bound ^{35}S-labeled OMTK1 (*A*) and ΔOMTK1 (*B*) protein were detected by autoradiography after SDS-PAGE (*top panel*). Similar amounts of GST and GST-MMK3 were used in the experiment, as shown by CBB staining of SDS-PAGE (*low panel*). Samples were analyzed on same SDS-PAGE gel. For clarity, lanes were assembled in separate panels.

plasts, we also investigated whether this interaction was direct or indirect. For this purpose, we performed *in vitro* binding assays. We transcribed/translated OMTK1 *in vitro* in the presence of [^{35}S]methionine and used ^{35}S-labeled OMTK1 to assay *in vitro* binding to GST and GST-fused MMK3 recombinant proteins adsorbed onto glutathione beads. As shown in Fig. 8*A*, OMTK1 associated clearly with GST-MMK3 but not with GST alone. Results of our *in vivo* interaction study suggested that MMK3 also directly interacts with the kinase domain of OMTK1. Indeed, this was found to be the case, because ΔOMTK1 could also associate with GST-MMK3 *in vitro* (Fig. 8*B*). However, although ΔOMTK1 still showed binding affinity to MMK3, the binding appeared to be significantly weakened when compared with the full-length OMTK1. These data support our previous results that the N-terminal domain of OMTK1 is necessary for full MMK3 activation. Taken together, these data indicate that complex formation of OMTK1 with MMK3 occurs through direct binding of the MAPKKK and MAPK.

DISCUSSION

Genomic sequence analysis of plants suggests an extraordinary complexity in MAPK signaling cascades. Work from several groups indicates that extensive cross-talk among MAPKs and other pathways exists which considerably complicates our understanding of signal transduction (2, 3). Moreover, it is becoming clear that a given MAPK component can perform very different functions in different pathways (2). This raises the question how specificity is achieved in signaling. Several studies in yeast and mammals revealed that scaffolding plays an important role in defining specificity. Besides identifying novel proteins with scaffolding functions, some components of MAPK modules were shown to function as scaffolds themselves. Until now, information that the *Arabidopsis* MAPKKK, MEKK1, can interact with the two *Arabidopsis* MAPKKs, MKK1 (formerly denoted as MEK1) and MKK2, and with the *Arabidopsis* MAPK, MPK4, in yeast (23), suggests that similar principles of scaffold molecules could also apply for plant MAPK cascades. In this study, a novel MAPKKK, OMTK1, is shown to have properties of a scaffold protein. OMTK1 directly interacts with MMK3 *in vitro* and is found in a protein complex with MMK3 *in vivo*.

Among a panel of different hormones and stress conditions, OMTK1 was found to be specifically activated by H_2O_2. By testing four possible downstream target MAPKs, OMTK1 was found to activate MMK3. Investigation of the activation mechanism of OMTK1 revealed that OMTK1 is regulated by protein stability. Treatment of protoplasts with H_2O_2 (Fig. 3), and the proteasome inhibitor MG115 (Fig. 4) resulted in accumulation of OMTK1. However, several observations suggest that additional post-translational mechanisms are also responsible for OMTK1 activation. Salt stress induced OMTK1 protein accumulation but no significant activation of MMK3 (Fig. 3*A*). Similarly, OMTK1 protein accumulated in response to 20 μM H_2O_2 but was not sufficient to result in pathway activation (Fig. 3*B*). Most significantly, MG115-induced accumulation of OMTK1 did not result in significant activation of the downstream MMK3 pathway but still required treatment with H_2O_2 (Fig. 4). These data suggest that mechanisms other than protein stability act on OMTK1. Likely candidates are protein phosphorylation or subcellular recruitment, and further studies are required to clarify the exact regulatory mechanism. Although the specific activity level of OMTK1 was dependent on post-translational activation other than protein stability, the absolute amounts of OMTK1 protein dictated absolute levels of OMTK1 activity. In other words, where there is no OMTK1 protein, there can be no OMTK1 activity and consequently no activation of downstream pathway components. Therefore, stabilization seems to be necessary but not sufficient for OMTK1 activation.

H_2O_2 is an active signaling molecule that is known to be involved in defense reactions against different kind of stresses (25). Plants are likely to respond to H_2O_2 in a dose-dependent manner. In some cases, H_2O_2 signaling contributes to recovery of damaged cells from stresses by activating detoxification and defense gene expression. On the other hand, H_2O_2 signaling results in programmed cell death. In *Arabidopsis*, the MAPKKKs ANP1 can activate MPK3 (a homolog of SAMK) and MPK6 (a homolog of SIMK) under H_2O_2 treatment and were shown to activate oxidative stress-inducible gene expression (26). In addition, constitutively active tobacco NPK1 (a homolog of ANP1)-overexpressing plants were shown to be more tolerant against multiple environmental stresses such as freezing, heat shock, and salt stress (26). Therefore, the ANP1/NPK1 signaling pathway seems to be more related to cell recovery than programmed cell death. In contrast, we found that

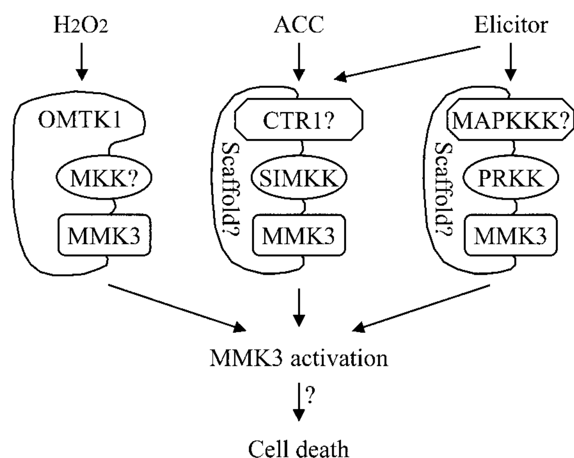

FIG. 9. **Schematic representation of MMK3 activation in different signal pathways.** MMK3 activation by H_2O_2 is dependent on OMTK1 that directly interact with MMK3. Presently it is unclear which MAPKK if any is required for H_2O_2 induced MMK3 activation. ACC and elicitor induced MMK3 activation is mediated by SIMKK and PRKK, respectively, however, it is not known which scaffolds or MAPKKKs are participating in these pathways.

OMTK1 clearly induces cell death through MMK3 activation (Figs. 5 and 6*B*), suggesting that the OMTK1 signaling pathway regulates programmed cell death rather than cell recovery.

MAPKKKs are composed of a conserved kinase domain and little conserved non-catalytic domains of considerable length (24). In the *Arabidopsis* MAPKKKs, CTR1 and ANP1, and the tobacco NPK1, a homolog of ANP1, deletion of the non-catalytic domains resulted in constitutively active kinases (26, 27). Deletion of the regulatory domain of MEKK1 was also thought to render MEKK1 constitutively active, although a comparison between truncated and full-length MEKK1 was not demonstrated (28). In contrast, ΔOMTK1, which lacks the N-terminal non-catalytic domain, was not constitutively active, and the kinase domain of OMTK1 was sufficient to transmit the H_2O_2 signal to the MMK3 pathway (Fig. 6*A*). However, compared with full-length OMTK1, ΔOMTK1 showed reduced activity to induce cell death. Furthermore, we found that MMK3 can still associate with the kinase domain of OMTK1 although at reduced levels (Figs. 7 and 8), suggesting that the N-terminal non-catalytic domain may contribute to MAPK binding, as suggested by analysis of MEKK1 with MPK4 (23).

Because the classic MAPK cascades are composed of MAPK, MAPKK, and MAPKKK, a certain MAPKK is supposed to function between OMTK1 and MMK3. Until now, three MAPKKs, MKK1, PRKK, and SIMKK, were isolated from alfalfa, and we analyzed whether they can fit into the pathway. Even though we found that all three MAPKKs can interact to a certain degree with OMTK1 *in vitro* (data not shown), addition of these MAPKKs did not enhance, but rather inhibited, MMK3 activation through OMTK1 in protoplasts (data not shown). Some endogenous *Arabidopsis* MAPKK is likely to function in between OMTK1 and MMK3. Because excess amounts of unsuitable exogenous MAPKKs can compete with the endogenous MAPKKs resulting in formation of unproductive MAPK modules that are unable to activate MMK3, our data are compatible with the notion that the correct MAPKK was not used. Because we found that immunoprecipitated active MMK3, which was activated through OMTK1 in protoplasts, was inactivated by *in vitro* λ phosphatase treatment (data not shown), MMK3 is likely to be activated through phosphorylation. MAPKKs are known to phosphorylate MAPKs on threonine and tyrosine residues in the TEY motif for MAPKs activation; therefore, we tested whether the TEY motif in MMK3 is phosphorylated or not. We could not detect phosphorylation of the TEY motif by Western blotting using phospho-specific TpEYp antibodies directed against mammalian ERK1/2 or alfalfa SIMK, which can recognize *in vitro* phosphorylated GST-MMK3 (data not shown). Although this result might indicate that MMK3 is not activated through MAPKKs, we cannot exclude the possibility that the phosphorylated form of MMK3 could not be recognized under our experimental conditions. To check the possibility that OMTK1 directly regulates MMK3 activity, we tested whether OMTK1 possesses kinase activity against MMK3. Immunoprecipitated OMTK1-Myc from protoplasts possessed kinase activity against MBP but could not phosphorylate GST-MMK3 (data not shown). This result suggests that OMTK1 cannot activate MMK3 through direct phosphorylation. However, it is still possible that MMK3 activity could be modified through binding to OMTK1, and further investigations will be necessary using kinase-inactive and MMK3 binding-deficient OMTK1 mutants.

Previously, we have shown that MMK3 is activated by ACC through SIMKK (6) and by fungal elicitor through SIMKK and PRKK (5). In the case of OMTK1-mediated H_2O_2 signaling, our data indicate that SIMKK and PRKK are not the functional upstream components of MMK3. So far, we have not been able to identify the MAPKK that mediates H_2O_2-induced MMK3 activation. In contrast, the newly described MAPKKK, OMTK1, is clearly involved in activating MMK3 in response to H_2O_2 but not when cells are treated by yeast elicitor or ACC (Fig. 3*A*). These data suggest that different signals activate MMK3 through different sets of MAPK modules (Fig. 9). The question arises then, how can signaling specificity be achieved or maintained? In the case of H_2O_2 signaling, our results strongly suggest that OMTK1 plays a key role in determining specificity through its scaffolding function. Considering that scaffolding functions were found to be essential for pathway specificity of yeast MAPK cascades, it is reasonable to assume that scaffolding proteins should also exist for ACC and yeast elicitor signaling pathways. Whether the scaffolding functions in these pathways will be conveyed by MAPKKKs or other proteins remains to be resolved (Fig. 9) but should provide important insight into the mechanisms of signal transduction in plants.

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