Mitogen-activated protein kinase (MAPK) cascades function in biotic and abiotic stress responses in plants. We analysed effect of oxidative stress on the activation of ATMPK6, an Arabidopsis thaliana MAPK, in Arabidopsis T87 cultured cells and rosette leaves using anti-ATMPK6 specific antibody. ATMPK6 in T87 cells was strongly activated by reactive oxygen species (ROS) such as H$_2$O$_2$ and KO$_2$. In leaves, ATMPK6 was activated by paraquat and 3-amino-1,2,4-triazole (a catalase inhibitor). These results indicate that ATMPK6 is one of the candidates for signal mediators in response to abiotic or biotic sources for ROS in Arabidopsis.

Key words: Arabidopsis — MAP kinase — Oxidative stress — Phosphorylation.

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; DTT, dithiothreitol; ERK, extracellular-regulated protein kinase; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; SA, salicylic acid; SIPK, salicylic-acid-induced protein kinase; TMV, tobacco mosaic virus; WIPK, wound-induced protein kinase.

Environmental stresses such as dehydration, salinity, chilling stress, wounding and pathogen infection are important factors that affect growth and metabolism of land plants (for reviews see Burdon et al. 1996, Lamb and Dixon 1997, Shinozaki and Yamaguchi-Shinozaki 1999).

Reactive oxygen species (ROS) such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radical (OH') are associated with a number of physiological disorders in plants. One of the important mechanisms by which plants are damaged during adverse environmental conditions is the excess production of ROS. Such oxidative stress has been shown to occur in plants exposed to high and low temperatures, particularly in combination with high light intensities, drought exposure to air pollutants (e.g. ozone or sulphur dioxide), ultraviolet light, and herbicides such as paraquat (Foyer and Mullineaux 1994). Superoxide and H$_2$O$_2$ can inactivate various macromolecules directly, but it is their conversion to OH', catalyzed by transition metals (i.e. the Haber–Weiss reaction), that accounts for their main toxicity. Hydroxyl radicals react instantaneously with proteins, lipids, and DNA, causing rapid cell damage.

H$_2$O$_2$ appears to play an important role in signal transduction during plant pathogen interactions. Not only in biotic stress response, ROS may have a role of signal molecule in abiotic stress. For instance, H$_2$O$_2$ levels were elevated during chilling of non-acclimated seedlings and during acclimation in maize (Prasad et al. 1994). It has further been shown that treatment of seedlings with H$_2$O$_2$ or menadione, a O$_2^-$-generating compound, at ambient temperature induces chilling tolerance. These results suggest a dual role for H$_2$O$_2$. During acclimation, H$_2$O$_2$ levels could be a signal that induces antioxidant enzymes, such as catalase, that subsequently protect the plant from excess H$_2$O$_2$ production at chilling temperature.

Several lines of evidence from biochemical and genetic studies of plant stress signaling indicate that phosphorylation and dephosphorylation of proteins are important in the regulation of physiological status and gene expression in response to various environmental stresses (for reviews see Mizoguchi et al. 1997, Ichimura et al. 2000a, Yang et al. 1997). A mitogen-activated protein kinase (MAPK) cascade is an intracellular signaling module ubiquitous among eukaryotic organisms (Herskowitz 1995). In plants, several MAPKs are activated by specific stresses. In tobacco cells, wounding stress induces the accumulation of mRNA of WIPK, a MAPK gene (Seo et al. 1995). The protein kinase activity of WIPK is stimulated by wounding (Seo et al. 1999), elicitors (Romeis et al. 1999, Zhang et al. 2000) and by infection with tobacco mosaic virus (TMV) (Zhang and Klessig 1998b). Salicylic-acid-induced protein kinase (SIPK), a homologue of MAPKs, is activated by salicylic acid (SA) (Zhang and Klessig 1997), elicitors (Zhang et al. 1998), wounding (Zhang and Klessig 1998a), TMV infection (Zhang and Klessig 1998b) and by hyperosmotic stress (Hoyos and Zhang 2000, Mikolajczyk et al. 2000).

We cloned and characterized nine MAPK genes, ATMPK1-9, from Arabidopsis thaliana (Mizoguchi et al. 1993, Mizoguchi et al. 1994, Mizoguchi et al. 1997). Furthermore, using the 2-hybrid analysis and a yeast complementation assay, we also identified a possible MAPK cascade (ATMEKK1 [a MAPKKK]→MEK1/ATM KK2 [MAPKKs]→ATMPK4

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[a MAPK]) (Ichimura et al. 1998, Mizoguchi et al. 1998). Our recent work showed that ATMPK4 and ATMPK6 in Arabidopsis leaves are rapidly and transiently activated by various environmental stresses such as low temperature, low humidity, hyperosmolarity, touch and wounding (Ichimura et al. 2000b). This suggests that ROS may be commonly produced by abiotic stresses, particularly low temperature and hyperosmotic stresses, which may induce activation of ATMPK4 and ATMPK6. In addition, it was suggested that ROS are potent activators for the transient expression of ATMPK6 in Arabidopsis protoplasts (by H$_2$O$_2$; Kovtun et al. 2000) and a 46-kDa ERK-type MAPK in tobacco cells (by ozone; Samuel et al. 2000).

To study whether ROS activates Arabidopsis MAPKs, we analyzed the alteration of ATMPK4 and ATMPK6 activities in Arabidopsis T87 cells that were exposed ROS. We report here that ATMPK6 but not ATMPK4 is activated by ROS. We also show that ATMPK6 in leaves was activated by treatment with paraquat and 3-amino1,2,4-triazole (3-AT). These results indicate that ATMPK6 is a possible mediator for ROS in abiotic stress.

To examine whether ATMPK4 and ATMPK6 are activated by ROS, we treated Arabidopsis T87 cells with H$_2$O$_2$ at final concentrations of 2 or 10 mM and KO$_2$ at a final concentration of 0.5 mM. As shown in Fig. 1A, H$_2$O$_2$ and KO$_2$ strongly activated ATMPK6 in T87 cells. When H$_2$O$_2$ was added into a T87 cell suspension at a final concentration of 2 mM, ATMPK6 activity gradually increased and peaked at 20 min after treatment, and then remained high for 2 h after treatment (Fig. 1A, D). With 10 mM H$_2$O$_2$, the activity of ATMPK6 peaked at 40 min after treatment, and then remained high for 3 h after treatment (Fig. 1B, E). Treatment of T87 cells with KO$_2$ at a final concentration of 0.5 mM rapidly activated ATMPK6, which peaked at 5 min. In contrast to kinase activation by H$_2$O$_2$, the ATMPK6 activity after KO$_2$ treatment rapidly decreased and reached a marginal level 60 min after treatment (Fig. 1C, F). Immunoblot analysis showed that the ATMPK6 protein was constant during the ROS treatments (Fig. 1G–I). This result suggests that post-translational modification such as phosphorylation is associated with the ROS-induced activation of ATMPK6 as previously described by Ichimura et al. (2000b) and Nühse et al. (2000).

The ROS preferentially activated ATMPK6, because the 49-kDa kinase in crude extracts specifically responded to ROS (Fig. 1A–F) and we have already confirmed that the 49-kDa kinase is ATMPK6 by immunodepletion (Ichimura et al. 2000b). Furthermore, an in-gel kinase assay of immunoprecipitates by anti-ATMPK4 antibody (Ab4CT1) (Ichimura et al. 2000b) showed that ATMPK4 was not activated by H$_2$O$_2$ (Fig. 1J) at the same concentration as in Fig. 1A and D. These results are consistent with our model that ATMPK6 and ATMPK4 may function in different MAP kinase cascades (Ichimura et al. 2000b).

It was reported that H$_2$O$_2$ is a strong activator for HOG1 MAPK in budding yeast and that activation of HOG1 is required for expression of CTT1, a catalase gene, and for maintaining survivability of yeast under oxidative stress (Schuller et al. 1994). Similarly, maize Cat1 gene encoding catalase is induced by H$_2$O$_2$, ABA and osmotic stress (Guan et al. 2000). Cat1-transcript accumulation in response to osmotic stress was shown to occur via an alternate ABA-independent pathway (Guan and Scandalios 1998). Thus, it is conceivable that ATMPK6 may function on induction of genes of the antioxidant systems against ROS when Arabidopsis cells were subjected to ROS.

Takahashi et al. (1997) demonstrated that hypoosmotic shock activated a MAPK-like kinase of 47 kDa and transiently mobilized intracellular Ca$^{2+}$ in tobacco BY-2 cells. Another MAPK-like kinase in tobacco cells was activated by H$_2$O$_2$ and hypoosmotic shock (Cazalé et al. 1999). Hypoosmotic shock imposed on Arabidopsis T-87 cells by diluting the cell suspension with an equal volume of distilled water had no effect on protein kinase activities in the immunoprecipitates (Fig. 1K).

Next we investigated whether the salicylic acid (SA) stimulates ATMPK6, because SIK, a tobacco MAPK, initially identified as a protein kinase activated by SA in suspension cells (Zhang and Klessig 1997) and ATMPK6 is an Arabidopsis homologue of SIK. Arabidopsis T87 cells were subjected to the SA treatments, and then protein kinase activities of ATMPK6 were analyzed by in-gel kinase assay coupled with immunoprecipitation (Fig. 1L). Although ATMPK6 exhibits high structural similarity to tobacco SIK which is activated in response to SA treatment (Zhang and Klessig 1997), no significant activation of ATMPK6 was detected after SA treatment (Fig. 1L). We also examined whether abscisic acid (ABA), jasmonic acid (JA) and hyperosmotic stress are potent activators of ATMPK6 in T87 cells. However, we detected no activation of ATMPK6 after these treatments (data not shown). In contrast to the case of T87, we recently reported that ATMPK6 in Arabidopsis leaves is activated by treatment of salt or dehydration stresses (Ichimura et al. 2000b). The difference on activation of ATMPK6 between T87 cells and leaves may be due to the difference of the cell types between T87 cells with proplastids and mature leaf tissues with functional chloroplast. In photosynthesis tissues, O$_2^-$ is produced by direct reduction of O$_2$ by photosynthesis system I in chloroplasts, and then converted to H$_2$O$_2$ and O$_2$ by superoxide dismutase. Thus, photosynthesis under salt and drought stresses may cause oxidative stress on leaf tissues by excess generation of ROS (Shikanai et al. 1998).

Stress-activated MAPKs such as p38 and SAPK/JNK in animal cells appear to be distant subgroups of the MAPK gene superfamily and are activated by hyperosmotic stress and other biotic and abiotic stresses (Han et al. 1994, Kyriakis et al. 1994). HOG1 and MPK1, budding yeast MAPK homologues, are stimulated by hyperosmotic shock and hypoosmotic shock, respectively. Several lines of evidence indicate that these stress-activated MAPKs in eukaryotes have pivotal roles in acquiring stress tolerance and are essential for cell differentiation and development in these organisms (Herskowitz 1995).
Our experiments showed that ATMPK6 activity is hardly affected by phytohormones or hypo/hyperosmotic stresses in T87 cells. This observation indicates that ATMPK6 functions in a highly specific signal pathway stimulated by ROS but not by phytohormones or osmotic stress. Alternatively, it is possible that T87 cells lost the upstream modules required for activation of ATMPK6 in the hormonal or osmotic signal pathway when the cell line was established.

Next, we investigated whether ATMPK6 is affected by intracellular production of ROS in the photosynthesis system. To produce intracellular ROS by inhibition of photosynthesis and photorespiration, we applied paraquat and 3-AT to Arabidopsis leaves (Fig. 2). Paraquat (methyl viologen) is a redox-cycle reagent widely used as source of superoxide radicals in the study of photosynthesis (Kurepa et al. 1998). 3-AT is an irreversible inhibitor of catalase, which scavenges H₂O₂ from the photorespiration system in leaves (Wang et al. 1999). We treated Arabidopsis leaves with paraquat at a final concentration of 50 μM under continuous illumination (Fig. 2A, C). Activation of ATMPK6 was detected at 2 h after treatment, peaking at 4 and 6 h after treatment (Fig. 2A, C, lanes 1–6). To examine whether the activation of ATMPK6 by paraquat is dependent on photosynthesis, we compared the activity of ATMPK6 in Arabidopsis leaves between dark and light conditions (Fig. 2B, D, lanes 1–5 vs. lanes 6–9). When paraquat was added, ATMPK6 was activated gradually in the light (Fig. 2C, D, lanes 6–9), but also in the dark (Fig. 2C, D, lanes 1–5). When 3-AT was added to the medium, ATMPK6 activity gradually increased (Fig. 2C, D, lanes 10–13). Inhibition of catalase possibly results in the accumulation of intracellular H₂O₂ in mesophyll cells and then the activation of a ROS-responsive signal pathway such as that involving ATMPK6. By contrast, paraquat treatment at a final concentration of 50 μM did not activate ATMPK6 in T87 cells (data not shown). T87 cells were grown in JPL medium containing sucrose as carbon and energy sources but do not require light for growth. Therefore, it is possible that paraquat was not able to produce ROS owing to an insufficient thylakoid system in the T-87 cells.
Oxidative stress-induced activation of ATMPK6

It was reported that treatment with H$_2$O$_2$ at 5 to 20 mM of final concentrations activates a MAP kinase-like enzyme at 44 kDa in Arabidopsis suspension cultured cells and that the kinase was cross-reacted with an anti-phosphotyrosine monoclonal antibody (Desikan et al. 1999). Thus, it is possible that ATMPK6 and/or the 44-kDa MAP kinase-like enzymes which are activated by H$_2$O$_2$ are involved in ROS-induced cell responses. Recently, Kovtun et al. (2000) demonstrated the activation of ATMPK6 by oxidative stress in a polyethylene glycol-mediated transient gene expression system using Arabidopsis mesophyll protoplasts. They have also developed a co-expression system of MAPKs and activators to investigate upstream factors in MAP kinase cascades and in MAPK-regulated gene expression using a protoplast transient expression system. Their study indicates that Arabidopsis NPK1-related protein kinase (ANP1) can activate ATMPK3 and ATMPK6 when the MAPK and ANP1 are introduced into protoplasts in a transient expression assay, and that ANP1 enhanced ROS-dependent activation of ATMPK6. Elicitors are potent activators for ATMPK6, as they are those for SIPK (Nühse et al. 2000). It is reasonable that ROS generated by elicitor treatment functions as a second messenger as well as an extracellular signals that activate ATMPK6, leading to the induction of defense response against pathogens. However, it remains to be clarified how ATMPK6 is involved in the ROS signaling.

In summary, ATMPK6 is an Arabidopsis MAPK mediating abiotic ROS signals, possibly involved in monitoring oxidative stress in photosynthesis organelles.

Arabidopsis T87 cells were cultured in JPL medium (Jouanneau and Péaud-Lenoel’s medium) containing sucrose at 1.5% (w/v) as the carbon source (Axelos et al. 1992). Fifty ml of 1-week-old T87 cell suspension was diluted in 85 ml of new JPL medium and turned at 100 rpm at room temperature overnight. Stress treatments were started by the addition of 15 ml of stress medium stock solutions containing reagents (H$_2$O$_2$, KO$_2$, sucrose, NaCl, or H$_2$O) or phytohormones (SA, JA, or ABA) at 10 times the final concentration. T87 cell suspension was diluted with an equal volume of H$_2$O (hypoosmotic shock). Fifteen ml of each culture was harvested by centrifugation at 500 g for 30 s and then frozen in liquid nitrogen. Mature rosette leaves from 6- to 8-week-old plants (A. thaliana, Columbia ecotype) were cut off and incubated in 10-cm-diameter dishes containing 1/10 MS medium without sucrose under continuous light illumination (3,000 lux) at 22°C for at least 10 h. The paraquat or 3-AT solutions were added gently into the dishes for stress treatments. At different time points, leaves were quickly transferred into liquid nitrogen and then stored at –80°C.

The cell homogenates or leaf extracts were centrifuged at 10,000g for 20 min at 4°C. Five hundred µg of protein from each supernatant was immunoprecipitated by the addition of 10 µl of anti-ATMPK6 N-terminus antibody (Ab6NT1) or anti-ATMPK4 C-terminus antibody (Ab4CT1) (Ichimura et al. 2000b). Twenty µl (50% [v/v] slurry) of protein A–Sepharose beads was added to the sample mixture. After gentle rotation at 4°C for 2 h, the protein A–Sepharose beads were recovered by centrifugation at 10,000g for 1 min and resuspended in 1 ml of lysis buffer, and then washed with 1 ml of washing buffer (50 mM Tris-Cl [pH 7.4], 1 M NaCl, 2 mM EGTA, 5 mM NaF, 1 mM Na$_3$VO$_4$, 0.1 mM PMSF, 1 mM DTT, 0.2% Triton X-100) three times. The immunoprecipitates were resuspended in SDS-PAGE sample buffers (Laemmli 1970). Protein kinase activity was analyzed by in-gel kinase assay according to Ichimura et al. (2000b).

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