Mastoparan Rapidly Activates Plant MAP Kinase Signaling Independent of Heterotrimeric G Proteins[1]

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It has long been known that mastoparan (MP), a cationic, amphiphilic tetradecapeptide isolated from wasp venom, is capable of directly stimulating the guanine nucleotide exchange reaction of the α-subunit of animal heterotrimeric G proteins via a mechanism analogous to that of G protein coupled receptors. This leads to a myriad of downstream events including the activation of mitogen-activated protein kinases (MAPKs). Here, we show that MP induction of plant MAPK signaling does not require the participation of either the Gα- or Gβ-subunits of the plant heterotrimeric G proteins, but is reliant on reactive oxygen species (ROS), a cognate MAPKK, and influx of extracellular Ca^{2+} ions. While this does not preclude a role for a heterotrimeric G protein in MAPK signaling, it raises concern about the conclusions drawn from published experiments using MP.

Gα-, Gβ-, and Gγ-homologs have been identified in Arabidopsis and other plant species (Ma et al., 1990; Gotor et al., 1996; Lee and Assmann, 1999; Saalbach, et al., 1999; Mason and Botella, 2000). In Arabidopsis, a single prototypical Gα-(Arabidopsis G PROTEIN, ALPHA SUBUNIT1 [GPA1]) and one prototypical Gβ-(Arabidopsis G PROTEIN, BETA SUBUNIT1 [AGB1]) subunit and potentially, two Gγ-subunits (Arabidopsis G PROTEIN, GAMMA SUBUNIT1 [AGG1 and AGG2]), are found. Interaction has been detected between the Arabidopsis Gγ- and Gβ-subunits (Mason and Botella, 2000; for review, see Jones, 2002), and evidence has been obtained for in vitro and in vivo interaction of GPA1 and Gβ (J.-G. Chen, J.S. Liang, and A.M. Jones, unpublished data).

Mutational and pharmacological studies have implicated plant heterotrimeric G protein subunits in numerous physiological processes and phenotypic changes, including auxin and gibberellin signaling, K^+ channel regulation, Ca^{2+} regulation, cell division, and stomatal function (Aharon et al., 1998; Jones et al., 1998; Saalbach et al., 1999; Ullah et al., 2001; Wang et al., 2001).

MP has been widely used to implicate G protein regulated processes in both plants and animals (Higashijima et al., 1988; Legendre et al., 1992; Legendre et al., 1993; Wise et al., 1993; Höller et al., 1999). For example, short-term responses to MP treatment reported for plants include increases in cellular Ca^{2+} ions, induction of an oxidative burst, stimulation of 1,4,5-inositol triphosphate turnover, and activation of phospholipase C, phospholipase D2, and myelin basic protein (MBP) kinases (Scherer, 1992; Kauss and Jeblick, 1996; Chahdi et al., 1998; Takahashi et al., 1998; Chahdi et al., 2003). Although it is generally assumed that these responses are mediated by the initial activation of heterotrimeric G proteins, there is limited direct evidence for this, particularly in plants.

MASTOPARAN-INDUCED ACTIVATION OF MAPKS IN ARABIDOPSIS HETEROTRIMERIC Gα AND Gβ LOSS-OF-FUNCTION GENOTYPES

Since the Gα-subunit is the classical target of MP in animal cells, we anticipated that loss of Gα function would interfere with its ability to activate downstream effectors such as terminal MAPKs. To test this, we employed both wild-type and loss-of-function mutant lines of Arabidopsis in which the genes encoding the prototypical heterotrimeric Gα (*gpa-1*) and Gβ (*agb-1*) proteins are disrupted. These well-characterized lines have provided insight into the role of plant G proteins in control of other plant processes such as cell division (Ullah et al., 2001, 2003) and stomate closure (Wang et al., 2001).

To determine if MP had the ability to induce the activation of an Arabidopsis protein kinase capable of phosphorylating MBP, a known substrate of eukaryotic MAPKs, we treated Arabidopsis wild-type seedlings (Columbia ecotype) with MP (5 μM) for 5 min, followed by in-gel analysis. As shown in Figure 1A, MP treatment led to strong MBP-phosphorylating activity by two proteins (approximately 44 and 46 kD). Anti-AtMPK6 antibodies (raised against the N-terminal peptide of AtMPK6) immunoprecipitated the 46-kD MBP-phosphorylating activity, suggesting that the 46-kD MAPK is most likely AtMPK6, the ortholog

[1] This work was supported by the Natural Sciences and Engineering Research Council of Canada (to B.E.), by the National Science Foundation (grant no. MCB-0209711), and by the National Institute of Health (grant no. GM65989 to A.M.J.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.103.037275.

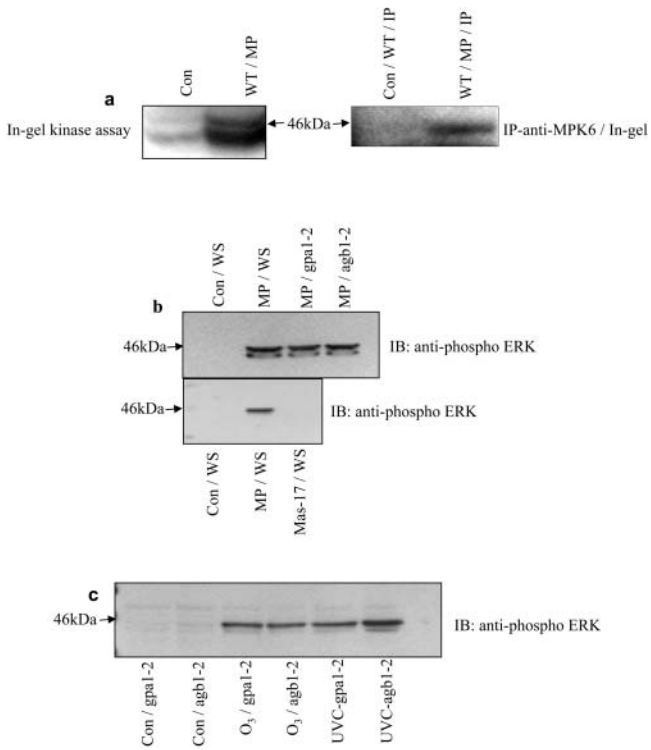

Figure 1. MP, ozone, and UVC induced the activation of MAPKs in wild-type and heterotrimeric (Gα, *gpa1-2* and Gβ, *agb1-2*) loss-of-function Arabidopsis seedlings. a, To ascertain if MP is capable of inducing the activation of an Arabidopsis MAPK, extracted proteins (40 μg) from MP-treated (5 μM for 5 min) samples, both crude and immunoprecipitated (using specific anti-AtMPK6 antibodies) were assayed for in-gel kinase activity. MBP-phosphorylation was detected in both the crude and immunoprecipitated samples but absent from the control cells. b and c, Seedlings were treated with MP or Mas-17 (5 μM, 5 min), ozone (200 ppb, 10 min), or UVC (10 J M^{-2}, total energy) and harvested as described earlier. Control tissue was from seedlings exposed to ambient air (ozone or UVC) or treated with water, the vehicle used for the MP solution. Protein (40 μg) extracts prepared from 2-week-old leaf tissue, isolated from MP-, ozone-, and UVC- exposed wild-type and heterotrimeric G protein loss-of-function seedlings, were resolved on a 10% SDS-polyacrylamide gel, blotted and probed with anti-pERK 1&2 antibodies (primary antibodies). Columbia wild-type seedlings, when treated with MP (5 μM, 5 min), ozone (200 ppb, 10 min), or UVC (10 J M^{-2}, total energy), gave the same results as the parental (wild-type) ecotype Wassilewskija seedlings (data not shown). All experiments were performed in triplicate.

of salicylic acid-induced protein kinase (SIPK) from tobacco (*Nicotiana tabacum*). The lower band has an activation profile that is similar to that of AtMPK3, the ortholog of the wound-induced protein kinase (WIPK) from tobacco. Treatment of *gpa-1*, *agb-1*, and WT seedlings with MP (5 μM) for 5 min, followed by immunoblotting of the extracted proteins, revealed that neither loss of the Gα-subunit, nor loss of the Gβ-subunit, interfered with the ability of MP to activate AtMPK6 (Fig. 1B, top). Neither the control nor treatment with Mas-17, the largely inactive analog of MP, was able to elicit a signaling response in these Arabidopsis seedlings (Fig. 1B, bottom).

ROS-induced modifications of animal heterotrimeric Gα proteins lead to their dissociation, followed by subsequent MAPK activation (Nishida et al., 2002). In light of our previous results, we hypothesized that G proteins might not be required for ROS-induced MAPK signaling in plants. To test this, we examined the ability of direct oxidant stress (ozone; 200 nL L^{-1}) and indirect oxidant stress from ultraviolet C radiation (UVC; 10 J M^{-2} [at 254 nm] total energy) to activate AtMPK6 in the *gpa-1* and *gpb-1* backgrounds. Immunoblot analysis of the proteins from the treated tissues was performed using anti-phospho-ERK 1& 2 antibodies, which identify the dual-phosphorylated, active form of SIPK (Samuel et al., 2000; Miles et al., 2002). As predicted, ROS-induced activation of the SIPK ortholog, AtMPK6, was unaffected by loss of either GPA1 or AGB1 (Fig. 1C).

MASTOPARAN-INDUCED MAPK PHOSPHORYLATION IN TOBACCO cv XANTHI nc SUSPENSION-CULTURED CELLS REQUIRES CA^{2+} IONS, A COGNATE MAPKK, AND ROS

The chemical and structural complexity of whole plant tissues can make it difficult to employ pharmacological probes. Cultured plant cells, on the other hand, provide a relatively homogeneous and dispersed tissue that is more easily manipulated and amenable to pharmacological studies.

To address the impact of MP on plant MAPK signaling, tobacco cv Xanthi nc suspension-cultured cells were employed. MP treatment of tobacco cells was previously reported to activate protein kinases (Takahashi et al., 1998). Although the kinases involved were not identified, examination of the published data suggested that two of the main species might be SIPK and WIPK, two MAPKs known to be activated by various environmental stresses, including ozone, wounding, and microbial elicitors (Samuel et al., 2000; for review, see Jonak et al., 2002; Miles et al., 2002).

To test this hypothesis, 1-week-old suspension-cultured tobacco cells were incubated for 1 min with either 5 μM MP or 5 μM mas-17, the inactive analog of MP. When protein extracts from treated cells were assayed in-gel for protein kinase activity, two bands (approximately 44 and 46 kD) displaying MBP-phosphorylating activity were detected in the extracts from MP-treated cells, but were absent from untreated or mas-17-treated cells (Fig. 2A). The position of these MBP kinases, and their ability to phosphorylate MBP, are consistent with the properties of MAPKs, while comigration with the 46-kD phosphorylation activity in UVC-irradiated tobacco cells (Miles et al., 2002) suggested that the upper band activity might be SIPK. Consistent with this, anti-SIPK antibodies were able to immunoprecipitate a 46-kD MBP-phosphorylating activity (Fig. 2A). Taken together, these results indicate that SIPK is activated by MP treatment in tobacco cells.

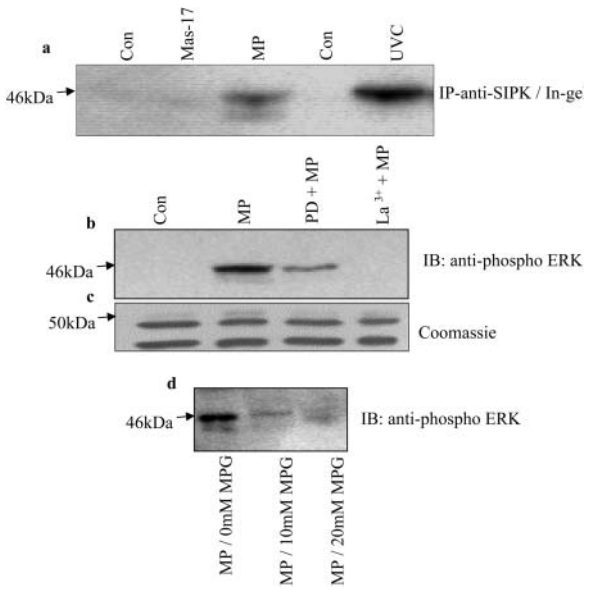

Figure 2. MP, but not the less active analog (Mas-17), rapidly activates an MAP kinase in wild-type tobacco suspension-cultured cells. a, Extracted proteins (40 μg) from these same samples were immunoprecipitated using specific anti-SIPK antibodies (5 μg), and the precipitate was assayed for in-gel kinase activity. MBP-phosphorylation was evident in MP-treated (5 μM for 1 min) tobacco cells, but absent from Mas-17 treated cells. Control cells were treated with water, the vehicle for MP treatment. UVC-irradiated tobacco cells also displayed MBP-phosphorylation activity and extracts of these cells were used as an in-gel size standard. Protein extracts from both controls, MP- and UVC-exposed cells, were analyzed by the MAPK phosphorylation assay. b, MP-induced activation of SIPK is dependent on Ca^{2+} ions, and an upstream MAPKK. Protein extracts (20 μg) from suspension-cultured tobacco cells that had been pretreated with either La^{3+} (5 mM, 15 min), or the MEK 1&2 inhibitor, PD98059 (100 μM, 60 min) or followed by MP treatment (5 μM, 1 min) were analyzed via immunoblotting using anti-pERK 1&2 antibodies. Pretreatment of tobacco suspension cultured cells with either La^{3+} or PD interdicted the ability of MP to induce the activation of SIPK. c, The Coomassie Brilliant Blue-stained membrane is used as a loading control. d, MP-induced activation of SIPK was also inhibited by pretreatment of the tobacco suspension-cultured cells with MPG (10 or 20 mM, 60 min) as detected by anti-pERK1&2 antibodies. All experiments were performed in triplicate.

In eukaryotic organisms, Ca^{2+} ions play important regulatory roles in a multitude of physiological processes (Lebrun-Garcia et al., 1998; Suzuki et al., 1999; Samuel et al., 2000; Miles et al., 2002). It has been shown that MP is able to induce a rapid intracellular increase in Ca^{2+} ion levels in both plants and animals (Okano et al., 1985; Franklin-Tong et al., 1996; Tucker and Boss, 1996; Takahashi et al., 1998). Ca^{2+} ions are also important in protein kinase signaling. We previously showed that oxidant-induced activation of SIPK in tobacco cells is strongly attenuated by pretreatment of the cells with either the Ca^{2+} channel blocker, La^{3+}, or the membrane-permeable Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-tetrakis (acetoxy-methyl) ester (Samuel et al., 2000; Miles et al., 2002). The report that MP induced activation of MAPK activity in tobacco is calcium-dependent (Takahashi et al., 1998) was confirmed in this study. When tobacco cells were pretreated with $LaCl_3$ (5 mM) for 15 min followed by incubation with MP (5 μM) for 1 min, immunoblot analysis revealed that La^{3+} blocked the MP-induced activation of SIPK (Fig. 2B). Since MP has been previously reported to induce a rapid influx of extracellular Ca^{2+} ions, our present data imply that this influx of extracellular Ca^{2+} ions mediates some of the downstream effects induced by the peptide, including MAPK activation.

While these results establish the importance of Ca^{2+} fluxes for signal transmission from MP to SIPK, the nature of the intervening steps remains to be defined. In canonical MAPK signaling modules, activation of a terminal MAPK is catalyzed by a cognate MAPKK. We previously reported that oxidant-induced activation of SIPK can be blocked by PD98059, a potent and specific MAPKK inhibitor (Samuel et al., 2000). Tobacco cells pretreated with PD98059 (100 μM) for 60 min followed by MP (5 μM) for 1 min had significantly reduced MP-induced SIPK activation, indicating that this activation involves one or more MAPKK (Fig. 2B). The Coomassie Brilliant Blue-stained membrane (Fig. 2C) is presented as a representative loading control.

It has been reported that MP can induce a rapid and transient accumulation of ROS in plant cells (Kauss and Jeblick, 1996), mimic an elicitor-induced oxidative burst in cultured soybean (*Glycine max*) cells (Legendre et al., 1992), induce the production of superoxide-anion formation in intact mammalian HL-60 cells (Klinker et al., 1994), and elicit a hypersensitive response in isolated *Asparagus sprengeri* mesophyll cells (Allen et al., 1999). Since it is well established that H_2O_2 and $^{\bullet}O_2^-$ can induce the activation of MAPKs in both plants and animals, we investigated the possibility that MP-induced ROS production might be involved in its ability to activate SIPK.

Extracted proteins (20 μg) from suspension-cultured tobacco cells that had been pretreated with the free radical scavenger, *N*-(2-mercaptopropionyl)-glycine (MPG; 10 or 20 mM) for 60 min, followed by MP (5 μM) for 1 min, were analyzed by immunoblotting using anti-pERK 1&2 antibodies. The control cells were treated only with MP (5 μM) for 1 min. MPG-treated cells showed a marked suppression of SIPK activation compared to control cells (Fig. 2D). The use of a different free radical scavenger, *N*-acetyl cysteine, gave similar results (data not shown).

CONCLUSION

Our findings suggest, at least with respect to the canonical heterotrimeric Gα and Gβ species in Arabidopsis, that neither MP action nor oxidant-induced activation of AtMPK6 requires a functional heterotrimeric G protein. While Arabidopsis has a single canonical Gα-subunit gene, there are three other genes (*XLGPA1-3*) that share some deduced amino acid sequence identity to GPA1 in their carboxy-terminal halves (Lee and Assmann, 1999; Assmann, 2002). By

extending our current understanding of G protein action from metazoans to Arabidopsis, we conclude that it is unlikely that the MP effects we observe here are dependent on one of these other unusual putative Gα-subunits. Since Gβ-subunits are required for Gα function, and there is a single Gβ gene in Arabidopsis, the observed MP effects in the absence of AGB1 (Fig. 1B), suggest a mode of action independent of a heterotrimeric G protein complex, regardless of the Gα-subunit composition. However, we emphasize that the results do not preclude a role for a heterotrimeric complex in MAPK signaling.

In addition, we have demonstrated in tobacco suspension-cultured cells that an influx of extracellular Ca^{2+} ions plays an essential role in the activation of SIPK, the AtMPK6 ortholog by both MP and ROS. However, the calcium-dependent step has not been identified. Plants contain many calcium-dependent protein kinases whose roles remain undefined, so it is possible that activation of one or more of these is required in order for the input signal to reach the MAPK module(s). It is also possible that an MP-induced Ca^{2+} influx could affect the intracellular redox environment by stimulating ROS formation through the calcium-regulated NADPH oxidases.

In aequorin-transformed tobacco cells, the MP-induced oxidative burst was inhibited by Ca^{2+} chelators or Ca^{2+} channel blockers (Chandra and Low, 1997), and movement of exogenous Ca^{2+} into the same cells initiated an oxidative burst in the absence of MP. Since addition of catalase had no effect on the influx of Ca^{2+} in these same tobacco cells, the Ca^{2+} ion influx was placed upstream of the ROS burst. By extension, this model would place the ROS signal generation, whether MP- or oxidant-induced, upstream of MAPK activation, which is fully consistent with the suppressive effect of free radical trapping reagents.

MP and its active analogs have been extensively employed in studies of both plant and animal signaling networks. The work reported here demonstrates that, at least in plants, MP clearly has the ability to activate a central signal pathway without requiring the involvement of a canonical heterotrimeric G protein. Hence, use of MP in studies of plant cells should consider other modes of signal transmission than the prototypical Gα protein.

ACKNOWLEDGMENT

Anti-SIPK antibodies were kindly provided by Y. Ohashi (Tsukuba, Japan).

Received December 5, 2003; returned for revision December 30, 2003; accepted December 30, 2003.

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