

Phosphatidic acid activates a wound-activated MAPK in *Glycine max*

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Summary

Many plant species demonstrate a systemic increase in phosphatidic acid (PA) levels after being wounded (Lee *et al.*, 1997). To understand the role of PA in wound signal transduction, we investigated if PA can activate protein kinases in soybean (*Glycine max* L.). We found that a MAPK is activated in soybean seedlings in both wounded and neighboring unwounded leaves. The wound-activated soybean kinase is specifically recognized by an antibody against the alfalfa MAPK, SIMK. When PA production is inhibited with *n*-butanol, an inhibitor of phospholipase D, the wound-induced activation of the MAPK is suppressed, suggesting that an elevation in PA levels is essential for its activation. Supporting this is the observation that exogenous PA activates the MAPK in suspension-cultured soybean cells. Activation of the 49 kDa MAPK occurs almost exclusively by PA, as other lipids are unable to or can only weakly activate the kinase. PA-induced activation of the MAPK is not a direct effect on the kinase but is mediated by upstream kinases. Our results suggest that PA acts as a second messenger in wound-induced MAPK signaling in plants.

Keywords: wound, phosphatidic acid, MAPK, soybean, SIMK

Introduction

Wounding in plants is typically caused by physical injury and herbivore or insect attack. When wounded, plants express several sets of defense-related genes that are involved in healing damaged tissues and protecting against pathogen infection and insect attack (Brederode *et al.*, 1991; Hemerly *et al.*, 1993; Lawton and Lamb, 1987; Memelink *et al.*, 1993). These genes are activated through signaling pathways that include various protein kinases (Bögre *et al.*, 1997; Mizoguchi *et al.*, 1996; Seo *et al.*, 1995; Zhang and Klessig, 1998a). Although some signal mediators participating in these pathways have been identified (Farmer and Ryan, 1992; Lee *et al.*, 1997; Narváez-Vásquez *et al.*, 1999; Ryu and Wang, 1996), many components are still missing. In particular, the upstream activators of the protein kinases have not been identified.

For many plant species, phosphatidic acid (PA) might act as a second messenger in wound signaling, as levels of PA increase rapidly and transiently both at the wound site and systemically (Lee *et al.*, 1997; Ryu and Wang, 1996). In addition, PA levels have been reported to increase after abscisic acid treatment (Jacob *et al.*, 1999; Ritchie and

Gilroy, 1998), drought (Chetal *et al.*, 1982; Frank *et al.*, 2000; Munnik *et al.*, 2000), freezing (Yoshida, 1979), elicitors (Van der Luit *et al.*, 2000), and hyper-osmotic stress (Munnik *et al.*, 2000). PA is also an important molecule in many signal transduction pathways of animal cells, as it controls intracellular Ca²⁺ levels, the organization of the cytoskeleton, and protein kinase activity (English, 1996; Ha and Exton, 1993; McPhail *et al.*, 1999; Munnik *et al.*, 1998). Thus, PA may play an important role in plant cell signal transduction (Chapman, 1998; Munnik *et al.*, 1998; Pappan and Wang, 1999). However, the mechanism by which PA acts remains to be elucidated.

Mitogen-activated protein kinases (MAPKs) may also be important in wound signal transduction in plants. Two different MAPKs, WIPK (wound-induced protein kinase) and SIPK (salicylic acid-induced protein kinase), are activated in tobacco plants after wounding (Seo *et al.*, 1999; Zhang and Klessig, 1998a). The kinase activity and mRNA levels of WIPK (tobacco) and its orthologs from alfalfa (SAMK) and Arabidopsis (AtMPK3) increase upon mechanical stress (Bögre *et al.*, 1997; Mizoguchi *et al.*, 1996; Seo

et al., 1995). Similar to tobacco SIPK, the alfalfa and Arabidopsis orthologs SIMK and AtMPK6 are also activated in response to wounding (Romeis *et al.*, 1999; Zhang and Klessig, 1998a; C. Jonak and H. Hirt, unpublished results). While WIPK is responsible for the production of wound-induced jasmonic acid (Reinbothe *et al.*, 1994; Seo *et al.*, 1995; Seo *et al.*, 1999), the role of SIPK in wound signaling is not yet clear.

Here we show that in soybean plants, wounding activates a SIMK-like MAPK. The wound-activation of the MAPK is inhibited when PA production is suppressed, and exogenously applied PA specifically activates the MAPK in suspension-cultured soybean cells. This suggests that PA participates as a second messenger in wound signal transduction by activating a specific MAPK cascade.

Results

Wounding activates a 49-kDa protein kinase in soybean leaves

To assess the role of PA in wound signal transduction, we studied the relationship between PA and MAPK activation. We used the soybean as our subject because the increase of PA due to wounding is more consistent and pronounced in the soybean compared with other plant species tested, including tomato, tobacco, pepper, sunflower and broad bean (Lee *et al.*, 1997). At both wounded and unwounded neighboring leaves of soybean, PA level, expressed as the percentage of PA in control leaves from non-wounded plants, was elevated (Figure 1a), and a protein kinase of 49 kDa was activated (Figure 1b). The kinetics of the protein kinase activation and the PA level elevation were very similar (Figure 1). Both were apparent as early as 2 min after wounding, and were stronger and more persistent at the wounded leaves than at unwounded neighboring leaves. The 49 kDa wound-activated protein kinase (WAPK) could phosphorylate both histone and myelin basic protein (MBP), but preferred MBP as a kinase substrate (data not shown). The molecular mass and substrate preference of the soybean WAPK are similar to those of MAPKs.

At least two MAPKs, SIPK and WIPK, are activated in tobacco after wounding (Mizoguchi *et al.*, 1996; Seo *et al.*, 1995; Zhang and Klessig, 1998a). In order to determine which group the soybean WAPK belonged to, we used antibodies specific for synthetic peptides corresponding to the C-terminal amino acids of alfalfa MAPKs SIMK, MMK2, MMK3 and SAMK (Jonak *et al.*, 1993; Jonak *et al.*, 1995; Jonak *et al.*, 1996). All of the antibodies except MMK3 antibody cross-reacted with proteins of the expected sizes from crude extracts of soybean leaves and suspension-cultured cells (Figure 2a). MMK3 antibody also cross-reacted with a protein of the expected size from soybean

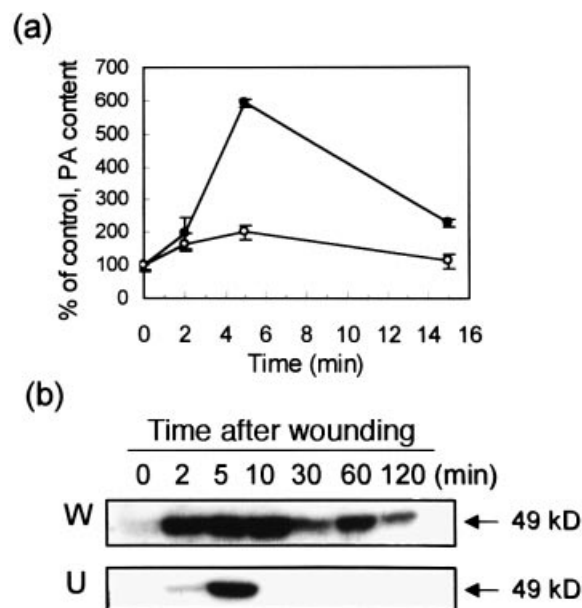


Figure 1. Wounding induces the elevation of PA levels and activates a protein kinase.

One leaf of a soybean plant at the two leaf stage was wounded, the plant was incubated for various periods, and the leaves were frozen in liquid nitrogen.

(a) Lipids were extracted from wounded (●) and neighboring unwounded (○) leaves and separated on a TLC plate. The PA levels were quantified with a phosphate assay and presented as a percentage of control (PA from control leaf from non-wounded plant). Data are mean \pm SE from 3 to 6 plants at each time point.

(b) Protein extracts were prepared from wounded (W) and neighboring unwounded (U) leaves and tested for kinase activity by the in-gel kinase assay using MBP as a substrate.

leaves, but not from suspension-cultured cells; MMK3-like protein kinase may be lacking in the cultured cells. Only the SIMK antibody immunoprecipitated a kinase that had been activated by wounding (Figure 2b).

PA activates the wound-activated 49 kDa protein kinase in soybean cells

To determine whether the WAPK activation is related to elevations in PA levels, we assessed the effect of *n*-butanol on soybean MAPK activation, using *sec*-butanol as a control. *n*-butanol has been widely used as an inhibitor of phospholipase D (PLD) in plant as well as animal systems (Chen *et al.*, 1997; Jacob *et al.*, 1999; Munnik *et al.*, 1995; Nofer *et al.*, 1997; Ritchie and Gilroy, 1998). PLD has been shown to be involved in systemic PA production in wounded castor bean leaves (Ryu and Wang, 1996). Two leaf-stage soybean seedlings were supplied with 0.1% of either *n*-butanol or *sec*-butanol through their cut stem for 30 min, and then one leaf was wounded. Five minutes after wounding, both wounded and unwounded neighboring leaves were harvested for measurement of either PA level

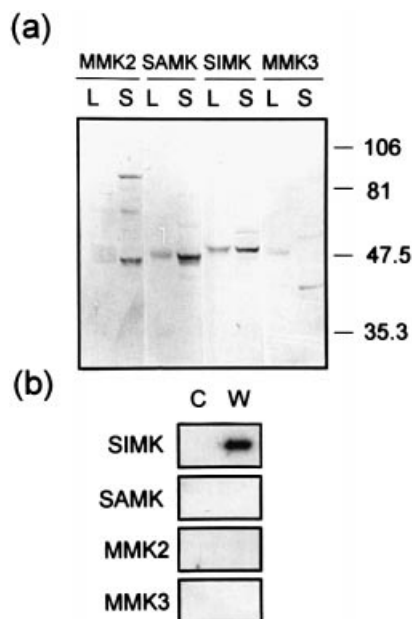


Figure 2. Wounding activates a SIMK-like MAPK in soybean leaves. (a) Crude extracts from soybean leaves (L) and suspension cells (S) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and then probed with polyclonal antibodies specific for the C-termini of the alfalfa MAPKs MMK2, SAMK, SIMK, and MMK3. Molecular weight standards are denoted to the right.

(b) Protein extracts from control (C) and wounded leaves (W) made 5 min after wounding, and immunoprecipitated with SIMK, SAMK, MMK2 and MMK3 antibodies. The kinase activity of the precipitates was determined by the in-gel kinase assay using MBP as a substrate.

or kinase activity. In the unwounded neighboring leaf, *n*-butanol completely inhibited both the PA increase (Figure 3a) and activation of MAPK (Figure 3b), while *sec*-butanol did not. However, in the wounded leaf, where PA levels are much higher than those seen systemically (Figure 1a, Lee *et al.*, 1997; Ryu and Wang, 1996), *n*-butanol suppressed neither PA production nor MAPK activity (data not shown). At the wound site, non-specific hydrolases released from broken cells, rather than PLD, may be the major enzymes that produce PA and activate the WAPK.

As this experiment suggests that PA is involved in the wound-activation of the soybean MAPK, we examined whether exogenously applied PA can also activate the WAPK. When soybean leaves were infiltrated with either PA or water as a control according to previously described methods (Zhang and Klessig, 1998a), both induced activation of the WAPK (data not shown), perhaps because the infiltration process imposes mechanical or osmotic stresses. Intact soybean leaves may thus not be suitable for testing PA activation of the MAPK in this manner. Due to the limitations of the infiltration experiment, we decided to use suspension-cultured soybean cells to assess the effect of exogenous PA on soybean protein kinase. We found that 50 μ M PA activates a 49-kDa protein kinase in these

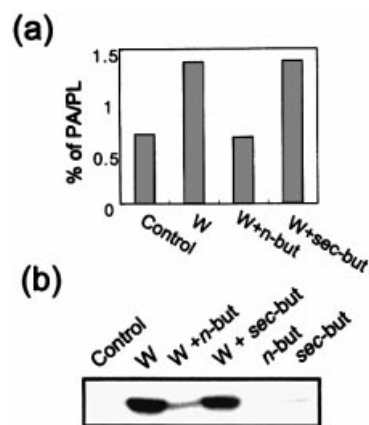


Figure 3. PA is required for wound-activation of the protein kinase in soybean leaves.

(a) Soybean seedlings labeled with 14 C-acetate were treated with 0.1% *n*- or *sec*-butanol (but) for 30 min before wounding (W). Five minutes after wounding, lipids were analyzed. PA level is presented as percentage of total phospholipids (PL). Data are representative of 3 different experiments with similar results.

(b) Soybean seedlings at 2 leaf stage were pretreated with either 0.1% *n*- or *sec*-butanol (but) for 30 min, and one leaf was wounded. Five min after, protein extract was prepared from unwounded neighboring leaves (W) and kinase activity was measured using the in-gel kinase assay with MBP as a substrate. Control was from non-wounded plants.

cells (Figure 4a). This protein kinase was similar to the 49 kDa WAPK, since it was immunoprecipitated by the SIMK but not the SAMK antibody (Figure 4b). Moreover, as with the seedling leaves, suspension-cultured soybean cells showed the 49 kDa WAPK activation in response to wounding, which was blocked by pretreatment with *n*-butanol (Figure 4c). In the suspension-cultured soybean cell system, PA activated the MAPK within 2–5 min after treatment (Figure 4a). The activity of the kinase peaked at 10 min and returned to basal levels 30 min after treatment. Control cells treated with water did not exhibit kinase activation at any time. The signals were not due to autophosphorylation because phosphorylation was not detected when MBP was omitted from the gel mixture (data not shown). The PA concentration-dependence of kinase activation showed a bell-shaped curve (Figure 5a); a PA concentration as low as 10 μ M activated the MAPK, and the effect was maximal at 50 μ M. At higher concentrations of PA (up to 200 μ M), kinase activity was still higher than the controls but much lower than at 50 μ M (Figure 5a). These observations indicate that the effect of PA on kinase activation is not due to a detergent effect.

PA selectively activates the soybean 49 kDa SIMK-like MAPK

To determine whether only PA can achieve activation of the soybean MAPK, soybean cells were treated for 5 min

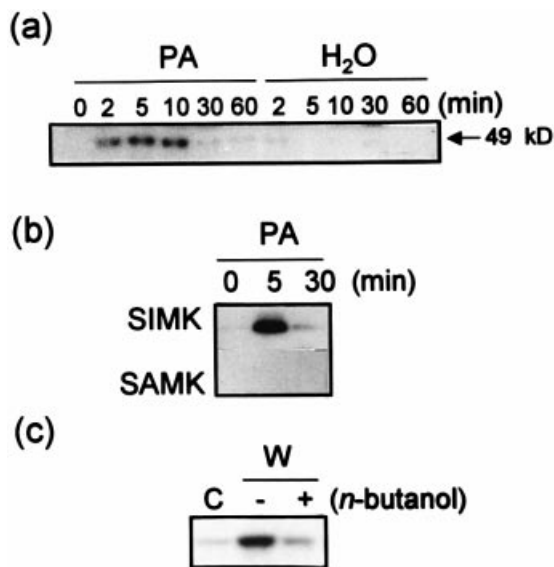


Figure 4. PA activates a SIMK-like MAPK in suspension-cultured soybean cells. (a) Suspension-cultured soybean cells were treated with 50 μM PA or an equal volume of water for various periods as indicated. PA: PA-treated; H₂O: water-treated. (b) Protein extracts before and 5 and 30 min after PA treatment were immunoprecipitated with SIMK and SAMK antibodies. (c) Suspension-cultured soybean cells were pretreated with 0.1% *n*-butanol for 10 min, wounded, and incubated for 5 min. C: control cell suspension, W: wounded cell suspension. In all cases, kinase activities were tested by the in-gel kinase assay using MBP as a substrate.

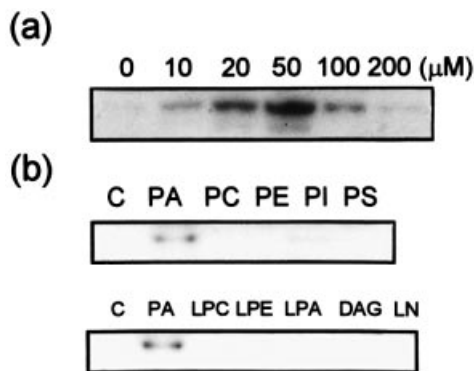


Figure 5. Concentration-dependence and lipid specificity of PA-induced MAPK activation. (a) Concentration-dependent activation of the MAPK by PA, in soybean cells treated with various concentrations of PA for 5 min. (b) Lipid specificity of soybean MAPK activation in suspension-cultured soybean cells treated with 50 μM of various lipids for 5 min. Kinase activities in all cases were determined by the in-gel kinase assay using MBP as a substrate. C: control; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; LPA: lysophosphatidic acid; DAG: diacylglycerol; LA: linoleic acid.

with 50 μM of other lipids, namely diacylglycerol and linoleic acid, and phospholipids such as lysophosphatidic

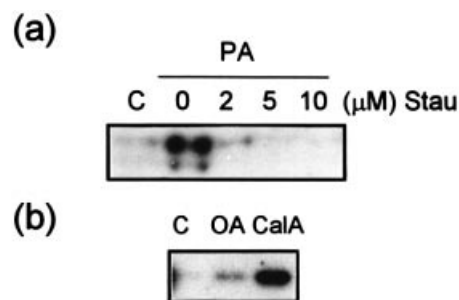


Figure 6. Inhibitors of protein kinases and phosphatases modulate PA-activated MAPK activity in suspension-cultured soybean cells. (a) Kinase activity in total cell extracts of suspension-cultured soybean cells that were pretreated with various concentrations of staurosporin (stau) for 10 min before PA treatment. (b) Kinase activity in suspension-cultured soybean cells that were treated with 1 μM of okadaic acid (OA) or calyculin A (Cal A) for 30 min in the absence of PA or wounding. Crude extracts containing 100 μg of total protein were immunoprecipitated with SIMK antibody. Kinase activities were determined by the in-gel kinase assay using MBP as a substrate. C: control, PA: PA-treated.

acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, and lysophosphatidylethanolamine. None of the lipids activated the protein kinase at levels comparable with that induced by PA, although slight activation was observed with lysophosphatidic acid and phosphatidylinositol (Figure 5b). The ineffectiveness of diacylglycerol, linoleic acid and lysophosphatidic acid shows that PA does not activate the protein kinase through its metabolites. However, it remains to be tested whether diacylglycerol pyrophosphate, a novel PA metabolite reported to form after PA signaling (Munnik *et al.*, 2000; Van der Luit *et al.*, 2000), activates the protein kinase. Dioctanoyl- and 1-palmitoyl, 2-linoleoyl-PA activated the protein kinase to similar extents as dipalmitoyl-PA did, suggesting that the length of the acyl chains in PA is not critical in its ability to activate the MAPK (data not shown).

PA-induced activation of the 49 kDa SIMK-like MAPK requires upstream phosphorylation

As MAPKs are activated by phosphorylation by upstream protein kinases, we tested whether staurosporin, a general protein kinase inhibitor, could alter PA-activation of the soybean MAPK. When suspension-cultured soybean cells were pretreated with 2 μM of staurosporin for 10 min, PA-induced activation of the 49 kDa protein kinase was completely suppressed (Figure 6a). We then reasoned that if PA activation of the protein kinase requires a phosphorylation step, activation might also be achieved by inhibiting dephosphorylation. Indeed, when soybean cells were treated for 30 min in the absence of PA with 1 μM calyculin A or okadaic acid (potent inhibitors of protein

phosphatases 1 and 2 A), a soybean protein kinase was activated (Figure 6b). Like the PA-activated protein kinase (Figure 4b), this kinase could be immunoprecipitated by the SIMK antibody and was 49 kDa in size.

Discussion

While PA has been shown to be involved in signal transduction pathways in plant cells (Jacob *et al.*, 1999; Munnik *et al.*, 2000; Ritchie and Gilroy, 1998), little is known about how PA acts as a signal mediator. Here, we show evidence that PA plays a critical role in wound signal transduction by activating a MAPK pathway.

Upon wounding, soybean leaves exhibit activation of a 49-kDa MAPK that crossreacts with an antibody that recognizes the alfalfa SIMK. Several lines of evidence support the involvement of PA production in the wound-induced activation of this MAPK. First, *n*-butanol, which inhibits the formation of PA by PLD, inhibits the wound-induced activation of the 49 kDa soybean protein kinase (Figures 3 and 4). Second, in correlation with PA levels in wounded plants (Figure 1a), the WAPK activity is higher in wounded than in unwounded neighboring leaves (Figure 1b). Third, treatment of suspension-cultured soybean cells with PA activates a 49-kDa SIMK-like MAPK that appears to be identical to the WAPK of soybean leaves, as both kinases are selectively recognized by anti-SIMK antibody (Figures 2 and 4), and their molecular weights and substrate preferences were identical. Fourth, elevation of systemic PA levels in wounded soybean parallels the activation of the 49 kDa WAPK (Figure 1). Also supporting our hypothesis, when PA is infiltrated into Arabidopsis leaves, it activates the 49 kDa AtMPK6 MAPK (our unpublished data).

Our data imply that elevations in PA levels are at least partly responsible for WAPK activation. This causal relationship can also be inferred from observations in other reports. In suspension-cultured alfalfa cells, hyper-osmotic stress activates SIMK (Munnik *et al.*, 1999), and stimulates PLD to raise PA levels with kinetics that are similar or slightly faster than those for SIMK activation (Munnik *et al.*, 2000). Thus, PA may activate SIMK not only in soybean and Arabidopsis, but also in alfalfa.

Lipid-activated protein kinases of plants have been reported previously. They belong to the Ca²⁺-dependent protein kinase (CDPK) family, and are activated by crude lipids and phospholipids such as lysophosphatidylcholine and lysophosphatidylinositol (Harper *et al.*, 1993; Schaller *et al.*, 1992). Recently a new type of CDPK that is specifically activated by PA has been reported (Farmer and Choi, 1999). However, the PA-activated 49 kDa protein kinase in soybean cells is clearly different from CDPKs: the activity of the PA-activated 49 kDa protein kinase is not dependent on Ca²⁺, as it remains active in the presence of 2 mM EGTA

(Figure 4), and is specifically recognized by an antibody against an alfalfa MAPK. In fact, our data strongly suggest that the 49 kDa protein kinase is an ortholog of the alfalfa SIMK MAPK, because it is recognized by antibodies against SIMK, but not by antibodies against three other alfalfa MAPKs (Figure 4).

The specific functions of most MAPKs in plants have not yet been fully unraveled (Meskiene and Hirt, 2000). However, several reports have implicated the class of SIMK/AtMPK6/SIPK MAPKs in signaling abiotic and biotic stresses (Mikolajczyk *et al.*, 2000; Munnik *et al.*, 1999; Nühse *et al.*, 2000; Romeis *et al.*, 1999; Zhang and Klessig, 1998a; Zhang and Klessig, 1998b; Zhang *et al.*, 1998). In animal cells, many MAPKs function as transcriptional regulators by coordinating the activity of transcriptional factors such as c-Jun, c-Fos and ATF-2 by phosphorylation (Treisman, 1996). MAPKs involved in transcriptional regulation are often localized in the nucleus, their site of action. SIMK in suspension-cultured alfalfa cells (Munnik *et al.*, 1999) and the PA-activated 49 kDa MAPK in soybean cells (our unpublished data) are also localized in the nucleus, suggesting that they may function as transcriptional regulators.

PA-activation of the MAPK is dependent upon upstream phosphorylation, as indicated by the effects of general protein kinase and phosphatase inhibitors (Figure 6). These results suggest that PA does not directly activate the soybean MAPK but does so by regulating other upstream protein kinases. In support of this idea, we could not observe PA-activation of the soybean MAPK when PA was added to the renatured protein present in a gel (our unpublished data). Furthermore, in animal cells, PA is known to activate protein kinases that are at the upstream end of MAPK pathways. For example, Raf, a MAPK kinase kinase, has a binding site for PA, and its binding is important for translocation of cytoplasmic Raf-1 to the plasma membrane, which is essential for Raf activation (Rizzo *et al.*, 1999; Rizzo *et al.*, 2000). In addition, some PKC isotypes, which function upstream of MAPK cascades in many signaling pathways in animal and yeast cells (Heinisch *et al.*, 1999; Kolch *et al.*, 1993; Seger and Krebs, 1995; Toda *et al.*, 1996), are also activated by PA (Limatola *et al.*, 1994; Yokozeki *et al.*, 1998).

To the best of our knowledge, this is the first report that characterizes the mechanism by which PA acts as a signal mediator and as an upstream regulator of a MAPK in plant cells. Elevation in PA levels and MAPK activation in response to wounding have been reported separately for many different plant species (Bögre *et al.*, 1997; Lee *et al.*, 1997; Mizoguchi *et al.*, 1996; Ryu and Wang, 1996; Seo *et al.*, 1995; Zhang and Klessig, 1998a), but a causal relationship between these two events has not yet been established. In the light of the present and previous results, PA-mediated activation of wound-induced MAPK

pathways may be a general mechanism in plants. Further studies on the role of PA and wound-induced MAPKs should help to understand wound signal transduction in plants better.

Experimental procedures

Plant materials

Soybean (*Glycine max* L., cv HwangKum) seeds were planted in vermiculite mixed with humus soil. The plants were grown in a greenhouse at 25 + 5°C with light/dark cycles of 16/8 h. Soybean plants were used at the two-leaf stage. Soybean cell suspensions were grown in Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 10% (w/v) casein enzymatic hydrolysate, 3 mg l⁻¹ 2,4-D and 0.1 mg l⁻¹ kinetin. Cells were subcultured every 7 d. Log phase cells were used 2–3 d after subculture. Lipids were purchased from Sigma, St. Louis, MO, USA, and dissolved in chloroform: methanol (1 : 1), except dipalmitoyl PA which was dissolved in chloroform: acetic acid (95 : 5, v/v) and diacylglycerol and linoleic acid which were dissolved in chloroform. Dissolved lipids were dried under nitrogen gas and then sonicated with water.

Wounding

Plants were wounded with pliers (soybean) by pinching the leaf across the vein at the distal side. The wound area covered about 1/3 of each wounded leaf. For wounding of suspension-cultured soybean cells, the cell suspension was mixed with an equal volume of glass beads (425–600 microns; Sigma) and vortexed for 2 min, 3 times. The wounded cells were mixed with 9 times their volume of the same type of cells and incubated for 5 min before protein preparation.

Butanol treatment

The roots of soybean seedlings were removed with a sharp razor blade in 1 mM Mes buffer (pH 6.5) containing 0.1 mM KCl and 0.2 mM CaCl₂. Seedlings were stabilized in the same buffer for at least 4 h. Before wounding, plants were transferred to vials containing 0.1% *n*- or *sec*-butanol in the buffer or the buffer alone, and incubated for 30 min. Five min after wounding, leaves were cut and frozen in liquid nitrogen. For lipid analysis, soybean seedlings were labeled with 555 kBq of ¹⁴C-acetate through their cut stem. After isotope uptake, the seedlings were incubated in the buffer described above for 4 h before wounding treatment.

Lipid analysis

Lipid extraction was performed as previously described (Lee *et al.*, 1997). The lipids were separated on a TLC plate (Merck F₂₅₄ 60, Darmstadt, Germany) with an upper phase of ethylacetate: isooctane: acetic acid: water = 13 : 2 : 3 : 10. The separated lipid bands were identified by comparison with known lipid standards using iodine staining and autoradiograms. The PA amount was analyzed by either phosphate assay of the separated lipid bands or by a phospho-imager.

Preparation of protein extracts

Wounded and control leaves were harvested by rapid freezing in liquid nitrogen. Suspension cells (3 ml) were harvested by vacuum filtration and frozen in liquid nitrogen. Proteins were extracted by homogenizing the samples in extraction buffer (50 mM Hepes pH 7.5, 5 mM EGTA, 2 mM EDTA, 2 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 10 mM MgCl₂, 50 mM β-glycerophosphate, 1 μg ml⁻¹ aprotinin, 1 μg ml⁻¹ leupeptin, 1 μg ml⁻¹ pepstatin, 100 μM phenylmethylsulfonyl fluoride). After centrifugation at 15 000 g for 20 min at 4°C, the supernatants (crude extracts) were saved for protein kinase assays. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA) using BSA as a standard.

In-gel kinase assay

Twenty to 50 μg of total protein were electrophoresed on a 10% SDS-polyacrylamide gel embedded with 0.25 mg ml⁻¹ myelin basic protein (MBP; Sigma, St. Louis, MO, USA) as a kinase substrate. After protein renaturation, the kinase reactions were carried out in the gel with γ-³²P-ATP as described previously (Zhang and Klessig, 1997). Relative kinase activities were analyzed by a phospho-imager. Protein kinase sizes were estimated by using prestained molecular mass markers (Bio-Rad).

Antibody production

Polyclonal rabbit antibodies were produced against synthetic peptides encoding the C-terminal amino acids of four alfalfa MAPKs, namely, FNPEYQQ of SIMK (Jonak *et al.*, 1993), VRFNPDPPNIN of MMK2 (Jonak *et al.*, 1995), LNFCKEQILE of MMK3 and LNPEYA of SAMK (Jonak *et al.*, 1996).

Immunoblotting

Crude extracts (50 μg and 70 μg of cell and leaf extracts, respectively) were separated on a 12% SDS-gel, and the proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, Postfach, Dassel, Germany). The membrane was blocked with TTBS buffer (25 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% BSA, and probed with polyclonal antibodies directed against alfalfa MAPKs in TTBS for 2 h at room temperature. Alkaline phosphatase-conjugated goat antirabbit IgG (1 : 2000 dilution; Promega, Madison, WI, USA) was used as a secondary antibody, and the reaction was visualized by hydrolysis of the substrate tetrazolium 5-bromo-4-chloro-3-indolyl phosphate (Promega).

Immunoprecipitation

Crude extract (100 μg) was incubated with the previously mentioned antibodies for 2 h at 4°C in immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 2 mM DTT, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 10 mM β-glycerophosphate, 2 μg ml⁻¹ aprotinin, 2 μg ml⁻¹ leupeptin, and 1% Triton X-100). Then protein A-agarose (RepliGen, Needham, MA, USA) was added, followed by incubation for another 1 h. The precipitates were washed three times with immunoprecipitation buffer and then re-suspended in the SDS sample buffer. Kinase activity of precipitated proteins was analyzed by the in-gel kinase assay as described previously.

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