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Abscisic Acid Induces Mitogen-Activated Protein Kinase Activation in Barley Aleurone Protoplasts

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Abscisic acid (ABA) induces a rapid and transient mitogen-activated protein (MAP) kinase activation in barley aleurone protoplasts. MAP kinase activity, measured as myelin basic protein phosphorylation by MAP kinase immunoprecipitates, increased after 1 min, peaked after 3 min, and decreased to basal levels after ~5 min of ABA treatment in vivo. Antibodies recognizing phosphorylated tyrosine residues precipitate with myelin basic protein kinase activity that has identical ABA activation characteristics and demonstrate that tyrosine phosphorylation of MAP kinase occurs during activation. The half-maximal concentration of ABA required for MAP kinase activation, 3×10^{-7} M, is very similar to that required for ABA-induced *rab16* gene expression. The tyrosine phosphatase inhibitor phenylarsine oxide can completely block ABA-induced MAP kinase activation and *rab16* gene expression. These results lead us to conclude that ABA activates MAP kinase via a tyrosine phosphatase and that these steps are a prerequisite for ABA induction of *rab16* gene expression.

INTRODUCTION

The phytohormone abscisic acid (ABA) influences many processes in plant physiology, including seed development and germination (Black, 1983; Walker-Simmons, 1987; Kermode, 1990). It plays an important role in inducing cell differentiation during the process of embryogenesis (Rajasekaran et al., 1987; Kiyosue et al., 1992). ABA also has a critical role in stress responses and enhances adaptation to various stresses, such as drought and salt stress (Hetherington and Quatrano, 1991; Holappa and Walker-Simmons, 1995). Many genes are induced by ABA; examples include the *rab16* gene from rice (Mundy and Chua, 1988), the *Em* gene from wheat (Marcotte et al., 1989), and the bifunctional α-amylase subtilisin inhibitor (*BASI*) gene from barley (Mundy and Rogers, 1986).

Much effort has been expended to unravel the ABA perception and signal transduction pathways. To date, Ca^{2+}, pH, and K^+ have been identified as possible mediators in ABA signaling (Gehring et al., 1990; Wang et al., 1991; Heimovaara-Dijkstra et al., 1994; Ward et al., 1995). Furthermore, strong evidence has been presented that protein (de)phosphorylation plays an important role in ABA action. The ABA-insensitive locus *ABI-1* from Arabidopsis has been found to be a PP2C-type phosphatase (Leung et al., 1994; Meyer et al., 1994). Furthermore, activity of this PP2C phosphatase seems essential for ABA regulation of K^+ fluxes in stomata (Armstrong et al., 1995). Recently, the involvement of tyrosine dephosphorylation in ABA-induced gene expression was shown by the use of the protein tyrosine phosphatase (PTP) specific inhibitor phenylarsine oxide (PAO) (Heimovaara-Dijkstra et al., 1996). This study piqued our interest, and we decided to investigate whether mitogen-activated protein (MAP) kinase activity is involved in ABA signaling.

MAP kinases constitute a widely distributed family of 40- to 45-kD serine/threonine kinases. MAP kinases, also known as extracellular signal-regulated protein kinases (ERK), have been shown to be activated by many different stimuli and to be involved in cell proliferation and differentiation (Blumer and Johnson, 1994). The MAP kinase gene family has been highly conserved during evolution, with members found in such diverse organisms as mammals, *Xenopus*, yeast, *Dictyostelium*, and several plant species (Nishida and Gotoh, 1993; Nishihama et al., 1995).

In mammalian cells, several different types of signal transduction cascades lead to the activation of MAP kinases. MAP kinase signaling continues via substrates, including transcription factors such as c-Myc, c-Jun, and STE12 from yeast (Lange-Carter et al., 1993). Other known substrates are protein kinases involved in regulation of gene expression, such as the 90-kD ribosomal S6 kinase II phosphoprotein $pp90^{rsk}$ and MAP kinase–associated protein MAPKAP kinase 2 (Burgering and Bos, 1995). MAP kinase activation requires the phosphorylation of a specific tyrosine and a threonine residue in the

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so-called TEY (Thr, Glu, Tyr) activation loop, which lies between regions VII and VIII of the kinase domain (Cano and Mahadevan, 1995). Several excellent reviews on the working mechanisms and scope of MAP kinase pathways have been published (see, for example, Jonak et al., 1994; Nishihama et al., 1995).

Currently, evidence is accumulating that MAP kinases play a similar, important role in plants. Several genes encoding MAP kinase homologs have now been cloned. In tobacco, two MAP kinase genes have been identified (Wilson et al., 1993; Seo et al., 1995). The tobacco *nft3* gene was cloned on the basis of conserved sequence domains of MAP kinase, and it is expressed in all tobacco tissues tested. The tobacco MAP kinase homolog wound-inducible protein kinase (*WIPK*) was cloned as a wound-induced transcript. Two MAP kinase homologs have been identified in Arabidopsis, *AtMPK1*, and *AtMPK2*. The genes are suggested to be involved in cell proliferation (Mizoguchi et al., 1994).

In alfalfa, three cDNA clones encoding MAP kinase homologs have been identified, *MsERK1*, *MMK1*, and *MMK2* (Duerr et al., 1993; Jonak et al., 1993, 1995). The MsERK1 protein, expressed in *Escherichia coli*, was phosphorylated on Tyr-215. The Tyr-215 residue was demonstrated to be essential for myelin basic protein (MBP) kinase activity of the protein (Duerr et al., 1993). The expression of *MMK1* has been demonstrated to be cell cycle dependent, indicating a role in proliferation. In pea, a MAP kinase homolog, *D5*, was cloned; however, no function was suggested (Stafstrom et al., 1993). In petunia, pMEK was cloned and found to be expressed in ovary tissue (Decroocq-Ferrant et al., 1995). Finally, ASPK9 cDNA, isolated from oat aleurone cells, shows high homology to *ERK1*, with 47% identity with the predicted protein sequence. The expression of this MAP kinase gene is strongly down-regulated by gibberellic acid (GA_3) in oat aleurones, indicating a possible involvement of MAP kinases in gibberellin-regulated processes (Huttly and Phillips, 1995).

In plants, other genes have been found that are known to be part of a MAP kinase signaling pathway in several organisms. One example is the identification of the ethylene response mutant *ctr1* gene as a putative Raf protein kinase (Kieber et al., 1993). Raf protein kinases are well-characterized upstream components of MAP kinase transduction pathways in many different organisms (Blumer and Johnson, 1994; Burgering and Bos, 1995). In addition, some groups have reported MBP kinase activity in plants. In some cases, this activity can be affected by external signals, such as elicitors, wounding, and auxin (Mizoguchi et al., 1994; Seo et al., 1995; Suzuki and Shinishi, 1995).

In this report, we show rapid and transient MAP kinase activation by ABA and present direct evidence for MAP kinase activation by the phytohormone ABA. This activation was brought about by physiological concentrations of ABA in vivo; submicromolar concentrations of ABA were sufficient to induce both MAP kinase activity and *rab16* gene expression. MAP kinase activity could be immunoprecipitated with antibodies recognizing phosphotyrosine residues. A correlation between ABA-induced MAP kinase activation and ABA-induced *rab16* gene expression was demonstrated by the use of PAO. PAO inhibits both ABA-induced MAP kinase activation and gene expression. These results have led us to propose a model of ABA signal transduction involving MAP kinase and tyrosine phosphatase activation.

RESULTS

Because earlier research demonstrated that phosphorylation events are involved in ABA signaling leading to gene expression, we developed a MAP kinase assay to measure MAP kinase activity in barley aleurone protoplasts. The MAP kinase assay is based on in vivo stimulation of protoplasts with saturating concentrations of ABA (10^{-5} M final concentration), followed by immunoprecipitation of MAP kinase, which preferentially phosphorylates MBP in vitro.

To determine whether MAP kinase protein levels change during ABA stimulation in the aleurone protoplasts, we used immunoblotting techniques to analyze protoplast protein preparations with varying stimulation times. Stimulation of protoplasts with 10^{-5} M ABA for up to 1 hr did not lead to any change in immunoreactive bands on an immunoblot probed with a polyclonal antibody recognizing MAP kinases from species such as mammals, *Xenopus*, and *Dictyostelium* (Figure 1). Several MAP kinase isoforms are probably present in barley aleurone protoplasts. Three positive bands between 40 and 43 kD were revealed by the anti-ERK1 antibody. The minor band of ~45 kD was shown not to be specific because it also appeared on the blot after treatment with a second antibody only and did

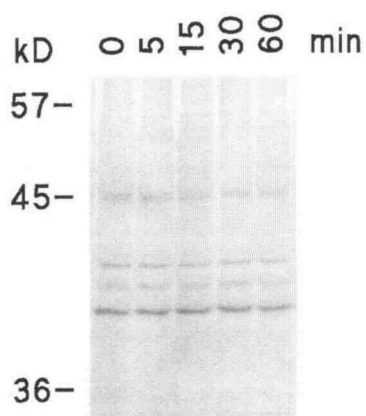

Figure 1. Protein Gel Blot Analysis of Barley Aleurone Protoplasts after Different Times of Stimulation with 10 μM ABA.

Cell equivalents (5×10^3) were loaded onto a polyacrylamide gel containing 12.5% acrylamide and 0.0625% bisacrylamide. MAP kinases were visualized by probing the resulting immunoblot with a polyclonal anti-ERK1 antibody.

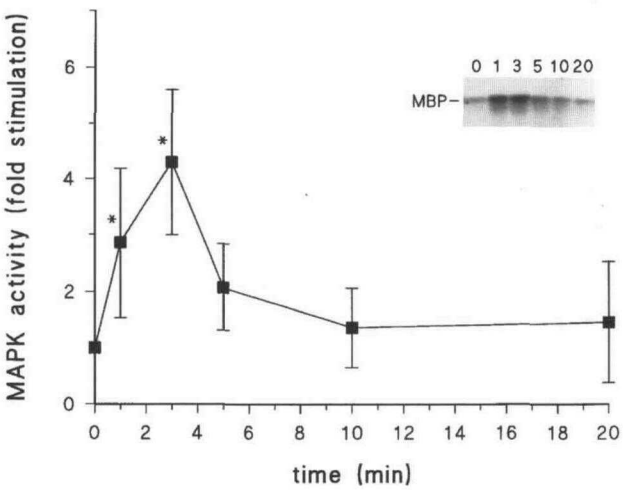

Figure 2. Time Course of ABA-Induced MAP Kinase Activity.

Protoplasts were treated with 10 μM ABA for the times indicated, after which MAP kinase (MAPK) activity was measured by determining MBP kinase activity on MAP kinase immunoprecipitates. MAP kinase activity is shown as the fold stimulation of the MAP kinase activity found at time zero. The mean values ± SD of seven independent experiments are shown. The inset shows MBP phosphorylation of a typical MAP kinase experiment at 0, 1, 3, 5, 10, and 20 min after ABA (10 μM) stimulation. (*) indicates significant difference with $t = 0$, as determined with the Student's t test ($P < 0.01$).

not appear in immunoprecipitates (data not shown). Furthermore, competition of the ERK1 antibody with a rat ERK1 "control" peptide did not reduce the intensity of this 45-kD band. Binding of the ERK1 antibody to the other bands could be competed with this peptide, confirming its specificity (data not shown).

We then investigated whether MAP kinase activity can be activated by ABA and, if so, what the kinetics of this stimulation are. Figure 2 shows clearly that ABA induces a rapid and transient stimulation of MAP kinase activity. Within 1 min of ABA treatment, stimulation of MAP kinase activity could be observed. MAP kinase activity peaked at 3 min of ABA stimulation and returned to basal levels after ~5 min. The inset in Figure 2, showing a typical MBP phosphorylation experiment, demonstrates that a basal level of MBP phosphorylation activity is present in immunoprecipitates of unstimulated cells. This basal level of MBP phosphorylation may be the result of coimmunoprecipitated MAP kinases, because the antibody used potentially recognizes multiple isoforms (Figure 1). ABA caused an activation of total MBP phosphorylation up to five times basal level. Stimulation of cells with saturating concentrations of the phytohormone GA_3, an antagonist of ABA action in seed germination (Black, 1983), did not lead to MAP kinase activation. In addition, GA_3 could not inhibit ABA-induced MAP kinase activation (data not shown).

MAP kinase is usually activated by dual phosphorylation on highly conserved threonine and tyrosine residues of the activational loop between regions VII and VIII of the kinase domain (Cano and Mahadevan, 1995). These residues are also conserved in the MAP kinases identified in plants (see, for example, Duerr et al., 1993). We tested whether MAP kinase activity in aleurone protoplasts can be immunoprecipitated by antibodies raised against phosphotyrosine residues. Indeed, phosphotyrosine antibodies immunoprecipitated MBP phosphorylation activity (Figure 3), which had the same ABA-inducible character as the MAP kinase response shown in Figure 2. The immunoprecipitation with the anti-phosphotyrosine antibodies of MBP phosphorylation activity could be competed by phosphotyrosine residues but not by phosphoserine or phosphothreonine residues, demonstrating the specificity of the antibody. The correlation between the MBP phosphorylation immunoprecipitated by the anti-MAP kinase antibodies and antibodies recognizing phosphotyrosine residues suggests that ABA induced tyrosine phosphorylation of MAP kinase.

To investigate whether ABA-induced MAP kinase activation can be linked to other ABA-induced responses, we studied the ABA dose dependence of the MAP kinase response (Figure 4). After 3 min of stimulation, submicromolar concentrations

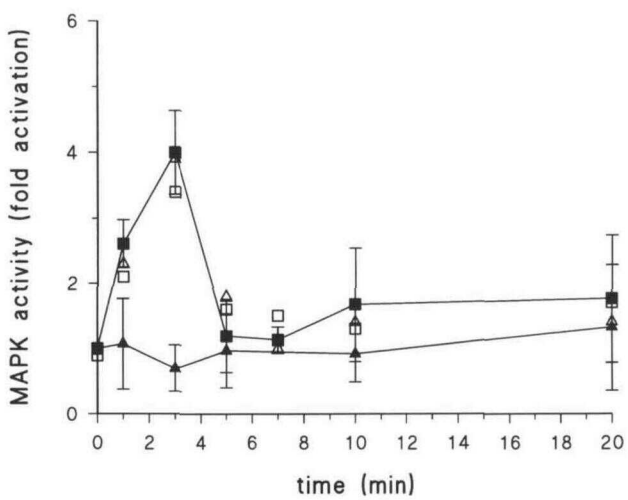

Figure 3. Time Course of ABA-Induced, Anti-Phosphotyrosine Antibody–Precipitated MAP Kinase Activity.

Protoplasts were treated with 10 μM ABA for the times indicated, after which MAP kinase (MAPK) activity was measured. MAP kinase was immunoprecipitated with anti-phosphotyrosine antibodies (solid squares) or with anti-phosphotyrosine antibodies in the presence of 0.1 mM phosphotyrosine residues (solid triangles), 0.1 mM phosphoserine residues (open squares), or 0.1 mM phosphothreonine residues (open triangles) as (non)competetive inhibitors during the immunoprecipitation. MAP kinase is presented as the fold stimulation of the MAP kinase activity found at time zero. Mean values ± SD of three independent experiments are shown. Competition with phosphothreonine and phosphoserine was performed once.

of ABA induced MAP kinase activation. The half-maximal concentration of ABA was ~3 × 10^{-7} M, and the maximal activation of MAP kinase was fivefold.

ABA-induced MAP kinase activation can be inhibited by micromolar concentrations of the tyrosine phosphatase inhibitor PAO (Figure 5; Garcia-Moralez et al., 1990). The half-maximal concentration of PAO was ~10^{-6} M. The inset in Figure 5 shows that PAO inhibited ABA-induced *rab16* expression. *Rab16* gene expression appeared after 15 min of ABA stimulation of aleurone protoplasts. Treatment of cells with ABA in the presence of micromolar concentrations of PAO completely inhibited ABA-induced *rab16* gene expression. Expression of the non–ABA-regulated control gene *GAPDH* was not affected, showing that this inhibition is rather specific and not caused by general inhibition of transcription. These results show that both ABA-induced MAP kinase activation and ABA-induced *rab16* gene expression are dependent on tyrosine phosphatase activity.

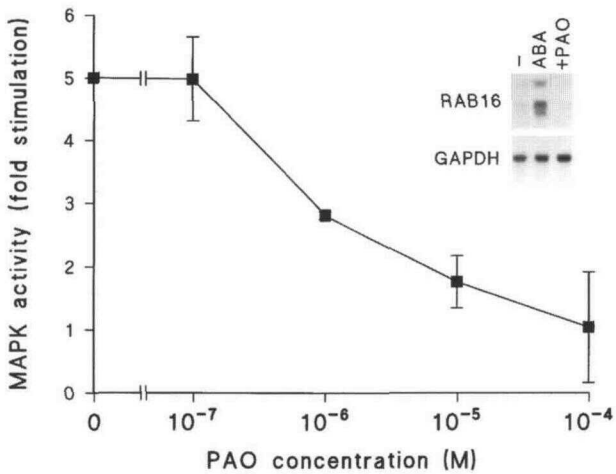

Figure 5. Inhibition of ABA-Induced MAP Kinase Activation by the Tyrosine Phosphatase Inhibitor PAO.

Protoplasts were incubated for 3 min with 10 μM ABA at the PAO concentrations indicated. MAP kinase (MAPK) activity is presented as the fold stimulation of the activity found in protoplasts not treated with ABA. Mean values ± SD of three independent experiments are presented. The inset shows PAO inhibition of ABA-induced *rab16* gene expression. RNA was isolated after the stimulation of protoplasts with water (−), 10 μM ABA (ABA), or 10 μM ABA and 1 μM PAO (+PAO). The *GAPDH* gene is shown as an internal control for RNA quantity.

DISCUSSION

In this report, we show that ABA induces MAP kinase activation in barley aleurone protoplasts. The characteristics of this ABA-induced MAP kinase activation correlate strongly with ABA-induced *rab16* gene expression. We propose a model in which MAP kinase may be involved in a signaling pathway linking ABA recognition to gene expression (Figure 6).

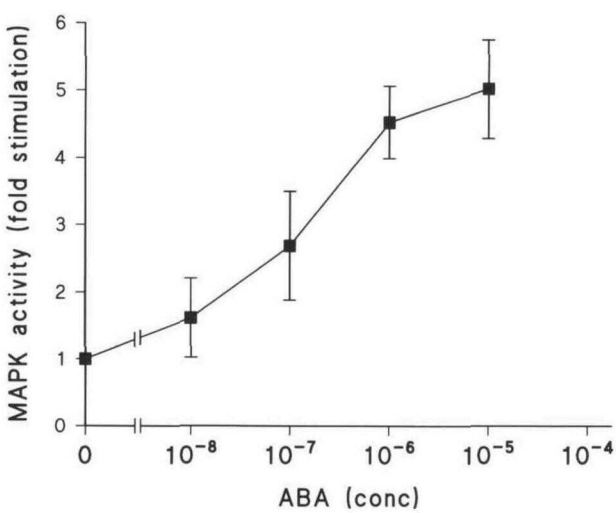

Figure 4. ABA Dose Dependence of MAP Kinase Activation.

Protoplasts were incubated with the indicated ABA concentration (conc) for 3 min, after which MAP kinase (MAPK) activity was measured. MAP kinase activation is presented as the fold stimulation of MAP kinase activity found in cells not stimulated with ABA. Mean values ± SD of four independent experiments are shown.

MAP kinases have been identified in several plant species (see Introduction), but only limited data on their function have been obtained. Strong data support the role of MAP kinases in wound responses; wounding rapidly induces the expression of the *WIPK* gene in tobacco (Seo et al., 1995). Inactivation of this MAP kinase in transgenic tobacco interferes with part of the typical wound-induced responses in tobacco. In tobacco, an MBP kinase activity has also been described that can be rapidly and transiently induced by wounding (Seo et al., 1995). Similar activation of tobacco MBP kinase activity was achieved with a fungal elicitor (Suzuki and Shinishi, 1995). Tyrosine phosphorylation is essential for the elicitor activation, suggesting that the MBP kinase activity is the result of MAP kinase activation.

Regarding phytohormone activation, the available data on MAP kinase activation concern auxin. Addition of the mitogenic hormone auxin to cell suspension cultures results in increased MAP kinase phosphorylation. Moreover, a correlation between 2,4-D addition and MBP kinase activation has been determined (Mizoguchi et al., 1994). GA_3 was shown to down-regulate the expression of a MAP kinase homolog in oat (Huttly and Phillips, 1995). However, we did not find an effect of GA_3 on the MAP kinase activity in barley aleurone. In most organisms in which MAP kinase action has been characterized, it appears to play a role in cell proliferation and defense mechanisms (such as the osmotic stress response in yeast), two areas in which ABA plays a major regulatory role in plants.

The use of barley aleurone protoplasts as a model system for ABA action has the advantage that only one cell type is present in this seed tissue. Furthermore, ABA can rapidly induce gene expression in these cells; barley *rab16* RNA was expressed after 15 min of ABA stimulation (van der Meulen et al., 1993). We found that treatment of aleurone protoplasts with ABA leads to a rapid and transient activation of an MBP kinase activity, which can be immunoprecipitated by an anti-ERK1 polyclonal antibody (Figure 2). Similar results were obtained with antibodies recognizing phosphotyrosine residues (Figure 3). These results make it very probable that MBP kinase activity is generated by a MAP kinase homolog. Immunoblot analysis demonstrated that three possible MAP kinase isoforms may be present in barley aleurone (Figure 1).

To investigate the possible role of ABA-induced MAP kinase activation in *rab16* gene expression, we performed a dose–response experiment. The ABA concentration that led to half-maximal activation of MAP kinase was $\sim 3 \times 10^{-7}$ M, which correlated well with the half-maximal ABA concentration of $\sim 5 \times 10^{-7}$ M for induction of *rab16* gene expression (van der Meulen et al., 1993). In addition, we showed that micromolar concentrations of the PTP inhibitor PAO (Garcia-Moralez et al., 1990) block MAP kinase activation by ABA (Figure 5). It has already been demonstrated that PAO can completely block ABA induction of *rab16* gene expression (Figure 5; Heimovaara-Dijkstra et al., 1996). All of these data led to a model (Figure 6) in which ABA stimulation of barley aleurone causes activation of a tyrosine phosphatase. This PTP can activate a protein kinase and subsequently MAP kinase by stimulating tyrosine phosphorylation of MAP kinase. The phosphorylated and activated MAP kinase can further transduce the signal, leading to increased gene expression.

The model suggests a vital role for tyrosine dephosphorylation upstream of a MAP kinase in regulating hormone-induced gene expression. Although MAP kinase cascades do not usually act via phosphatase, there are several examples of such pathways. In early *Xenopus* oocyte development, an Src homology 2 (SH2) domain containing PTP is vital for fibroblast growth factor signaling (Tang et al., 1995). This PTP leads to activation of Ras, thereby activating the protein kinase cascade and resulting in MAP kinase activation. In rat embryo fibroblasts, a receptor PTP leads to activation of the tyrosine kinase c-Src. c-Src in turn can activate MAP kinase (Zheng and Pallen, 1994). This MAP kinase activation leads to the phosphorylation of the c-Jun transcription factor and consequently to developmental gene expression. In human embryonic kidney cells, PTP2C acts upstream of MAP kinase in the epidermal growth factor signal transduction pathway (Zhao et al., 1995). The activated MAP kinase can phosphorylate and deactivate PTP2C, resulting in negative feedback on its own activation (Peraldi et al., 1994).

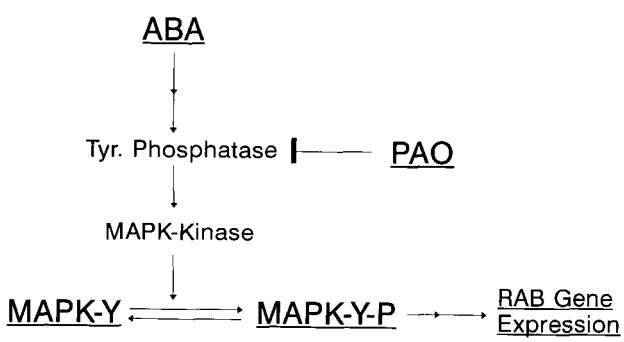

Figure 6. Model of ABA Signaling Involving MAP Kinase and Leading to *rab16* Gene Expression.

Recognition of ABA leads to the activation of a tyrosine phosphatase (Tyr. Phosphatase). This PTP leads via a protein kinase (MAPK-kinase) to tyrosine phosphorylation and activation of MAP kinase (MAPK-Y → MAPK-Y-P). The activated MAP kinase can subsequently result in increased *rab16* gene expression.

All of these examples indicate that the model proposed in this report has analogies to signaling in other organisms. The exact mode of action of MAP kinase in regulation of plant gene expression is still unclear. MAP kinase could be translocated to the nucleus upon activation and directly phosphorylate transcription factors or components of the transcription machinery. MAP kinase may also be indirectly involved in the regulation of gene expression by phosphorylation of other protein kinases that exert their function in the nucleus, such as the $pp90^{rsk}$ and MAPKAP kinase 2 (Burgering and Bos, 1995). Many questions concerning the pathway by which ABA controls physiological events remain unanswered, but we are convinced that these data contribute to further understanding of the ABA signal transduction cascade. One question to answer is whether ABA can induce MAP kinase translocation to the nucleus.

METHODS

Materials

γ-^{32}P-ATP came from Amersham. The anti-ERK1 rabbit polyclonal antibody and the ERK1-control peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G–Sepharose 4B–fast flow was obtained from Pharmacia. The polyclonal antibody recognizing phosphotyrosine residues was a generous gift from J.A. Maassen (Department of Medical Biochemistry, Leiden University, The Netherlands). Peroxidase-labeled goat anti–rabbit antibody was from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Tablets of complete protease inhibitors were from Boehringer Mannheim (Mannheim, Germany).

Barley Aleurone Protoplast Isolation and Treatment

Barley (*Hordeum vulgare* cv Himalaya, harvest 1985; Department of Agronomy, Washington State University, Pullman) grains were de-embryonated and cut into halves longitudinally. Grains were sterilized, aleurones were isolated, and protoplasts were prepared as described

by Heimovaara-Dijkstra et al. (1994). The buffer used for washing and incubation of protoplasts consisted of 2% glucose, 10 mM L-arginine, 20 mM $CaCl_2$, 10 mM Mes, and 0.5 M mannitol, pH 5.4, the osmolarity being ~800 mosmol.

Protein Gel Blot and Immunoblot Analysis

Aleurone protoplasts were resuspended in sample buffer (60 mM Tris, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 80 mM SDS) and analyzed on 12.5% polyacrylamide gels containing 0.0625% bisacrylamide. Protein was blotted to nitrocellulose. Immunoblots were probed with a 1:1000 dilution of rabbit polyclonal anti-ERK1 antibody at 4°C for 16 hr. Bands were visualized by peroxidase-labeled goat anti-rabbit antibody, followed by detection with diaminobenzidine staining.

MAP Kinase Activity Assay

Approximately 2×10^4 protoplasts per sample were treated with 10 μM abscisic acid (ABA) for various times, as indicated in Figures 2 to 5. Each stimulation was stopped by lysing the cells in buffer A (20 mM Tris, pH 8.0, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and one tablet of complete protease inhibitors per 50 mL). The Triton-insoluble particles were removed from the lysates. Each lysate was incubated with the polyclonal anti-extracellular signal-regulated protein kinase (ERK) antibody, or anti-phosphotyrosine antibody, precoupled to protein G–Sepharose to immunoprecipitate MAP kinase. The washed immunoprecipitate was incubated with 0.5 μCi γ-^{32}P-ATP, 30 mM Tris, pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, and 7.5 μg of myelin basic protein as substrate for 30 min at 22°C. The mixture was electrophoresed on a 15% polyacrylamide gel, which was subsequently autoradiographed. Bands were quantified using an LKB Ultroscan densitometer (LKB Produkter AB, Bromma, Sweden).

RNA Isolation and RNA Gel Blot Analysis

Aleurone protoplasts were incubated with water, 10 μM ABA, or 10 μM ABA and 1 μM PAO for 15 min. RNA was isolated and blotted to a Genescreen membrane (Du Pont, Boston, MA), which was subsequently probed with *rab16* and *GAPDH*.

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