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Activation of MAPK homologues by elicitors in tobacco cells

Angela Lebrun-Garcia*, Fatma Ouaked, Annick Chiltz and Alain Pugin

Unité Associée I.N.R.A., Université de Bourgogne, Laboratoire de Phytopharmacie et Biochimie des Interactions Cellulaires, BV 1540, Dijon Cedex, 21 034, France

Summary

Elicitors of plant defence reactions (such as cryptogein, an elicitor produced by *Phytophthora cryptogea*, or oligogalacturonides (OGs)), induced in tobacco cell suspensions (*Nicotiana tabacum* var *Xanthi*) a rapid and transient activation of two protein kinases (PKs) with apparent molecular masses of 50 and 46 kDa, respectively. These PKs activated and phosphorylated at tyrosine residues, phosphorylated myelin basic protein (MBP) at serine/threonine residues. Both are recognized by anti-MAPK antibodies. The two MBP kinases possessed the same kinetics of activation, and their activation depended, to the same extent, on different exogenously applied compounds (staurosporine, lanthanum, EGTA). We demonstrate here that the activation of the MBP kinases is calcium dependent and sensitive to staurosporine, a protein kinase inhibitor which annihilates all known responses of tobacco cells to cryptogein. The activation of MBP kinases appeared to be independent of the production of active oxygen species (AOS) and insensitive to calyculin A, a protein phosphatase type 1 and 2A inhibitor. The activation of MAPKs is discussed in relation to the early responses induced by cryptogein.

Introduction

Signal transduction pathways initiated by plant–pathogen interactions, and leading to plant defence, include a wide variety of biochemical events such as modifications in the phosphorylation or redox status of cellular components, rapid ion fluxes and depolarization of the plasma membrane. Cytoplasmic signals are translocated to the nucleus where transcription of plant defence related genes is activated (for review see Yang *et al.*, 1997). These events have been studied either by inoculations with pathogens or by treatment with purified elicitors which are released from invading pathogens or from plant cell walls. In signal

transduction pathways, initiated by pathogen interactions or by abiotic stresses, protein phosphorylation plays a central role in propagating signals from the cell surface to the nucleus. A receptor-like kinase (RLK) with a transmembrane domain has been identified as the product of the resistance gene (*R* gene) which confers resistance to *Xanthomonas oryzae* pv *oryzae* (Song *et al.*, 1995), while Pto, an intracellular serine/threonine kinase of tomato, is involved in resistance to *Pseudomonas syringae* pv *tomato* (Martin *et al.*, 1993) and interacts directly with the avirulence gene product AvrPto (Tang *et al.*, 1996). Pto also interacts with another serine/threonine kinase, Pti, promoting the hypersensitive response (Zhou *et al.*, 1995). In plants, cascades of phosphorylation that amplify the original signal also involved the mitogen-activated protein kinase (MAPK) pathway, establishing a link between the perception of elicitor molecules by a receptor and transcriptional activation (for review, see Hirt, 1997). In animal cells or in yeast, such cascades are promoted by extracellular signals such as growth hormones or stress conditions sensed by specific receptors. Different cascades are possible depending on the stimulus but, alternatively, different stimuli can share part of a pathway. MAPK cascades are composed of three functionally interlinked protein kinases: MAPKs are serine/threonine kinases that are activated by dual phosphorylation on tyrosine and threonine residues, a reaction catalyzed by MAPK kinases (MAPKK), themselves under the control of MAPKK kinases (MAPKKK).

In plants, MAPK involvement after a specific signaling event was reported in an increasing number of cases (Hirt, 1997). These PKs are involved in cell proliferation and differentiation (Jonak *et al.*, 1993; Mizoguchi *et al.*, 1994; Wilson *et al.*, 1997), in stress or elicitor responses (Adam *et al.*, 1997; Bögre *et al.*, 1996, 1997; Mizoguchi *et al.*, 1996; Seo *et al.*, 1995; Suzuki and Shinshi, 1995; Usami *et al.*, 1995; Zhang *et al.*, 1998), in phytohormone signaling (Knetsch *et al.*, 1996), and in transduction pathway involving salicylic acid (SA) (Zhang and Klessig, 1997, 1998). Activation of MAPKs are correlated with an increase in their tyrosine phosphorylation level, consistent with the phosphorylation requirement of the tyrosine residue present in the conserved activation sequence TXY, and located between subdomains VII and VIII of the kinase catalytic domain. MAPKs are activated by a post-translational mechanism although *de novo* protein synthesis seems to be necessary to negatively regulate MAPK activity (Adam *et al.*, 1997; Bögre *et al.*, 1997; Usami *et al.*, 1995). Moreover, mRNA levels corresponding to MAPK genes are increased following stress applications (Bögre *et al.*, 1997;

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*For correspondence (fax +33 0380 633265; e-mail lebrun@epoisses.inra.fr).

Jonak *et al.*, 1996; Mizoguchi *et al.*, 1996; Seo *et al.*, 1995). However, inhibition of transcription by α -amanitin did not prevent MAPK activation but further impaired MAPK inactivation (Bögge *et al.*, 1997). Indeed, expression of a protein phosphatase type 2C (PP2C), acting at the MAKKK level, correlates with the inhibition of the alfalfa stress-activated MAPK (Meskiene *et al.*, 1998).

In this context, our first aim was to determine whether a MAPK cascade is involved in connecting parts of the signal transduction pathway induced by cryptogein, an elicitor of the elicitor family constituted by low-molecular weight proteins secreted by *Phytophthora* sp. and, second, which elements of the signal transduction pathway influences activation of the PKs. In tobacco plants, cryptogein triggered a hypersensitive-like response (HR) and induced systemic resistance against pathogens (Ricci *et al.*, 1989). Initial steps of the cryptogein transduction pathway, that presumably lead to plant defence reactions, were studied using *Nicotiana tabacum* var Xanthi cell suspensions. Our results showed that after binding of cryptogein to high affinity sites of the plasma membrane (Wendehenne *et al.*, 1995), rapid phosphorylation events occurred (Viard *et al.*, 1994) which preceded a large influx of calcium (Tavernier *et al.*, 1995). This influx of calcium triggered many early events including changes in extracellular and cytosolic pH (Blein *et al.*, 1991; Pugin *et al.*, 1997) and transient and fast production of AOS (Tavernier *et al.*, 1995). Activation of transcription was also reported as an early response of tobacco cells to cryptogein treatment (Petitot *et al.*, 1997; Suty *et al.*, 1995, 1996).

In this study, we show the activation of two tobacco MAPK homologues in response to cryptogein, whose activities are shown to be dependent on Ca^{2+} influx and occur independently of the AOS burst. These two MAPK homologues were also activated by an unrelated elicitor, namely oligogalacturonides, in a Ca^{2+} -dependent fashion. Recently, Zhang and co-workers demonstrated the activation of MAPKs in response to both SA and cryptogein (Zhang *et al.*, 1998). Our results differ in the number of PKs activated by cryptogein treatment and in their kinetics of activation. The relation between the activation of MAPKs and the activation of other early responses known to be induced by cryptogein is discussed in this paper.

Results

Activation of MBP kinases by cryptogein and OGs

In-gel kinase assays, performed with MBP as a substrate, revealed that cryptogein activated two PKs in tobacco cells (Figure 1a). The two PKs showed relative molecular masses of 50 and 46 kDa (abbreviated p50-PK and p46-PK). Both PKs were activated with the same kinetics: their activation was detectable within the first minutes of treatment, peaked

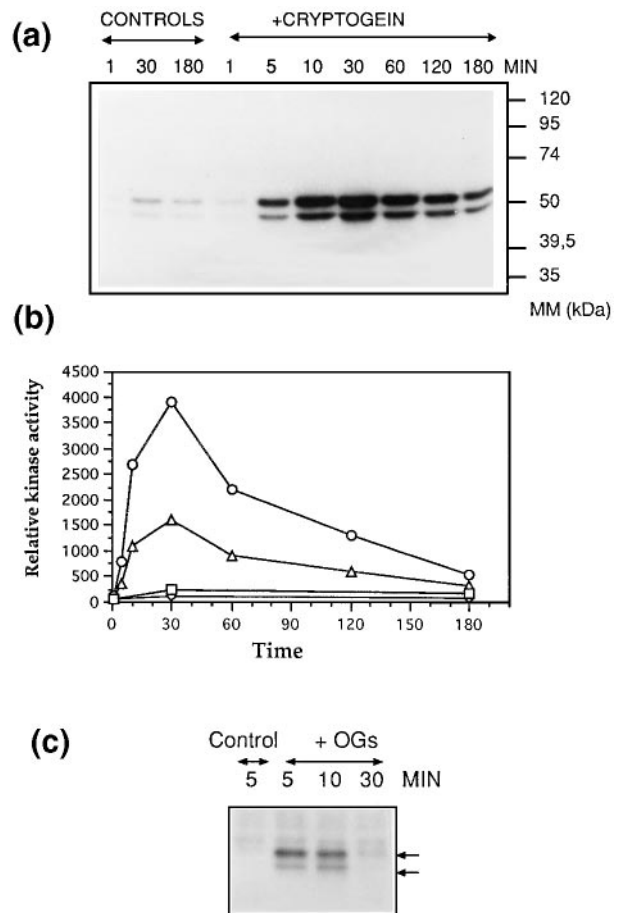


Figure 1. Time course activation of MBP kinases with 50 nm cryptogein (a) and (b) or with 50 $\mu\text{g ml}^{-1}$ oligogalacturonides (OGs), (c). Figure 1(a and c) represent in-gel kinase assays performed with the two elicitors. (b) Relative kinase intensities corresponding to the quantification of the bands obtained for the p50-PK in control cells (\square) or in cryptogein-treated cells (\circ), and for the p46-PK in control cells (\diamond) or in cryptogein-treated cells (\triangle).

at 30 min and then decreased slowly. After 180 min of treatment, activities had not yet returned to the basal level (Figure 1a,b). The maximal relative intensities of the upper (p50-PK) and lower (p46-PK) bands compared to the intensities of the control bands corresponded to 17-fold and 12-fold increases, respectively. The intensity of the lower band represented $35\% \pm 11\%$ of the upper band. When treated with 5–50 nm cryptogein during 30 min, a plateau for MBP kinase activation was reached with 10 nm for both PKs. However, activation of both PKs was faster with 50 nm cryptogein (data not shown). These two MBP kinases were also activated after tobacco cell treatment with OGs used at 50 $\mu\text{g ml}^{-1}$ (Figure 1c). However, the activation of MBP kinases with this elicitor lasted less than 30 min. The maximal relative intensities obtained with OGs for both PKs at time 5 min corresponded to 16% of those reached with cryptogein at time 30 min.

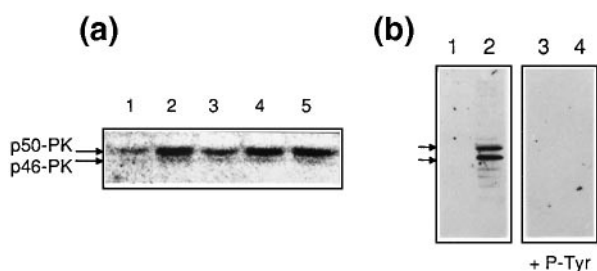


Figure 2. Phosphotyrosine content of activated MBP kinases.

(a) Immunoprecipitation of protein crude extracts with an anti-phosphotyrosine antibody, followed by an in-gel kinase assay. Lane 1: untreated cells. Lanes 2–5: cells treated during 30 min with cryptogein. During the immunoprecipitation step, samples from lanes 3–5 contained 1 mM phosphotyrosine, phosphothreonine or phosphoserine, respectively. (b) Western blot analysis performed with the same antibody. Two blots were performed: one incubated with the anti-phosphotyrosine antibody, the other supplemented with 1 mM phosphotyrosine during the primary antibody incubation step (indicated below the blot: + P-Tyr). Lanes 1 and 3: protein extract from untreated cells. Lanes 2 and 4: protein extract from cells treated during 30 min with cryptogein. Antibodies were used at 1:2000 and 1:10 000 dilutions for the primary and secondary antibodies, respectively. Forty μ g of protein was loaded. Arrows indicate the bands corresponding to the molecular mass of p50- and p46-PKs.

MBP kinase activation by tyrosine phosphorylation and phosphorylated amino acids in MBP

Proteins from control or treated cell extracts were immunoprecipitated using a mouse monoclonal antibody directed against phosphotyrosine residues, in the presence or absence of competitors, phosphotyrosine, phosphothreonine or phosphoserine residues. The immunoprecipitate was then submitted to an in-gel kinase assay (Figure 2a). Bands corresponding to the two MBP kinases were present in cryptogein-treated cell extracts (lane 2) and showed higher intensities than those detected in non-treated cell extracts (lane 1). When immunoprecipitation was performed in presence of the competitor phosphotyrosine (1 mM, lane 3), band intensities were comparable to those obtained in control cell extracts (lane 1) suggesting that immunoprecipitated PKs from cryptogein-treated cells were phosphorylated on tyrosine residues. On the other hand, addition of phosphothreonine (lane 4) or phosphoserine (lane 5) did not produce a decrease in kinase activities which were comparable to those from cryptogein-treated cells (lane 2), indicating that these PKs were specifically precipitated due to their phosphotyrosine content. In addition, Western blot analysis of protein extracts using the anti-phosphotyrosine antibody showed that only protein extracts from cryptogein-treated cells contained two major bands whose electrophoretic mobility corresponded to the p50- and p46-PKs (Figure 2b). When blots were incubated with 1 mM phosphotyrosine together with the anti-phosphotyrosine antibody, no band was detected (Figure 2b).

To identify the MBP phosphorylated residues, alkali treatment which removes phosphate from serine and threonine residues (Cooper *et al.*, 1983) was performed after the in-

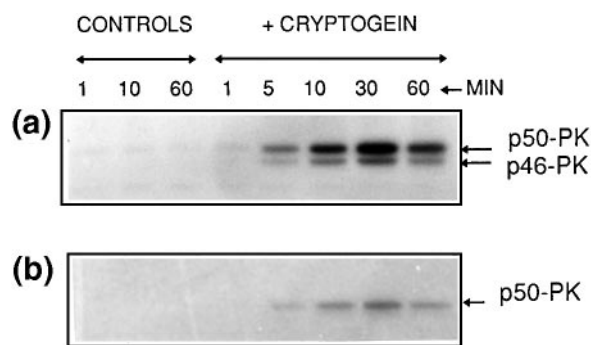


Figure 3. Radioactivity incorporated in MBP after an in-gel kinase assay (a) and remaining after alkali treatment of the gel (b).

The same samples were loaded on each gel and they corresponded to protein extracts from control cells or cells treated with cryptogein during different times.

gel kinase assay. This treatment eliminated most of the incorporated radioactivity (Figure 3b, compared to Figure 3a), indicating that MBP was phosphorylated on Ser or Thr residues by the activated p50-PK and p46-PK.

Detection of MAPK homologues in protein extracts from tobacco cells

Activation of both PKs by tyrosine phosphorylation and their substrate specificity (phosphorylated MBP on serine or threonine residues) suggested that cryptogein-activated PKs may belong to the MAPK family. Therefore, we tested the cross-reactivity of two different specific antibodies raised against human ERK1/ERK2 (members of the MAPK family) and a MAPK-activated form (antibody raised against a phosphotyrosine peptide containing the TEY sequence of the human ERK1). Western blots performed with the antibody against ERK1/ERK2 revealed several identical bands in both control cells and cryptogein-treated cells. Two of these bands showed electrophoretic mobilities comparable to the MBP kinases, detected after an in-gel kinase assay, and had comparable intensities in protein extracts from control cells and cryptogein-treated cells (Figure 4a), suggesting that these proteins are homologues of human MAPKs and are constitutively expressed. Immunodetection performed with the antibody against the activated form of MAPKs showed that two bands were detected only in lanes corresponding to protein extracts from cryptogein-treated cells (Figure 4b). These bands had relative molecular masses similar to the bands immunorevealed with the human ERK1/ERK2 antibody. The band intensities decreased after 1 h of cryptogein treatment, in agreement with the decrease of MBP kinase activity. Thus, consistent with in-gel kinase assays, activated forms of MAPK homologues were only present in protein extracts from cryptogein-treated cells.

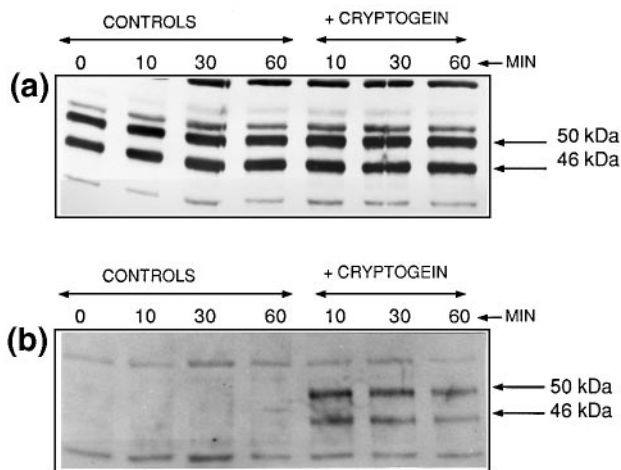


Figure 4. Immunodetection with anti-MAPK antibodies of proteins present in extracts from untreated or cryptogein-treated cells during different times. (a) Western blot analysis using an anti-human ERK1/ERK2 antibody. (b) The same samples used in (a) were tested with an antibody specific to MAPK activated form. Arrows indicate the bands corresponding to proteins with the same electrophoretic mobility and detected after in-gel kinase assays.

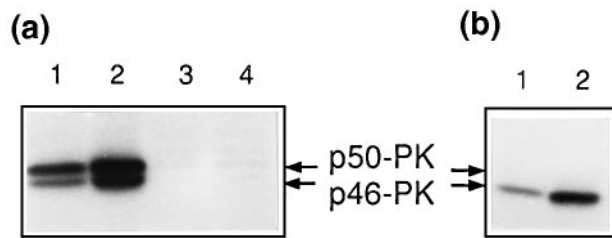


Figure 5. Staurosporine effect on the activation of MBP kinases *in vivo* (a) or on MBP kinase activity *in vitro* (b). Lane 1: cells treated with 25 nM cryptogein and 1.5 μM staurosporine. Lane 2: cells treated with 25 nM cryptogein. Lane 3: cells treated with 1.5 μM staurosporine. Lane 4: control cells. Cells were treated during 30 min. Arrows indicate the relative position of p50-PK and p46-PK. Gels in (a) or (b) correspond to the same treatments except that 1.5 μM staurosporine was added during the radioactive labeling step *in vitro* (b).

Sensitivity of MBP kinase activation to protein kinase/ phosphatase inhibitors

Early events, such as calcium influx, extracellular alkalinization and AOS production, monitored after treatment of tobacco cells with cryptogein, were suppressed with the protein kinase inhibitor staurosporine (Pugin *et al.*, 1997; Tavernier *et al.*, 1995; Viard *et al.*, 1994), indicating a signal transduction pathway dependent on phosphorylation/ dephosphorylation events. Consequently, it was interesting to test whether staurosporine inactivation acted upstream or downstream from the potential MAPK pathway. When staurosporine was added, together with cryptogein to tobacco cell suspensions, both p50- and p46-PKs showed a reduced activity: intensities of bands were reduced by about 65% compared to their intensities in cryptogein-treated cells (Figure 5a). To determine whether these tobacco PKs were directly inhibited by staurosporine, an

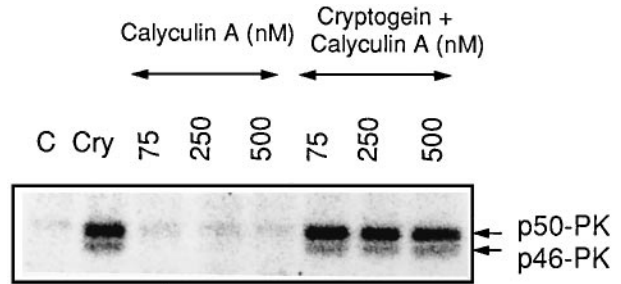


Figure 6. Effect of calyculin A on the activation of MBP kinases analyzed by in-gel kinase assays. Tobacco cells were treated during 30 min with calyculin A used at different concentrations (75, 250 or 500 nM), or co-treated with calyculin A and 25 nM cryptogein. C: control tobacco cells. Cry: tobacco cells treated with 25 nM cryptogein.

in-gel kinase assay was performed where staurosporine was added to the reaction buffer containing the radioactive ATP substrate (Figure 5b), and compared to the same experiment without staurosporine (Figure 5a). While the p50-PK was completely inhibited, the p46-PK still showed 60% of the original activity obtained in protein extracts from cryptogein-treated cells (Figure 5b, lane 2), indicating that both PKs were direct targets of staurosporine *in vitro* but showed different sensitivities. Thus, when tobacco cells were treated with staurosporine, inhibition of MBP kinases could result from direct or indirect effects.

The effect of calyculin A, a potent inhibitor of protein phosphatases 1 and 2A that mimics some effects of elicitors (Chandra and Low, 1995; Felix *et al.*, 1994), was tested on the activation of MBP kinases. In our system, calyculin A induced Ca²⁺ influx, AOS production and extracellular pH alkalinization (our unpublished results). As shown in Figure 6, calyculin A, used at concentrations between 75 and 500 nM, was not able to activate the MBP kinases, suggesting that the protein phosphatases, whose inactivation promotes the effects described above, are not involved in the activation of MBP kinases. When mixed with cryptogein, no additional effect of calyculin A was detected, neither activating nor inhibiting the cryptogein-induced PKs.

The activation of MBP kinases depends on Ca²⁺ influx

Ca²⁺ influx is required in the elicitor transduction pathway and is one of the first steps which is sensitive to staurosporine (Tavernier *et al.*, 1995). The effect of La³⁺ (a Ca²⁺ channel blocker) and EGTA (a complexing agent) were tested in order to determine if Ca²⁺ influx was a prerequisite for the activation of MBP kinases. A strong, dose-dependent inhibition of PK activation was observed with both La³⁺ (used in a concentration range from 0.25 to 1 mM) and EGTA (used in a concentration range from 0.25 to 2 mM), suggesting that Ca²⁺ influx acted upstream to the pathway leading to the activation of MBP kinases (Figure 7a,b). When oligogalacturonides, which also induce Ca²⁺ uptake

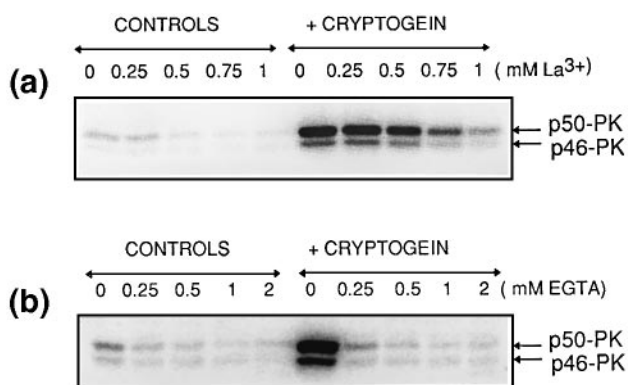


Figure 7. Ca^{2+} -dependent activation of the MBP kinases analyzed by in-gel kinase assays.

Dose-dependent inhibition of the activation of MBP kinases by increasing concentrations of La^{3+} (a) or increasing concentrations of EGTA (b). Control tobacco cells were treated in the presence of La^{3+} or EGTA at different concentrations during 30 min, indicated above each lane (controls). The same concentrations of La^{3+} or EGTA were used for tobacco cells co-treated with 25 nM cryptogeiin.

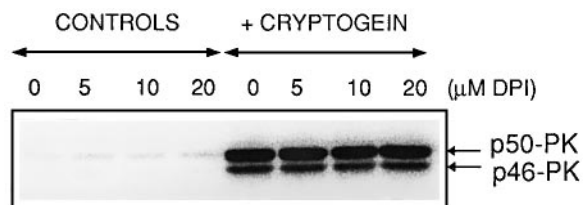


Figure 8. Effect of DPI on the cryptogeiin-induced activation of MBP kinases analyzed by in-gel kinase assays.

Different concentrations of DPI were used in control cells (0, 5, 10 and 20 μM). The same concentrations were used in co-treatment of tobacco cells with 25 nM cryptogeiin. Cells were treated during 30 min.

(Binet *et al.*, 1998) and MBP kinase activation (Figure 1c), were used in the presence of La^{3+} (2 mM), no activation of MBP kinases was observed (data not shown).

Relationship between AOS production and activation of MBP kinases

Diphenylene iodonium (DPI), known to be an inhibitor of the mammalian neutrophil NADPH oxidase, suppresses the AOS production induced by cryptogeiin (Simon-Plas *et al.*, 1997). When used in a concentration range (0–20 μM) that inhibits AOS production in cryptogeiin-treated cells, DPI did not affect the activation of MBP kinases (Figure 8). These results indicate that these kinases are not acting downstream from AOS production.

Discussion

Signal transduction, leading to plant defence reactions, is under current investigation in our laboratory and has been essentially studied using cryptogeiin, an elicitor which promotes hypersensitive-like and SAR responses. Different steps have been characterized, from the initial interaction

of cryptogeiin with a receptor site located in the plasma membrane (Wendehenne *et al.*, 1995) to the transcriptional activation of genes (Galiana *et al.*, 1997; Keller *et al.*, 1996a; Petitot *et al.*, 1997; Suty *et al.*, 1996). Early physiological events were observed following cryptogeiin treatment of tobacco cells that are connected to the signal transduction pathway. These include calcium influx, Cl^- and H^+ efflux, AOS production, extracellular and cytosolic pH changes, plasma membrane depolarization and activation of the pentose phosphate pathway (Blein *et al.*, 1991; Pugin *et al.*, 1997; Tavernier *et al.*, 1995). All these effects were inhibited by staurosporine indicating that protein phosphorylation is an upstream event which triggers subsequent events and precedes Ca^{2+} influx. One important pathway which connects the perception of extracellular stimuli to gene transcription is represented by the MAPK cascade. This cascade, composed of three interlinked PKs, can be activated through receptors with seven transmembrane domains, receptor tyrosine kinases, or membrane-anchored histidine kinases (Nishihama *et al.*, 1995; Wurgler-Murphy and Saito, 1997). In plant cells, this cascade is activated by wounding, elicitors, abiotic stresses or phytohormones (reviewed by Hirt, 1997).

In this paper, we show a rapid activation of two PKs in response to cryptogeiin treatment of tobacco cells, and in response to oligogalacturonides also (Figure 1). These PKs possessed features of MAPKs: they phosphorylated MBP on serine or threonine residues and were themselves phosphorylated on tyrosine residues when activated, as shown by immunoprecipitation with an anti-phosphotyrosine antibody (Figure 2a). In Western blot experiments, this antibody also recognized two bands whose electrophoretic mobilities were compatible with MBP kinase mobilities (Figure 2b). Furthermore, using antibodies directed against human ERK1/ERK2 or against a phosphorylated peptide corresponding to the consensus sequence of the activated ERK1 form, two bands, again with similar electrophoretic mobilities to those detected by in-gel kinase assays, were immunorevealed with both antibodies (Figure 4). These two bands were present only in protein extracts from cryptogeiin-treated cells when immunoblots were probed with the antibody corresponding to the activated form of ERK1. This correlates with the increased activity observed in the gel. Taken together, these results clearly support activation of the MAPK pathway in response to cryptogeiin.

The activated PKs have apparent molecular masses of about 46 and 50 kDa, which is in agreement with the usual molecular mass range of the MAPK family. The p50-PK appeared to be more activated than the p46-PK, although interestingly they share identical properties with one exception, i.e. when staurosporine treatment was performed *in vitro* (Figure 5). Kinetics of activation were similar for both PKs: activation was detectable after 5 min, reached a maximum at 30 min and decreased slowly. The identical

time course activation of the two tobacco MAPK homologues favors the hypothesis of a regulation by common factors; a situation already encountered in yeast, where in response to the mating pheromone STE7, a MAPKK member, phosphorylates MAPKs FUS3 and KSS1 (Gartner *et al.*, 1992). Recently, it was shown that overexpression of MTK1, a human MAPKKK homologue to yeast Ssk2 and Ssk22 MAPKKK, stimulated both the p38 and the jun-N-terminal kinase (JNK) pathway (Takekawa *et al.*, 1997). In animal cells, stimulation of at least two ERKs (ERK1 and ERK2) was reported in response to insulin or nerve growth factors (Boulton *et al.*, 1991): ERK1 and ERK2 are activated by either MEK1 or MEK2 (Cobb and Goldsmith, 1995). Thus, in plant cells, as in animal cells, one signal can lead to the activation of several MAPKs via a common mediator.

We analyzed the sensitivity of tobacco MBP kinases to staurosporine, a general PK inhibitor, known to block early physiological events in tobacco cells when treated with cryptogein (Tavernier *et al.*, 1995; Viard *et al.*, 1994) and with oligogalacturonides (Mathieu *et al.*, 1996). This effect has also been observed in soybean treated with oligogalacturonides (Chandra and Low, 1995) and in tomato cells treated with a yeast extract (Felix *et al.*, 1991). Staurosporine treatment of tobacco cells suppressed the calcium influx (Tavernier *et al.*, 1995) and, according to our results, should suppress the calcium-dependent MBP kinase activation. However, addition of staurosporine during cryptogein treatment inhibited both PKs by 65% only. Remaining MBP kinase activities could be due to undetectable residual calcium influx or to the inhibition of other staurosporine targets, possibly connected to the MAPK pathway, that resulted in partial MBP kinase activation. When staurosporine was assayed *in vitro* (added to the reaction medium used for in-gel kinase assays), the signal corresponding to the p50-PK disappeared, whereas the p46-PK signal was reduced by 40% (Figure 5). The different sensitivities of MBP kinases to staurosporine treatment *in vitro*, together with their equivalent inhibition *in vivo*, suggest that the staurosporine effect *in vivo* is exerted mainly on a protein acting upstream from the MBP kinases.

Inhibitors of serine/threonine protein phosphatases are able to mimic elicitor action in plant cells (Chandra and Low, 1995; Felix *et al.*, 1994). In tobacco cells, calyculin A, an inhibitor of PP1 and PP2A, induces a Ca²⁺ influx, an extracellular pH alkalization and an AOS burst (our unpublished results). Consequently, it appears that some kinases are constitutively activated, being negatively regulated by protein phosphatases. To verify if the MBP kinases were under the control of a negatively regulated protein, we treated tobacco cells with calyculin A and performed in-gel kinase assays. This inhibitor alone had no effect on the activation of MBP kinases, indicating that kinases upstream from MBP kinases are not constitutively activated and/or regulated by protein phosphatases sensitive to

calyculin A. Although calyculin A mimics elicitor-induced physiological changes, additional events are necessary for the activation of MBP kinases. Moreover, calyculin A did not affect the cryptogein-induced activation of the MBP kinases (Figure 6), even when assays were performed over longer periods of time (2 and 3 h, data not shown). Thus, calyculin A did not sustain the cryptogein-induced MAPK activation as reported when tobacco cells were treated with fungal elicitor or were wounded (Suzuki and Shinshi, 1995; Usami *et al.*, 1995).

In order to establish a relationship between MAPK activation and Ca²⁺ influx, experiments were performed with various concentrations of EGTA, a Ca²⁺ chelator, and with various concentrations of La³⁺, an inhibitor of Ca²⁺ channels. Both compounds, known to inhibit AOS production (Tavernier *et al.*, 1995), plasma membrane depolarization, cytosol acidification and chloride efflux (Pugin *et al.*, 1997), also inhibited MAPK activation, indicating that Ca²⁺ influx is necessary for this pathway. In a second step, we verified the connection between the Ca²⁺-dependent AOS production and MAPK activation by using DPI, an inhibitor of the mammalian neutrophil NADPH oxidase. AOS suppression by DPI had no effect on MAPK activation. Consequently, the MAPK pathway is either upstream or acts independently of the AOS burst, both being under the control of Ca²⁺ influx.

In tobacco, MAPK activations have been demonstrated in relation to some plant/pathogen interactions. A fungal elicitor derived from the cell wall of *Phytophthora infestans* and a harpin from *Erwinia amylovora* activated MBP kinases with molecular masses of 47 kDa and 49 kDa, respectively (Adam *et al.*, 1997; Suzuki and Shinshi, 1995). Recently, it was reported that a tobacco MAPK (SIPK, molecular mass 48 kDa) was activated by exogenously applied SA, used at the optimal concentration of 500 µM, in tobacco cell suspensions (Zhang and Klessig, 1997). This MAPK is also activated in response to a cryptogein treatment (Zhang *et al.*, 1998). In this last study, cryptogein also induced two other MBP kinases (apparent molecular mass 44 and 40 kDa) whose activation was delayed and peaked at nearly 4 h (Zhang *et al.*, 1998). Although the SIPK and the p44 kDa MBP kinase may be the same as those characterized here, striking differences are observed in comparison to our results: the kinetics of activation differed and the p40 kDa MBP kinase activity was not detected in our assays. The identity and the number of MAPKs, their sustained activation, and their regulation in general may be determinant points directing downstream events, since elicitors or other signals (SA, wounding, cold, drought, etc.) may share some steps of signal transduction pathways, including MAPK pathways. In cryptogein-treated cells, the fast MAPK activation may be independent of SA. Although SA has been shown to be required in cryptogein-induced SAR (Keller *et al.*, 1996b), SA accumulation is hardly detect-

able within the first few hours (3–8 h) in response to chemicals inducing resistance to tobacco mosaic virus (TMV), or after infection of tobacco by TMV (Malamy *et al.*, 1996; Mur *et al.*, 1997). Moreover, in non-elicited tobacco cell suspensions or tobacco plants, the natural concentration of SA is very low (near or below $1 \mu\text{M}$) (Conrath *et al.*, 1995; Malamy *et al.*, 1996; Mur *et al.*, 1997), which renders unlikely activation of the cryptogein-induced MAPK pathway by SA in the beginning of the elicitation process, and before SA accumulation.

In future, further identification of proteins implicated upstream in the transduction pathway will provide information about the regulation of the output response. In addition, targets of MAPKs have to be identified in order to complete the study of a specific plant/pathogen interaction.

Experimental procedures

Plant material and elicitors

Nicotiana tabacum var Xanthi cell suspensions were grown in Chandler's medium (Chandler *et al.*, 1972) on a rotary shaker (150 rpm, 25°C, photon flux rate of $30\text{--}40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Cells were maintained in exponential phase and subcultured 1 day prior to utilization. Elicitins were purified according to the method of Bonnet *et al.* (1996). Purified oligogalacturonides were a gift of M.A. Rouet-Mayer (Gif/Yvette, France) and were used as a mixture of oligomers with degrees of polymerisation of 7–20.

Elicitor and chemical treatments

Cells from cultures in exponential growth phase were collected, washed by filtration in a suspension buffer containing 175 mM mannitol, 0.5 mM CaCl_2 , 0.5 mM K_2SO_4 , and 2 mM HEPES adjusted with KOH to pH 5.75. Cells were resuspended at 0.1 g fresh weight (FW) ml^{-1} of suspension buffer and equilibrated during 2 h on a rotary shaker (150 rpm, 24°C). Tobacco cells were then treated with 5–50 nM cryptogein or $50 \mu\text{g ml}^{-1}$ oligogalacturonides. Other components were added where indicated (LaCl_3 , EGTA, staurosporine, calyculin A or DPI) at different concentrations and for various times. Control tobacco cells were incubated under the same corresponding conditions without any treatment.

Preparation of protein extracts

After treatment, cells (0.25 g) were harvested by filtration on GF-A glass filters, frozen in liquid nitrogen and ground in a mortar. The powder was thawed in 400 μl of extraction buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 1 mM Na_3VO_4 , 10 mM NaF, 50 mM β -glycerophosphate, $5 \mu\text{g ml}^{-1}$ leupeptin, $5 \mu\text{g ml}^{-1}$ antipain, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 10 000 g, supernatant aliquots were stored at -80°C or recovered under Laemmli buffer conditions. The protein concentration in the extracts was quantified according to Bradford (1976) with bovine serum albumin (BSA) as the standard.

In-gel protein kinase assay

The in-gel kinase assay was performed as previously described by Kameshita and Fujisawa (1989), with minor modifications.

Extracts containing 20–30 μg of proteins were electrophoresed on 10% SDS-polyacrylamide gels embedded with 0.25 mg ml^{-1} MBP in the separating gel as a substrate for the kinases. After electrophoresis, SDS was removed by washing the gel for 1 h with a solution containing 50 mM Tris-HCl pH 8.0, 20% 2-propanol, and then again for 1 h with a 50 mM Tris-HCl pH 8.0 buffer, containing 5 mM β -mercaptoethanol. The separated proteins were denatured for 1 h in 6 M guanidine-HCl, 50 mM Tris-HCl pH 8.0, 5 mM β -mercaptoethanol, and then allowed to renature by five successive washes at 4°C over 16 h with 50 mM Tris-HCl pH 8.0, 5 mM β -mercaptoethanol and 0.04% (v/v) Tween-40. The gels were then equilibrated for 30 min at room temperature in 40 mM HEPES pH 7.5, 0.1 mM EGTA, 20 mM MgCl_2 and 2 mM DTT and then for 1 h in the same buffer supplemented with 25 μM ATP and 0.37–0.925 MBq [γ - ^{32}P]-ATP (Amersham). Where indicated, the gels were equilibrated prior to and during phosphorylation with 1.5 μM staurosporine added to the above buffer. The reaction was stopped by extensive gel washing with 5% (w/v) trichloroacetic acid, containing 1% (w/v) potassium pyrophosphate. The gels were dried on chromatography paper and exposed to X-Omat AR film (Kodak). MBP kinase activity was quantified using a PhosphorImager (Molecular Dynamics Inc). Pre-stained molecular markers from Sigma or Bio-Rad were used to estimate the apparent molecular mass of the MBP kinases after in-gel kinase assays.

The presence of phosphorylated residues of MBP susceptible to alkali was verified by incubating the gel, after the in-gel kinase assays with 1 M KOH at 60°C for 2 h. The gel was then neutralized with 10% acetic acid prior to drying.

Immunoblot analysis

Protein extracts (30–60 μg protein) were subjected to 10% SDS-polyacrylamide electrophoresis and thereafter transferred to 0.45 μm nitrocellulose membrane (HybondC, Amersham) by semi-dry electroblotting using a buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.0375% (w/v) sodium dodecylsulfate and 20% (v/v) methanol. The membrane was blocked for 1 h in 10 mM Tris-HCl pH 7.5, 150 mM NaCl buffer supplemented with 1% BSA. Probing and detection of blots were performed as described in the ECL Western detection kit (Amersham). The following primary antibodies were used: monoclonal antiphosphotyrosine IgG (clone PT-66, Sigma), polyclonal p44/42 MAP kinase (ERK1, ERK2) antibody (New England Biolabs) and polyclonal phospho-specific MAPK antibody (New England Biolabs), raised against a synthetic phosphotyrosine peptide corresponding to residues 196–209 of human p44 MAP kinase (ERK1). Horseradish peroxidase antirabbit (Bio-Rad) or antimouse IgG (Sigma) were used as secondary antibodies to perform the ECL detection.

Immunoprecipitation with anti-phosphotyrosine antibody

Proteins (50 μg) from untreated or treated cell extracts were immunoprecipitated using monoclonal anti-phosphotyrosine IgG (clone PT-66, Sigma) for 2 h at 4°C in an immunoprecipitation buffer containing 10 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4 , 10 mM NaF, 10 mM β -glycerophosphate, $5 \mu\text{g ml}^{-1}$ leupeptin, $5 \mu\text{g ml}^{-1}$ antipain, 0.5% (v/v) Nonidet P40, 1% (v/v) Triton X-100. Samples with competitor (1 mM phosphotyrosine) or non-competitor (1 mM phosphoserine or 1 mM phosphothreonine) phosphorylated amino acids were also assayed to check the specificity of the antibody. Anti-mouse IgGs coupled to agarose (Sigma) were added to the mixture and gently agitated overnight at 4°C. The immunoprecipitate was washed three times

with the immunoprecipitation buffer, resuspended in Laemmli buffer and incubated for 10 min at 85°C. After centrifugation, the supernatant was loaded on a SDS-polyacrylamide gel containing MBP for an in-gel kinase assay.

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