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Harpin induces mitogen-activated protein kinase activity during defence responses in *Arabidopsis thaliana* suspension cultures

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Abstract. Elicitation of *Arabidopsis thaliana* (L.) Heynh. suspension cultures with the bacterial protein harpin (from *Pseudomonas syringae* pv. *syringae*) induced the activation of two kinases of 39 and 44 kDa, as demonstrated by in-gel kinase assays using myelin basic protein (MBP) as a substrate. Both these kinases appeared to be tyrosine-phosphorylated upon activation, as demonstrated by treatment with tyrosine phosphatase and immunoprecipitation using an anti-phosphotyrosine monoclonal antibody. An inhibitor of mammalian mitogen-activated protein kinase (MAPK) activation, PD98059, inhibited harpin-induced MBPK activation, but did not inhibit the activity of these kinases. PD98059 also inhibited harpin-induced programmed cell death and defence gene expression, suggesting the involvement of harpin-induced MAPKs in defence responses in *Arabidopsis thaliana*.

Key words: *Arabidopsis* – Defence response – Harpin – Inhibitor (PD98059) – Mitogen-activated protein kinase – Tyrosine phosphorylation

Introduction

Plants mount a range of defence responses following challenge by potential pathogens, which includes transcriptional activation of genes encoding proteins with defensive functions as well as the Hypersensitive Response (HR), a form of localised programmed cell death (PCD) (Benhamou 1996; Dangl et al. 1996). Recognition of microbial pathogens and subsequent activation of defence responses require a complex interplay of signalling cascades, in which elicitor perception, generation of reactive oxygen species (ROS) such as $O_2^{\cdot-}$ and H_2O_2, and reversible protein phosphorylation have central roles (Yang et al. 1997).

Mitogen-activated protein kinase (MAPK) cascades are intracellular signalling modules highly conserved across all eukaryotes, that function to integrate and transduce a range of extracellular signals (Hirt 1997; Kultz 1998). Mitogen-activated protein kinases are serine/threonine kinases with both cytoplasmic and nuclear substrates, and are activated via dual phosphorylation on threonine and tyrosine residues by a MAPK kinase (MAPKK), itself in turn activated by a MAPKK kinase (MAPKKK). A large number of MAPK signalling components have now been cloned from plants and it is likely that they mediate responses to a variety of stimuli (Hirt 1997; Mizoguchi et al. 1997). It has recently become apparent that MAPKs are essential signalling components in plant-pathogen interactions. For example, Ligterink et al. (1997) have shown that elicitation of parsley cells with a fungal elicitor activates a MAPK which subsequently translocates to the nucleus. In tobacco, several MAPKs have been shown to be activated in response to bacterial (Adam et al. 1997) and fungal elicitors (Suzuki and Shinshi 1995; Zhang et al. 1998; Romeis et al. 1999), as well as salicylic acid (Zhang and Klessig 1997).

Using *Arabidopsis thaliana* suspension cultures as a model system, we have shown that the bacterial elicitor harpin (derived from *Pseudomonas syringae* pv. *syringae*) induces the rapid generation of H_2O_2, such signalling requiring protein phosphorylation (Desikan et al. 1996), and that harpin initiates PCD and activates the expression of various defence-related genes (Desikan et al. 1998a,b). Here, we present novel data which demonstrate the activation of MAPK-like enzymes by harpin in *Arabidopsis* suspension cultures. We show that harpin rapidly activates two protein kinases of 39 and 44 kDa, with typical characteristics of MAPKs. Additionally, an inhibitor of MAPKK (PD98059), and thus

Abbreviations: DMSO = dimethyl sulfoxide; GST = glutathione *S*-transferase; HR = hypersensitive response; MAPK(K) = mitogen-activated protein kinase (kinase); MBP = myelin basic protein; PAL = phenylalanine ammonia-lyase; PCD = programmed cell death; ROS = reactive oxygen species

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MAPK activation, inhibits programmed cell death and defence gene expression and also inhibits activation of the 39-kDa kinase.

Materials and methods

Treatment of A. thaliana suspension cultures. Cell-suspension cultures of *Arabidopsis thaliana* (L.) Heynh. var. Landsberg *erecta* were maintained as described previously (Desikan et al. 1996). For elicitor treatment, harpin$_{Pss}$ was isolated and purified as described in Desikan et al. (1998a). For the inhibitor experiments, PD98059 (Calbiochem, Nottingham, UK) was dissolved in dimethyl sulfoxide (DMSO) and added to the cells at the indicated concentrations 20 min prior to the addition of elicitors. Controls included the mock-treatment of cells with appropriate volumes of sterile distilled water or 0.5% (v/v) DMSO. Viability of treated *Arabidopsis* cells was determined 6 h after addition of elicitors, using Evan's Blue dye and light microscopy (Desikan et al. 1998a).

Protein extraction. *Arabidopsis* cells were treated with harpin in the presence or absence of inhibitors for the appropriate lengths of time, and frozen in liquid nitrogen. Frozen cells (ca. 0.5 g) were ground with a mortar and pestle using liquid nitrogen, followed by homogenisation at 4 °C with 2 volumes of protein extraction buffer [100 mM Hepes (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM α-glycerophosphate, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin]. The ground slurry was then centrifuged at 10,000 *g* for 20 min at 4 °C, in a microcentrifuge. Supernatants were aliquotted into clean tubes, snap-frozen in liquid nitrogen and stored at −80 °C for later use. Protein concentrations were estimated using the method described by Bradford (1976).

In-gel protein kinase assays. Forty micrograms of protein from *Arabidopsis* cell extracts was electrophoresed on 10% SDS-polyacrylamide gels embedded with either 0.5 mg mL^{-1} myelin basic protein (MBP) from bovine brain (Sigma, Dorset, UK), dephosphorylated casein (1 mg mL^{-1}) or histone (1 mg mL^{-1}; type III S-S, Sigma) in the resolving gel as substrates for the kinase. Pre-stained molecular weight markers (New England Biolabs, Hitchin, UK) were used as standards. After electrophoresing at 100 V for 2 h, SDS was removed from the gel by washing the gel with 100 mL of washing buffer [25 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg mL^{-1} BSA, 0.1% Triton X-100] three times for 30 min each at room temperature with gentle shaking. The proteins were then denatured by incubating the gel in 100 mL of denaturation buffer [6 M guanidine-HCl, 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol] for 1 h at room temperature. Subsequently, the proteins were renatured overnight at 4 °C in 200 mL of renaturation buffer [25 mM Tris-HCl (pH 8), 1 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF] with at least three changes of the buffer. The gel was then incubated at room temperature in 30 mL of reaction buffer [25 mM Tris-HCl (pH 8), 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4] for 30 min. Phosphorylation was performed for 1 h at room temperature in 15 mL of the same reaction buffer supplemented with 50 μM ATP (Sigma) and 1.85 MBq [γ-^{32}P]ATP (specific activity: 110 TBq/mmol; Amersham, Little Chalfont, UK). Unincorporated radioactivity was subsequently removed by washing the gel for 5–6 h at room temperature with several changes of 5% (w/v) trichloroacetic acid, 1% (w/v) sodium pyrophosphate. The gel was dried onto Whatman 3 MM paper and subjected to autoradiography.

In-vitro *treatment of protein extracts with protein tyrosine phosphatase*. For protein tyrosine phosphatase treatment, 40 μg of protein from harpin-treated cells was isolated as described above and incubated with 5 U of protein tyrosine phosphatase β (Upstate Biotechnology Inc., Lake Placid, N.Y., USA) in phosphatase buffer [25 mM Hepes (pH 7.2), 50 mM NaCl, 5 mM DTT, 2.5 mM EDTA, 100 μg mL^{-1} BSA] at 30 °C for 30 min. The samples were subsequently denatured and in-gel assays performed as described above.

Immunoprecipitation and in-vitro kinase activity assay. Fifty micrograms of protein from harpin-treated cells was incubated by shaking for 2 h at 4 °C with 5 μg of anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology) in immunoprecipitation buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM α-glycerophosphate, 5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin, 1 mM PMSF, 0.5% Triton-X 100] in the presence or absence of phosphoaminoacid (1 mM final concentration) as competitor. About 30 μl packed volume of protein G-Sepharose (Sigma) was added and incubated for a further 2 h. The Sepharose bead-protein complexes were pelleted by gentle centrifugation (600 *g*), and subsequently washed twice in wash buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1% Triton-X 100] and once in kinase assay buffer [25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM Na_3VO_4]. Following washing, in-vitro kinase activity assay of the immunoprecipitated proteins was perfomed in 15 μl of kinase buffer containing 0.5 mg mL^{-1} MBP, 74 kBq [γ-^{32}P]ATP, and 0.1 mM ATP at room temperature for 30 min. The reaction was stopped by the addition of SDS sample loading buffer; the samples were then denatured and electrophoresed on a 15% SDS-polyacrylamide gel. The gel was subsequently stained in Coomassie Blue to confirm equal protein loading, destained in 14% methanol, 10% acetic acid, and then dried and subjected to autoradiography.

Isolation of RNA and northern analysis. Isolation of RNA and northern analysis were performed as described in Desikan et al. (1998a) with the following modifications. RNA blotted onto nylon membrane was pre-hybridised and hybridised at 42 °C overnight in a formamide buffer containing 5× SSPE (a 20× SSPE solution contains 3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M Na_2EDTA, pH 7.4), 5× Denhardt's solution (a 50× Denhardt's solution contains 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA Fraction V, 1% (w/v) Ficoll 400), 1% (w/v) SDS, 50 mM NaH_2PO_4 (pH 6.8), 10% dextran sulphate, 100 μg mL^{-1} denatured salmon sperm DNA, and 50% formamide. A *PAL1* genomic clone and a *GST* PCR product (Desikan et al. 1998a) were used as hybridisation probes. Equivalent RNA loadings were confirmed by ethidium bromide staining of the gel.

Results

Harpin induces myelin basic protein (MBP) kinase activity. Extracts of control or harpin-treated *Arabidopsis* cells were subjected to in-gel protein kinase assays utilising MBP as substrate. As shown in Fig. 1, harpin$_{Pss}$ at a concentration of 1 μg mL^{-1} induced the rapid and transient activation of two kinases, one at ca. 44 kDa and a second at ca. 39 kDa. Activation of these kinases occurred maximally between 15 and 30 min, and reduced to control levels within 60 min. Control treatments had no effect on the activity of these kinases. In addition to the inducible protein kinases, constitutive kinase activities with molecular masses of ca. 60 and 30 kDa were also present. The 60-kDa kinase possessed both auto- and casein-phosphorylating activity, whilst the 30 kDa kinase phosphorylated histone (data not shown). However, relative to their MBP phosphorylating activity, the inducible kinases (44 and 39 kDa) possessed no appreciable auto-, casein- or histone-phosphorylating activity (data not shown).

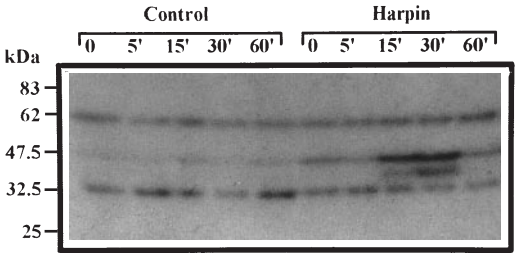

Fig. 1. Induction of MBP kinase activity by harpin in *Arabidopsis* suspension cultures. Protein extracts from cells treated with either water (*Control*) or 1 μg mL^{-1} harpin for different lengths of time (indicated in minutes), were subjected to in-gel protein kinase assay using MBP as substrate. The constitutively active kinases are visible at 60 and 30 kDa; molecular weight markers (in kDa) are indicated on the left

To demonstrate the dose-dependency of the harpin-activated kinases, *Arabidopsis* cells were treated with increasing amounts of harpin and protein extracts subjected to in-gel kinase assays. Figure 2 shows that the activity of harpin-induced kinases increased with increasing doses of harpin, at concentrations previously shown to elicit defence responses (Desikan et al. 1998a,b).

Harpin-activated protein kinases are tyrosine-phosphorylated. One of the characteristics that distinguishes MAP kinases from other protein kinases is that their activation is dependent on tyrosine phosphorylation. Incubation of protein extracts from harpin-treated cells with tyrosine phosphatase (which specifically dephosphorylates only proteins that are phosphorylated on tyrosine residues) prior to in-gel kinase assay substantially reduced harpin-induced MBP-kinase activity of both the 44-kDa and 39-kDa kinases (Fig. 3A). However, the activity of the constitutively active 30 kDa and 60 kDa kinases remained relatively unchanged.

To demonstrate further that the harpin-activated kinases are tyrosine-phosphorylated, protein extracts from harpin-treated cells were immunoprecipitated with

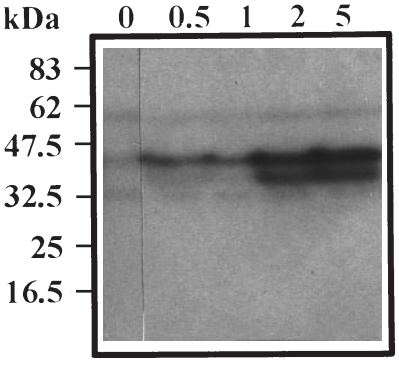

Fig. 2. Dose-response of harpin-induced MBP kinase activity. *Arabidopsis* cells were exposed to increasing amounts (0–5 μg mL^{-1}) of harpin, harvested after 15 min, and crude protein extracts subjected to in-gel kinase assay using MBP as substrate. Molecular weight markers (in kDa) are indicated on the left

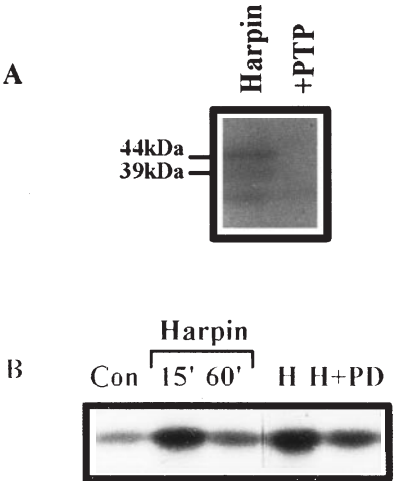

Fig. 3A,B. Tyrosine phosphorylation of harpin-activated MBP kinases. **A** Protein extracts from *Arabidopsis* cells treated with harpin (2 μg mL^{-1}) for 15 min were incubated in the presence or absence of protein tyrosine phosphatase (*PTP*) prior to in-gel kinase assay using MBP as substrate. The 44-kDa and 39-kDa kinases are indicated; the lowest band represents the 30-kDa constitutive kinase. **B** Immunoprecipitation with the anti-phosphotyrosine antibody 4G10 and in-vitro MBP kinase activity assay were performed on protein extracts from cells treated with DMSO (*Con*), harpin (1 μg mL^{-1}) for the indicated times (in min), and harpin (2 μg mL^{-1}) in the absence (*H*) or presence (*H + PD*) of 10^{-4} M PD98059 for 15 min. (Cells were pre-treated with with PD98059 for 20 min before addition of harpin)

the phosphotyrosine-specific monoclonal antibody 4G10; the resulting protein complexes were assayed for kinase activity using [γ-^{32}P]ATP and MBP as substrates, and subsequently fractionated using SDS-PAGE. As shown in Fig. 3B, control treatments revealed basal levels of MBP phosphorylation. However, treatment with harpin resulted in an increase in immunoprecipitated MBP-kinase activity in a transient manner, with an almost 2-fold increase in activity within 15 min, and a return to control levels after 60 min (Fig. 3 and as determined by scanning densitometry; data not shown).

Phosphoaminoacids were used as competitors in the immunoprecipitation experiments described above in order to demonstrate the specificity of the anti-phosphotyrosine antibody. Whilst phosphoserine and phosphothreonine had little effect on the efficiency of 4G10 at immunoprecipitating harpin-activated protein kinases, excess phosphotyrosine efficiently inhibited 4G10-immunoprecipitation of harpin-activated MBP kinases (Fig. 4).

The involvement of individual kinases in 4G10-immunoprecipitated MBP kinase activity was further investigated by fractionating the immunoprecipitated protein extracts on MBP-embedded gels followed by in-gel kinase assays. This revealed that both the harpin-induced kinases (44 and 39 kDa) are tyrosine-phosphorylated (Fig. 5).

Effect of a MAPKK inhibitor on harpin-induced programmed cell death. In previous work we have shown that harpin induces PCD in *Arabidopsis* suspension

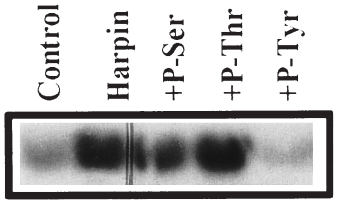

Fig. 4. Phosphotyrosine specificity of 4G10. Protein extracts from *Arabidopsis* cells treated with water (*Conrol*) or harpin (2 μg mL^{-1}) for 15 min were immunoprecipitated with the anti-phosphotyrosine antibody 4G10 in the absence (*Control*, *Harpin*) or presence of 1 mM phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*) or phosphotyrosine (*P-Tyr*), and the immunocomplexes subjected to in-vitro MBP kinase activity assay

cultures (Desikan et al. 1996, 1998a). In an attempt to determine the physiological role of MAP kinase signalling cascades in harpin-induced defence responses, the effects of a MAPKK inhibitor, PD98059, on harpin-induced PCD were determined. In mammalian systems, PD98059 has been widely used as an inhibitor of specific MAPKKs, and thus MAPK activation, to establish the physiological role of MAPKs in various cell types (Cohen 1997). Cells were treated with harpin in the presence or absence of PD98059, and viability determined after 6 h, the time point at which cell viability has been shown to decline (Desikan et al. 1998a). As shown in Fig. 6, treatment of *Arabidopsis* cells with 10^{-4} M PD98059 alone had little effect on cell viability while there were no effects at lower concentrations (data not shown). However, pre-treatment of the cells with PD98059 at 10^{-4} M for 20 min significantly inhibited harpin-induced cell death (Fig. 6). At concentrations lower than this, the effects of PD98059 were less apparent. Such pre-treatments with PD98059 in the same concentration range did not inhibit or delay harpin-induced H_2O_2 generation in *Arabidopsis* cells (data not shown).

Effect of the MAPKK inhibitor on harpin-induced defence gene expression. Harpin induces the expression of several defence-related genes including those encoding glutathione-*S*-transferase (GST) and phenylalanine

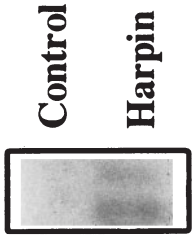

Fig 5. Tyrosine phosphorylation of both the harpin-activated MBP kinases. Protein extracts from *Arabidopsis* cells treated with water (*Control*) or harpin (2 μg mL^{-1}) for 15 min were immunoprecipitated with the anti-phosphotyrosine antibody 4G10 and then subjected to in-gel protein kinase assay using MBP as a substrate. The upper and lower bands represent the 44- and 39-kDa kinases, respectively

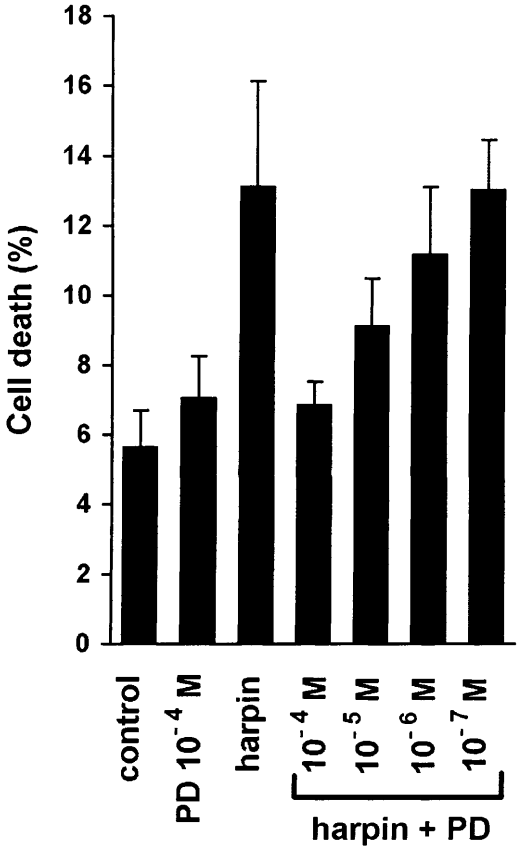

Fig 6. Effect of PD98059 on harpin-induced programmed cell death. *Arabidopsis* cells were treated with DMSO (*control*), harpin (1 μg mL^{-1}) or PD98059 (*PD10*$^{-4}$ M), or were pre-treated for 20 min with PD at the indicated concentrations before addition of harpin at 1 μg mL^{-1} (*harpin + PD*), and viability of the cells determined after 6 h. Values represent the mean ± SE ($n = 9$)

ammonia-lyase (PAL) (Desikan et al. 1998a). To determine the role of MAPK-based signalling in harpin-induced defence responses, the effect of the MAPKK inhibitor PD98059 on harpin-induced *PAL* and *GST* expression was determined (Fig. 7). At 10^{-5} M, PD98059 enhanced the expression of *PAL* mRNA in control treatments; a corresponding increase was also observed in the expression of harpin-induced *PAL* mRNA in the presence of PD98059 at this concentration (Fig. 7B). This effect of 10^{-5} M PD98059 was specific to *PAL* mRNA as there was no effect on *GST* expression in control treatments (Fig. 7A; the same blot was used). However, pre-treatment of cells with 10^{-4} M PD98059 considerably reduced harpin-activated expression of both *PAL* and *GST* (Fig. 7A,B).

An inhibitor of MAPKK activation reduces harpin-induced MBP kinase activation. In order to determine if the effects of PD98059 on cell death and gene expression were associated with MAPK activation, the effect of PD98059 on harpin-induced MBP kinase activation was determined. Protein extracts from *Arabidopsis* cells treated with harpin in the presence of varying amounts of PD98059 were subjected to in-gel kinase assays (Fig. 8). At 10^{-4} M PD98059, there was

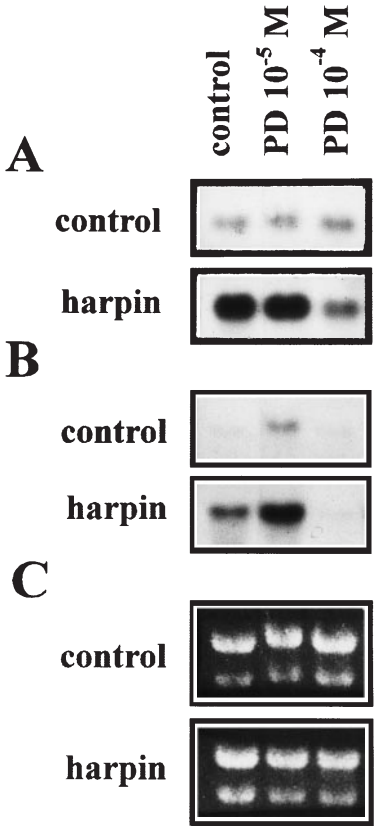

Fig. 7A–C. Effect of PD98059 on harpin-induced defence gene expression. *Arabidopsis* cells were treated with water (*Control*) or harpin (1 μg mL^{-1}) for 2 h in the presence or absence of a pre-treatment with PD98059 (*PD*) at the indicated concentrations, and total RNA extracted and subjected to Northern analysis using ^{32}P-labelled *GST* (**A**) or *PAL* (**B**) probes. Equal RNA loading was confirmed by staining the gel with ethidium bromide (**C**)

some inhibition of activation of the 44-kDa kinase with greater inhibition of the 39-kDa kinase (Fig. 8). The constitutively active 30-kDa and 60-kDa (not shown) kinases did not appear to be inhibited at this concentration of the inhibitor. At lower concentrations, PD98059 had less effect on the activation of the two harpin-activated kinases. To demonstrate that PD98059 had no effect on the activity of the harpin-activated kinases, extracts were fractionated on MBP-embedded gels and the gels were incubated in 10^{-4} M PD98059 or 2×10^{-6} M K-252a for 30 min prior to kinase assay. PD98059 had no effect on the in-gel activity of the harpin-activated kinases, whereas treatment with the general serine/threonine protein kinase inhibitor K-252a completely abolished it (data not shown).

To investigate the effect of PD98059 on tyrosine phosphorylation of the harpin-activated MBP kinases, protein extracts from cells treated with harpin in the presence or absence of PD98059 (10^{-4} M) were subjected to immunoprecipitation with the anti-phosphotyrosine antibody 4G10, followed by in-vitro MBP kinase assays (Fig. 3B). The inhibition of harpin-induced MBP kinase activation by PD98059 (see Fig. 8) correlated with the decrease in 4G10-immunoprecipitation of harpin-activated MBP kinases (Fig. 3B).

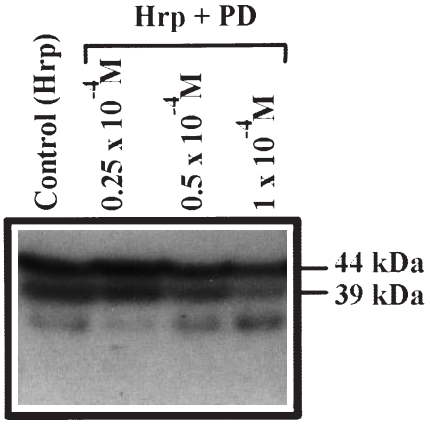

Fig. 8. Effect of PD98059 on harpin-induced activation of MBP kinases. Protein extracts from cells treated with harpin (2 μg mL^{-1}) in the absence [*Control* (*Hrp*)] or presence of a pre-treatment with PD98059 (*Hrp + PD*) at the indicated concentrations were subjected to in-gel kinase assay using MBP as substrate. The 44-kDa and 39-kDa kinases are indicated; the lower band represents the constitutive 30-kDa kinase

Discussion

The HR is a key phenomenon occurring during plant-pathogen interactions, and is a culmination of several underlying signalling mechanisms occurring within the host plant. Emerging paradigms of the HR suggest that MAPKs play a pivotal role in some of the defence-related responses (Somssich 1997). Although several MAPKs and their upstream components have been cloned from *Arabidopsis thaliana* (Hirt 1997; Mizoguchi et al. 1997) there have been no reports yet in the literature relating MAPKs with defence responses in this model plant. Here, we present novel data which demonstrate that the proteinaceous bacterial elicitor harpin induces the activation of MAPK-like enzymes in *Arabidopsis*.

Harpins have been isolated from phytopathogenic bacteria such as *Erwinia amylovora* (Wei et al. 1992) and *Pseudomonas syringae* pv. *syringae* (He et al. 1993); these harpins can mediate HR-related events such as changes in ion fluxes and the oxidative burst (Baker et al. 1993), and tissue necrosis (He et al. 1994). It has also been suggested that harpins might serve to deliver bacterial avirulence gene products into plant cells (Collmer 1998). In previous work we have established that harpin induces an oxidative burst in *Arabidopsis* cells, a process requiring protein phosphorylation (Desikan et al. 1996). Here we show that harpin induces a MAPK pathway potentially involved in defence responses in *Arabidopsis* suspension cultures. It has been demonstrated recently that harpin from *Erwinia amylovora* induces an MBP kinase in tobacco that has the characteristics of a MAPK (Adam et al. 1997). Our data are in accordance with this, in that harpin does indeed induce the activity of *Arabidopsis* MBP kinases. However, the activation of the kinases induced by harpin from *Pseudomonas syringae* pv. *syringae* in *Arabidopsis* cells differs from that reported by Adam et al. (1997). Whereas Adam et al. (1997)

observed the activation of a 49-kDa MBP kinase by $harpin_{Ea}$, which was also activated by water infiltration of tobacco leaves, we observed the activation of two kinases, one of ca. 44 kDa and another of ca. 39 kDa, by $harpin_{Pss}$ (Fig. 1).

The MBP kinases activated by harpin in *Arabidopsis* cells have a number of features typical of MAPKs. Firstly, the molecular mass of the 44-kDa kinase is within the appropriate size range of plant MAPKs identified so far (Kultz 1998). Zhang et al. (1998) have shown that both proteinaceous and carbohydrate fungal elicitors activate 48- and 44-kDa kinases in tobacco, both these kinases being tyrosine-phosphorylated. They also reported a 40-kDa kinase activated solely by proteinaceous elicitors; this could be similar to the 39-kDa MBP kinase that is activated by $harpin_{Pss}$ in *Arabidopsis*. There are at least nine MAPK genes in *Arabidopsis*, encoding potential enzymes that can be classified into four sub-groups based on their predicted amino acid sequences (Mizoguchi et al. 1997). It is possible that the harpin-activated MBP kinases might be related to one or more of the MAPKs cloned so far from *Arabidopsis*.

Secondly, the harpin-activated kinases in *Arabidopsis* preferentially utilise MBP as a substrate in vitro, a property which has been used to characterise several plant MAPKs (Adam et al. 1997; Ligterink et al. 1997; Zhang and Klessig 1997). Furthermore, both the kinases are rapidly and transiently activated. Zhang et al. (1998) observed the activation of at least three kinases in tobacco, by different elicitors, and one of these appeared to be activated at a much later stage with a duration of hours rather than minutes, interpreted by the authors as indicating different regulatory roles of the kinases in cellular responses. It remains to be determined whether the two harpin-activated kinases or other kinases are activated at later time periods in our system. It is also not yet known whether these two kinases regulate similar or different cellular responses.

An important characteristic of the harpin-activated MBP kinases that suggests that they might be members of a MAPK family, is that both the 44-kDa and 39-kDa proteins are tyrosine phosphorylated. Two lines of investigation demonstrated this: tyrosine phosphatase treatment substantially reduced their MBP kinase activity, and both these kinases were immunoprecipitated by the anti-phosphotyrosine antibody 4G10, an antibody used by previous workers (Zhang and Klessig 1997). The activation and inactivation of the harpin-activated kinases correlated with the immunoreactivity of these kinases to 4G10, implying that tyrosine phosphorylation is essential for activation of the MBP kinases, one of the hallmarks of MAPKs. Additional experiments revealed that an anti-MAPK (anti-ERK) antibody also recognises proteins of the same molecular weight in *Arabidopsis* extracts (data not shown), further confirming that proteins immunologically related to MAPKs are present in *Arabidopsis*.

In an attempt to determine the physiological significance of harpin-induced MAPK activation in *Arabidopsis*, a pharmacological approach was employed. In mammalian systems, the compound PD98059 is proving to be a very useful tool with which to elucidate the involvement of MAPK-based signalling in various physiological responses (Cohen 1997). Its mode of action appears to be highly specific, in that it binds only to the non-phosphorylated form of certain MAPKKs, thereby preventing their activation and subsequent ability to phosphorylate and thus activate their particular substrate MAPKs (Alessi et al. 1995). It has been recently reported that PD98059 abolished the activation of both salicylic acid and wound-induced MAPKs in tobacco cell cultures challenged with a fungal pathogen (Romeis et al. 1999), suggesting that PD98059 does target MAPK pathways in plant cells. However, at least six different MAPKK genes have been cloned from *Arabidopsis* (Jouannic et al. 1996; Mizoguchi et al. 1997; Morris et al. 1997), and it is possible that PD98059 will bind to a broader range of MAPKK enzymes in plants than in mammalian cells. Nevertheless, we have demonstrated here that at 10^{-4} M, PD98059 inhibits both tyrosine phosphorylation and the activation of at least one of the harpin-activated MBP kinases. However it does not inhibit the activity of these kinases (i.e. the phosphorylated form), which is in accordance with its mode of action in mammalian systems.

At 10^{-4} M, PD98059 significantly inhibited harpin-induced PCD, and substantially reduced harpin-induced expression of both *PAL* and *GST*. However, the inhibitory effects of PD98059 on the activation of the two MAPKs were not as pronounced. Thus, even though activation and inhibition of the 39-kDa MAPK suggest that its action may be involved in defence responses, it is also possible that PD98059 has other effects, including inhibition of other MAPKs not detected in our assays. The specific positive effect of 10^{-5} M PD98059 on *PAL* gene expression suggests that this might be the case. Other studies have correlated the inhibition of activation of MAPKs by the broad-spectrum serine/threonine protein kinase inhibitors K-252a or staurosporine with activation of defence responses such as the HR (Adam et al. 1997; Suzuki et al. 1999) and defence gene expression (Zhang et al. 1998). Although we observe that at low concentrations of K-252a, both harpin-induced ROS generation and PCD can be inhibited in our system (Desikan et al. 1996, and data not shown), PD98059, a specific inhibitor of MAPK activation, does not inhibit ROS generation induced by harpin, although PCD is inhibited. This suggests that different kinases are involved in the harpin-induced activation of ROS generation and the downstream effects of ROS.

Although PCD in plants is now the focus of intensive research, there is currently little detail of the mechanisms by which it is executed and regulated (Dangl et al. 1996; Pennell and Lamb 1997). In mammalian systems it is clear that the regulation of cell death is highly complex, and may involve the opposing effects of different MAPK pathways (Anderson 1997). Our data provide evidence that harpin-induced MAPK activity occurs during defence responses in *Arabidopsis* cells. On-going research in our laboratory aims to determine whether H_2O_2, generated in response to elicitor and microbial challenge,

plays a role in the activation of any MAPKs which may be involved in H_2O_2-induced defence responses. It will also be important to determine the identity of the harpin-induced MAPKs so that their biological function can be tested directly via mutant and antisense approaches.

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