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RESEARCH PAPER

Involvement of mitogen-activated protein kinases in the symbiosis *Bradyrhizobium–Lupinus*

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Abstract

In plants, mitogen-activated protein kinases (MAPKs) are involved in signalling to hormones, cell cycle regulation, stresses, and plant defence responses. In this work, several MAPKs were detected by immunoblotting in roots and nodules of *Lupinus albus* produced by inoculation with *Bradyrhizobium* sp. (*Lupinus*). *In vitro* kinase assays showed that inoculation of seedling roots with *B.* sp. (*Lupinus*) activates salt stress-inducible and stress-activated MAPKs after 5 min of incubation. By contrast, inoculation with dead *B.* sp. (*Lupinus*) or the heterologous bacteria *Sinorhizobium meliloti* did not induce salt stress-inducible and stress-activated MAPK activities. *In vivo* experiments showed that inoculation with *B.* sp. (*Lupinus*) induced the activation of MAPKs in roots. The maximal activation was in the region of the root tip with emerging hairs, which corresponds to the infection zone. The p38 MAPK inhibitors SB 202190 and SB 203580 blocked these kinase activities. Experiments with SB 202190 and the MAPKK inhibitor UO 126 altered the pattern of nodulation in the main root, decreasing the number and weight of nodules produced in the upper sites while increasing the nodule number in the younger lower root zone. These data suggest that MAPK inhibition blocks early events in the susceptible root zone to rhizobial infection, delaying nodulation, and support a role for MAPKs in the infection and nodulation of *L. albus* by *B.* sp. (*Lupinus*).

Key words: Legume infection, nodulation, protein phosphorylation, signal transduction, symbiotic interaction.

Introduction

Mitogen-activated protein kinase (MAPK) signalling cascades are one of the major pathways by which extracellular stimuli are transduced into intracellular responses in mammals, yeast, and plants. MAPK cascades consist of three functionally linked protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK). Activation of MAPKs occurs by phosphorylation of both threonine and tyrosine residues in the sequence TXY that is catalysed by an upstream MAPKK. In turn, this kinase is phosphorylated by its own upstream MAPKKKs. Following activation, MAPKs translocate from the cytoplasm into the nucleus and phosphorylate a number of transcription factors leading to changes in gene expression (Whitmarsh and Davis, 1998).

MAPKs are involved in cell differentiation, division, and stress responses (Robinson and Cobb, 1997). In plants, a number of studies have demonstrated that MAPKs signal

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Abbreviations: DMSO, dimethylsulphoxide; DTT, dithiothreitol; MAPK, mitogen-activated protein kinases; MBP, myelin basic protein; MMK2 and MMK3, *Medicago* MAPK2 and MAPK3, respectively; SAMK, stress-activated MAPK; SIMK, salt stress-inducible MAPK; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

abiotic and biotic stresses, including cold and drought (Jonak *et al*., 1996), wounding (Seo *et al*., 1995; Bögre *et al*., 1997; Zhang and Klessig, 1998), hormone action (Hirt, 1997; Ligterink and Hirt, 2001), and plant pathogen attack. Extensive research has been done to elucidate the role of MAPKs in plant–pathogen interactions and several members of the MAPK family have been shown to be involved in plant defence responses (Ligterink *et al*., 1997; Cardinale *et al*., 2000; Zhang and Klessig, 2001, Asai *et al*., 2002). The alfalfa MAPKs, salt stress-inducible MAPK (SIMK) and stress-activated MAPK (SAMK), which were originally identified as inducible by osmotic stress (Munnik *et al*., 1999) and wounding (Bögre *et al*., 1997), respectively, were later also found to be activated by various fungal elicitors (Cardinale *et al*., 2000). Two other alfalfa MAPKs, MMK2 and MMK3 (*Medicago* MAPK2 and MAPK3, respectively), are involved in cell growth and division (Bögre *et al*., 1999) but are also activated by elicitors (Cardinale *et al*., 2000). So far, little is known about MAP kinases in *Rhizobium*–legume symbiosis. SIMK has been cloned as MsERK1 from a cDNA library prepared from the infection zone of *Rhizobium*-inoculated alfalfa roots (Duerr *et al*., 1993) and another alfalfa MAPK, called TDY1, is expressed in roots and nodules (Schoenbeck *et al.*, 1999), suggesting a possible role of MAPKs in nodule initiation and development.

Nitrogen fixation in legumes takes place in root nodules, highly specialized organs that result from the symbiosis between the host plant and the rhizobia. Interaction for the correct recognition between the symbiotic partners begins with the exchange of multiple signals between legume (flavonoids, hormones) and rhizobia (Nod factors, exopolysaccharides, and lipopolysaccharides). Flavonoids secreted by the host plants induce the *nod* genes in the bacteria that encode the nodulation factors (Nod factors). These molecular signals are essential for initiating root morpho- and organogenesis, leading to the formation of nodules (Dénarie *et al*., 1996). There is evidence that intracellular calcium changes (Felle *et al*., 1999; Wais *et al*., 2002), G-proteins, and protein phosphorylation (Pingret *et al*., 1998) are involved in the Nod factor signal transduction mechanism (Stougaard, 2000).

At first sight, symbiotic and pathogenic interactions appear to be different. However, common strategies have been found in the early plant response to infection by pathogenic and symbiotic bacteria, leading to the proposal that pathogenesis and symbiosis are variations on a common theme (Baron and Zambryski, 1995; Parniske, 2000; Herouart *et al*., 2002). Defence responses in plant–pathogen interactions involve ion fluxes, oxidative burst, and reversible protein phosphorylation (Yang *et al*., 1997). Asai *et al*. (2002) have described a complete plant MAPK cascade, suggesting that signalling events initiated by diverse pathogens converge into a conserved MAPK cascade. In this framework, it is interesting to consider the involvement of MAPKs in the symbiotic interaction as good candidates to integrate a variety of signals triggered by the two partners.

In this work, *Lupinus albus* was used to analyse the putative role of MAPKs in the rhizobia–legume symbiosis. Although *Lupinus–Bradyrhizobium* is not considered as a model symbiosis, it has been successfully employed to determine new aspects of the functioning of legume nodules: mechanism and visualization of diffusion barrier operation (de Lorenzo *et al*., 1993; Iannetta *et al*., 1993); presence of nitric oxide synthase in nodules (Cueto *et al.*, 1996); and the presence and role of aldehyde oxidase in nodule morphogenesis (Fedorova *et al*., 2005). MAPKs were detected in roots and nodules of *Lupinus albus* inoculated with *Bradyrhizobium* sp. (*Lupinus*). Kinase assays using immunoprecipitated MAPKs of root seedlings showed that inoculation with *B.* sp. (*Lupinus*) transiently activates SIMK, and SAMK and MAPK inhibitors negatively affected lupin root nodulation. Taken together, the results indicate that MAPKs play a role in the establishment of symbiosis.

Materials and methods

Plant culture

To obtain nodules, lupin plants (*Lupinus albus* L. cv. Multolupa), uninoculated or inoculated with *Bradyrhizobium* sp. (*Lupinus*) strain ISLU 16, were grown in autoclaved pots filled with vermiculite supplemented with a nutrient solution without nitrogen (Lang *et al*., 1993) in an acclimated growth chamber. The conditions in the growth chamber were: a 16/8 h light/dark photoperiod; 25/15 °C day/night temperature; 58% relative humidity; an irradiance level of 200 μm m^{-2} s^{-1}.

For early interaction experiments of MAPK's phosphorylation, sterilized seeds were allowed to germinate for 3 or 4 d on moist filter paper in Petri dishes at 28 °C. Uniform seedlings (10–15) were selected and four different groups established. Each group was inoculated by incubation for different times (5–60 min) with a bacterial suspension (10^8 cfu ml^{-1}) of *B.* sp. (*Lupinus*), *Sinorhizobium meliloti* 2011, or *B*. sp (*Lupinus*) which had been killed by autoclaving. Germinated seedlings incubated in Vincent growth medium (Vincent, 1970) were used as controls. Root segments, ~3.5 cm in length, were frozen in liquid nitrogen and stored at −80 °C. Samples were taken under red light avoiding mechanical stress. To study enzyme distribution, roots were excised, using a razor blade, in to three segments corresponding to the mature zone, the medium zone with young hairs, and the root tip (Fig. 1A). Likewise, tips of root segments (~15 mm) were separated into the zone with emerging hairs, the elongation zone, and the apical meristem plus the root cap, according to the studies on root development described by Ishikawa and Evans (1995).

Nodulation experiments in the presence of inhibitors were carried out. Roots of 3-d-old seedlings were incubated with the corresponding inhibitor for 8 h in darkness at 28 °C. After treatment, the seedlings were transferred to small autoclaved Leonard jars filled with vermiculite, inoculated with *B.* sp. (*Lupinus*) and grown in the acclimated chamber. At sampling, 16 d post-inoculation, 10–15 plants with an intact main root were taken and the number and fresh weight of the nodules measured. The sampling was carried out as follows: nodules located up to 5.5 cm (Fig. 1B) from the main root base (zone 1) were separated from the rest of the nodules located

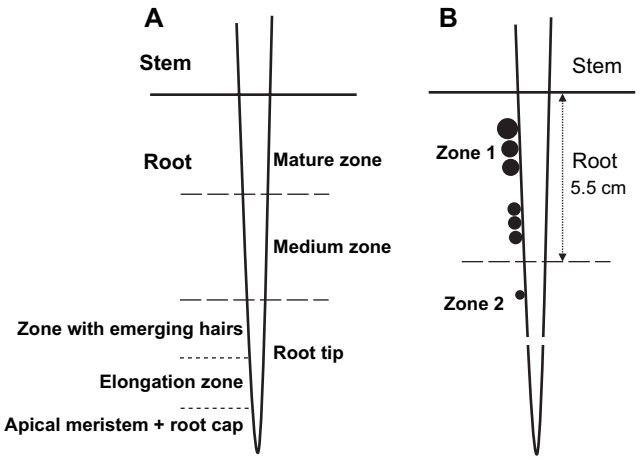

Fig. 1. (A) Scheme of a 4-d-old lupin root germinated on moist filter paper in Petri dishes at 28 °C showing the zones excised to analyse MAPK distribution. (B) Scheme of a nodulated lupin root grown in Leonard jars, showing zones 1 and 2 of nodulation, used to determine the effects of MAPK inhibitors on nodulation.

at a longer distance (zone 2) which were formed later. The inhibitors (Calbiochem) SB 202190 and its inactive analogue SB 202474 (40 and 100 μM), and the inhibitor UO 126 and the corresponding negative control UO 124 (1 and 10 μM) were dissolved in dimethylsulphoxide (DMSO) and diluted in a buffer solution (15 mM MES pH 6.0 containing 0.75 mM KCl, 0.75 mM $CaCl_2$, 0.150 mM $MgSO_4$) to reach the indicated concentrations in a final DMSO concentration of 0.05%. Controls were incubated in DMSO (0.05%) in the same buffer without inhibitors. Previously, different concentrations of DMSO and inhibitors were tested in order to avoid a negative effect on plant growth (number of leaves and fresh plant weight were routinely measured). Inhibitor concentrations were adjusted to be in the range described for plants (Desikan *et al*., 2001; Samaj *et al.*, 2002), that was higher than that used for animal tissues (Ward *et al*., 2000). The time of root pretreatment with inhibitors was tested at 1 h and 8 h. Although 1 h of incubation had already affected the number and weight of nodules, the values were more significant at 8 h.

Preparation of enzyme extracts

Using a mortar and pestle, frozen samples of roots and nodules were homogenized in extraction buffer [30 mM HEPES pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 10 mM glycerophosphate, 0.2 mM Na_3VO_4, 3 mM $MgCl_2$, 1 mM dithiothreitol (DTT), protease inhibitors (PMSF 10 μg ml^{-1}, leupeptin 10 μg ml^{-1}, pepstatin 5 μg ml^{-1}, E-64 10 μM)] and PVPP (polyvinylpolypyrrolidone; 30% w/w). The homogenate was centrifuged twice at 12 100 *g* for 10 min and the cleared supernatant was used as a crude extract.

Immunoblotting and immunoprecipitation

The polyclonal antibodies, M23, M24, H140, and H141, raised against the alfalfa MAP kinases, SIMK, SAMK, MMK2, and MMK3, respectively, have been used to detect the corresponding MAPK in roots and nodules of lupin. These antibodies were produced against synthetic peptides, encoding the C-terminal amino acids FNPEYQQ of SIMK (Jonak *et al*., 1996), LNPEYA of SAMK (Bögre *et al*., 1999), VRFNPDPPNIN of MMK2 (Munnik *et al*., 1999), and LNFCKEQILE of MMK3 (Jonak *et al*., 1995), and purified by protein A column chromatography. The specificity of the antibodies was tested by immunoblotting glutathione-*S*-transferase–MAPK fusion proteins that were prepared as described previously (Jonak *et al*., 1996). These antibodies cross-react with MAPKs from several plant species (Ligterink *et al*., 1997; Link *et al*., 2002).

Crude extracts from non-inoculated, inoculated lupin roots, young, and mature nodules were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), loading 30 μg protein per well in a 12% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (0.45 μm; Bio-Rad, Trans-blot transfer medium), for 2 h at 25 V, using a Semidry Transfer Cell from Bio-Rad, as described in Fernandez-Pascual *et al*. (1996). The blots were incubated for 1 h in blocking solution (5% defatted milk powder in sterilized PBS–0.05% Tween 20) followed by overnight incubation with the antibodies diluted in the blocking solution. Antibody dilutions were 1:5000 for M23 and M24 and 1:2000 for H141 and H142. Primary antibody was removed by washing five times in sterilized PBS–0.05% Tween 20. The blots were then placed for 2 h in the secondary antibody (whole molecule peroxidase conjugated anti-rabbit IgG, Sigma) diluted 1:5000 in blocking solution. Secondary antibody was removed again by washing in PBS–Tween. Protein detection was performed using an enhanced chemiluminescence system (Amersham, ECL kit, RPN 2106) followed exposure to an X-ray film (Kodak X-OMAT).

Crude extracts containing 100 μg of total protein were immunoprecipitated with 5 μg protein A-purified alfalfa antibodies in immunoprecipitation buffer (10 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 10 mM glycerophosphate, 1 mM NaF, 0.5% Nonidet P-40, 1% Triton X-100, 10 μg ml^{-1} each of PMSF and leupeptin, 5 μg ml^{-1} pepstatin, 1 mM DTT) at 4 °C for 1 h. Protein A–agarose (30 μl of 50%, Sigma) was added to the mixture and gently shaken for 1 h. The immunoprecipitate was washed twice with immunoprecipitation buffer and once with kinase buffer (20 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT).

In vitro *kinase activity assays*

Protein kinase activity was measured in the immunoprecipitate by phosphorylation of myelin basic protein (MBP) as substrate. Kinase reaction of the immunoprecipitated proteins was performed in 100 μl of kinase buffer containing okadaic acid (25 nM), MBP (0.2 mg ml^{-1}), 20 μM ATP, and 1 μCi of [γ-^{32}P]ATP for 30 min at 25 °C. The reactions were stopped by the addition of Laemmli sample buffer and, after centrifugation, electrophoresed on 10% SDS-PAGE. The dried gel was subjected to autoradiography and MBP kinase activity was quantified by scanning densitometry.

Activation of MAP kinases upon inoculation

To test whether the functional MAPKs detected in lupin tissues could be activated by the inoculation of the rhizobia bacteria, 4-d-old seedlings of lupin were incubated for 5–60 min with *B.* sp. (*Lupinus*), the specific nodulating bacteria of lupin, dead cells of *B.* sp. (*Lupinus*), and *Sinorhizobium meliloti*, which does not nodulate lupin. Uninoculated roots treated with Vincent growth medium were used as controls. The roots were homogenized and immunoprecipitated with M23 and M24, antibodies specifically recognizing alfalfa SIMK and SAMK, and assayed by phosphorylation of the substrate MBP. The kinase activities were analysed by *in vitro* phosphorylation and SDS-PAGE analysis.

Results

Detection of MAPKs in lupin nodules by immunoblotting

The antibodies M23, M24, H140, and H141 that recognize the constitutively expressed MAP kinases, SIMK, SAMK,

MMK2, and MMK3, respectively, in alfalfa have been used to detect MAPKs in lupin nodules. It has been shown that these antibodies cross-react with MAPKs from parsley (Ligterink *et al*., 1997) and tomato (Link *et al*., 2002) cells.

Immunoblotting of protein extracts of different *L. albus* tissues with the alfalfa MAPK antibodies M23, M24, H140, and H141 indicated cross-reaction with lupin MAPKs (Fig. 2). SIMK, a protein of 46 kDa involved in general hyper-osmotic responses (Munnik *et al*., 1999), SAMK (44 kDa), induced by different forms of stress and activated rapidly and transiently by wounding (Bögre *et al*., 1997), and MMK2 (44 kDa), involved in cell growth, were detected in both roots and nodules of lupin (Fig. 2A–C).

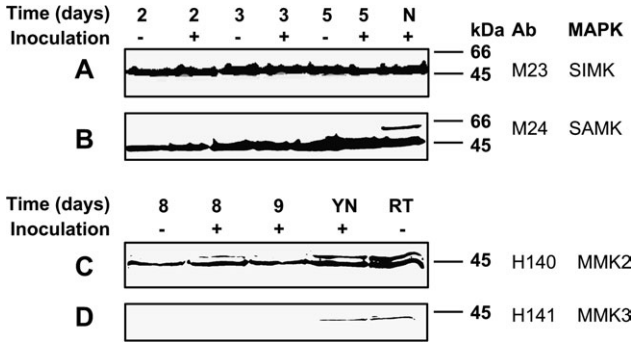

Fig. 2. Immunodetection of MAPKs in lupin plant organs. SDS-PAGE and western blot of protein extracts (35 μg) from uninoculated roots (–), inoculated roots (+), young and mature nodules, and root tips probed with alfalfa MAPK antibodies (A, M23; B, M24; C, H140; D, H141). Detection was performed by ECL. Numbers indicate days after inoculation. YN, nodule (10–15-d-old); N, nodule (20-d-old); RT, root tip.

MMK3, a 44 kDa kinase activated in late mitosis and assumed to participate in cell division (Bögre *et al*., 1999), was detectable in the root apical tissues and in young nodules (Fig. 2D).

Activation of MAP kinases upon inoculation

Figure 3A and B shows the time-course of activation of SIMKs and SAMKs in roots after inoculation with living and dead *B.* sp. (*Lupinus*) and *S. meliloti*. Inoculation with living *B.* sp. (*Lupinus*) was able to activate SIMK and SAMK. Both MAPKs showed similar activation profiles. They were rapidly and transiently activated after 5 min of incubation with *B.* sp. (*Lupinus*) reaching a peak, SIMK at 10 min and SAMK at 20 min, and decreasing thereafter. No differences were observed between incubation with dead *Bradyrhizobium* and Vincent growth medium, used as a control. However, incubation with *S. meliloti* decreased phosphorylation to basal levels. The amount of SIMK and SAMK proteins was constant among the samples (Fig. 3C, D).

To identify the site of MAPK activation in the seedling roots, after treatment the roots were divided into three segments, corresponding to the mature zone, the medium zone with young hairs, and the root tip. In the same way, root tips were subdivided into a zone with emerging hairs, an elongation zone, and the zone of the apical meristem plus the root cap (Fig. 1A). Table 1 shows SIMK and SAMK kinase activities in the different regions of the roots after incubation with *B.* sp. (*Lupinus*). Compared with controls in inoculated roots, phosphorylation increased gradually from the mature region to the tip. SIMK activity was nearly 5-fold

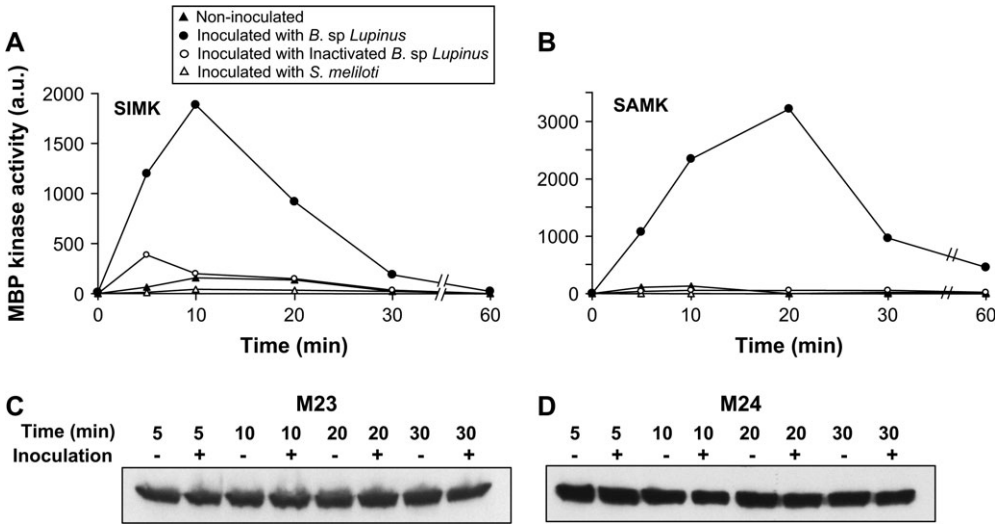

Fig. 3. Activation of SIMK and SAMK in primary lupin roots inoculated with rhizobia. Four day-old seedling roots were incubated for the indicated periods of time with *B.* sp (*Lupinus*), inactivated *B.* sp (*Lupinus*), *S. meliloti*, and with growth medium as controls. Root extracts (100 μg of protein) were immunoprecipitated with 5 μg of antibody M23 (A) or M24 (B). Kinase reactions were performed with MBP as a substrate, and 20 μM [γ-^{32}P]ATP in a final volume of 0.1 ml as described in Materials and methods. An aliquot of the reaction mixture (20 μl) was fractionated by SDS-PAGE and the phosphorylation of MBP was analysed by autoradiography and the kinase activities were quantified by scanning densitometry. (C, D) Western blot analysis of SIMK and SAMK with M23 and M24 antibodies, respectively, in lupin roots inoculated with *B.* sp (*Lupinus*). Results show the mean of two experiments.

Table 1. *Distribution of SIMK and SAMK activities in the main root of lupin after inoculation with* B. *sp. (*Lupinus*)*

Four-day-old seedling roots incubated for 10 min with rhizobia and controls incubated with growth medium, were divided in three segments corresponding to mature zone, medium zone with young hairs, and root tip (Fig. 1A). Likewise, root tips were subdivided into a zone with emerging hairs, elongation zone, and a meristematic zone plus cap. Protein extracts of samples were immunoprecipitated (I.P.) with M23 and M24 antibodies and then the kinase reaction performed. After SDS-PAGE and autoradiography, the kinase activities were quantified by densitometry. Results show the mean ±standard deviation of three experiments.

Zones of main root	MBP kinase activity (arbitrary units)			
	SIMK (I.P. M23)		SAMK (I.P. M24)	
	Control	Inoculated	Control	Inoculated
Mature	348±11	994±54	326±7	1451±72
Medium with young hairs	365±33	1173±118	340±40	1501±134
Tip	322±24	1590±141	339±31	2177±96
Zone with emerging hairs	87±10	747±55	105±7	1023±104
Elongation zone	126±71	557±24	88±2	784±91
Apical meristem and cap	109±11	286±14	156±52	370±5

and SAMK more than 6-fold higher in the tip of inoculated roots than in controls. In the three regions of the tip, the distribution of SIMK and SAMK activity was similar, showing that the zone of cells with emerging hairs has most kinase activities, and the apical meristem including the cap the least (47% and 18% for SIMK, and 47% and 17% for SAMK, of the total tip activity). It is worth mentioning that maximal activation coincides with the legume infection zone (Bhuvaneswari *et al*., 1981).

The effect *in vitro* of several specific inhibitors of MAPK, PD 98059, SB 202190, and SB 203580, were examined (Davies *et al*., 2000). PD 98059 blocks the activity of MAPKK and has been used as an inhibitor of plant MAPKs (Desikan *et al*., 2001). SB 202190 and SB 203580 are pyridinyl imidazole derivatives that act as potent inhibitors of the mammalian p38 MAPK, which is activated by both bacterial lipopolysaccharides and hyperosmolarity (Ward *et al*., 2000). Before the kinase reaction started, the immunoprecipitated MAPKs were incubated for 15 min with the inhibitor (2 μM) using DMSO as a control and the inactive analogue SB 202474 as a negative control. Figure 4 shows the effect of the inhibitors on the SIMK activity of roots at 10 min after incubation with *B*. sp. (*Lupinus*). PD 98059 did not inhibit SIMK activity. However, SB 202190 and SB 203580 inhibited its activity by 78% and 58%, respectively. Similar results were obtained on SAMK activity (data not shown).

Effect of MAPK inhibitors on nodulation

The mammalian MAPK inhibitors, which had been shown to be effective inhibitors of SIMK and SAMK activities *in vitro*, were examined to find out if they could also affect nodulation in the lupin root. Figure 5 shows the pattern of nodulation and the effect of the pretreatment of

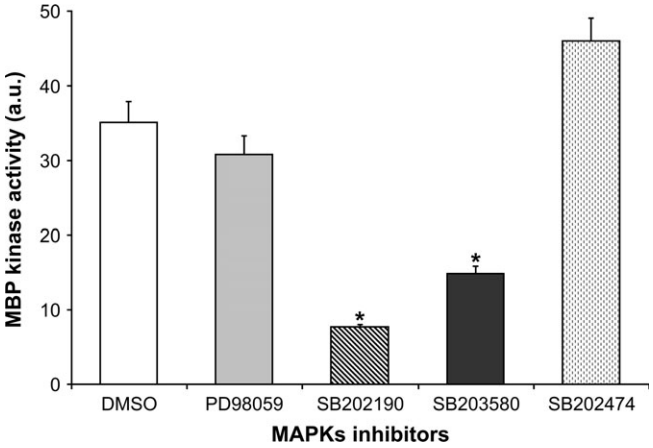

Fig. 4. Effect of MAPK inhibitors on SIMK activity. Protein extracts of primary roots inoculated for 10 min with *B*. sp. (*Lupinus*) were immunoprecipitated with M23 and were pretreated with the inhibitors PD 98059, SB 202190, SB 203580, and SB 202474, at a 2 μM concentration for 15 min before kinase reactions were started. Controls received the same concentration of DMSO as the samples incubated with the inhibitors. Kinase reaction assays were performed as described in Fig. 2. Results show the mean ±standard deviation of three experiments. Treatments with an asterisk differ significantly from the control at $P < 0.05$.

root seedlings with SB 202190 and its negative control SB 202474 (40 and 100 μM) (A, C) and UO 126 and the corresponding negative control UO 124 (1 and 10 μM) (B, D). The compound UO 126 is a MAPKK inhibitor (Favata *et al*., 1998) that has been used in studies on cytoskeleton organization and SIMK distribution in plant root hairs (Samaj *et al*., 2002). To distinguish between the effect on the early and late nodulation (when the inhibitor was probably already metabolized), nodules located in zone 1 (Fig. 1B) were separated from later formed nodules located in zone 2. The treatment 100 μM SB 202190 significantly decreased the number and fresh weight of nodules located in zone 1 of the main root by 54% and 68% (least significant difference at $P < 0.05$), respectively, while the inhibitor tended to increase the number and weight of nodules located in zone 2. Although the total nodule number was not altered, the total fresh weight decreased significantly by 58% ($P < 0.05$) with 100 μM of SB 202190. Changes in the nodule ultrastructure (data not shown) were not observed and the reduced weight was due to a delay in nodulation. The first nodules formed on the main root were located further from the base of the root in the treated plants than in the controls, 3.5±0.09 cm in the control plants and 3.78±0.20 and 4.18±0.38 cm in the plants treated with 40 and 100 μM of SB, respectively (the values are the means ±standard deviation of three independent experiments).

UO 126, used at 1 and 10 μM, affected nodulation in a similar way to SB 202190, although the potency was 100-fold higher (Fig. 5C, D). Treatment of seedlings for 8 h with 1 μM was already sufficient to decrease

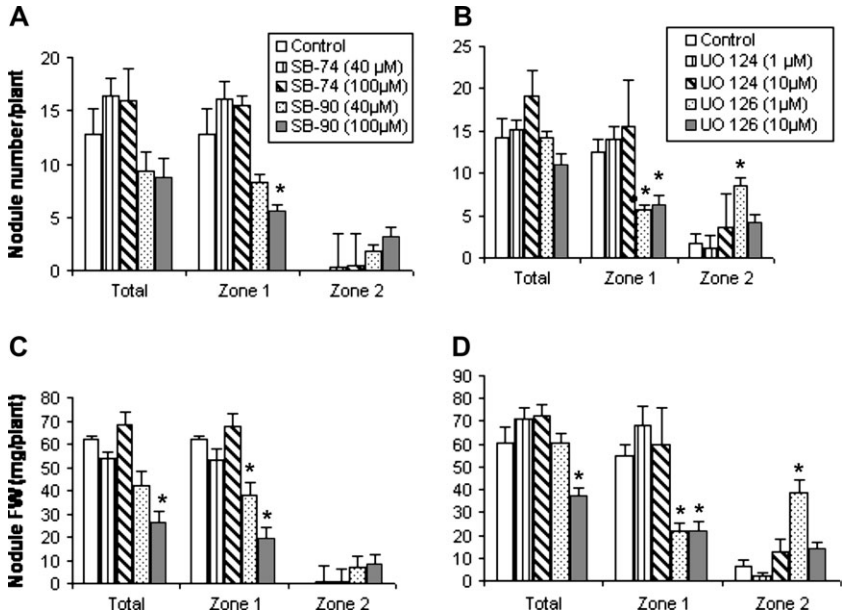

Fig. 5. Effect of the MAPK inhibitors on lupin nodulation. Three-day-old seedling roots were incubated, before inoculation with *B. sp*. (*Lupinus*), with the inhibitors SB 202190 HCl (40 μM and 100 μM; A, C) and UO 126 (1 μM and 10 μM; B, D) and their respective negative controls (SB 202474 and UO 124) dissolved in DMSO and diluted with buffer (Materials and methods) to the desired concentration. Incubations were performed for 8 h at 28 °C in darkness. Controls were incubated in 0.05% DMSO. After 16 d of growth in Leonard jars, 10–15 plants with the main root intact, were sampled and nodules located up to 5.5 cm from the root base (zone 1) were separated from nodules at a longer distance (zone 2). Nodule number (A, B), nodule fresh weight (C, D), and distance from the base of the main root to nodules were measured. Results from a representative experiment are shown; replicate experiments yielded similar results. Data represent mean ±standard error. Values were analysed by the least significant difference test. Treatments with an asterisk differ significantly from controls at $P < 0.05$.

significantly ($P < 0.05$) the number and weight of nodules in zone 1 of the main root by 55% and 60%, respectively, compared with the control. Contrarily, the number and weight of nodules in zone 2 increased significantly ($P < 0.05$) five and six times, respectively, compared with controls. The total number and weight of nodules were not affected. Higher concentrations did not increase the inhibitory effect on nodulation, 10 μM of UO 126 decreased the number and weight of nodules in zone 1 by 49% and 60%, respectively ($P < 0.05$). However, the increase in number and weight of nodules of zone 2 was not significant. The location of nodules on the main root also appeared, as in the case of SB 202190, at a longer distance from the base of the root in treated versus control plants (3.02±0.34 cm in the control and 3.38±0.22 and 3.31±0.26 in the treated plants). No such effects were found using the inactive analogues SB 202474 and UO 124. Taken together, these data indicate a delay in nodule formation as a result of MAPK inhibition in some early step of infection.

Discussion

The mitogen-activated protein kinases, SIMK and SAMK, can be activated by a number of diverse stimuli. SIMK is activated by high salt concentrations (Munnik *et al*., 1999) and SAMK by cold, drought, and wounding (Bögre *et al*., 1997). Cardinale *et al*. (2002) have shown that both MAPKs are activated by yeast cell wall-derived elicitors and observed that a given elicitor can activate several distinct MAPKs. In this work, evidence for the activation of both SIMK and SAMK in the roots of *L. albus* after infection by *B.* sp. (*Lupinus*), the symbiotic bacteria which form root nodules in lupin, is provided.

The results obtained by immunoblotting show the presence in lupin of the protein kinases SIMK and SAMK. Inoculation of seedling roots of lupin with *B.* sp. (*Lupinus*) activated both SIMK and SAMK transiently and rapidly 5 min after inoculation. Neither incubation with the dead bacteria of *B*. sp. (*Lupinus*) nor with a non-compatible bacterium like *S. meliloti* was able to induce activation of SIMK and SAMK. These data reveal that activation of these MAPK pathways is a specific response of the host cells to bacteria that may lead to a successful symbiotic interaction, suggesting that MAPKs may take part in the recognition of compatible partners. In connection with this, the expression of effector proteins has been described in bacterial pathogens of animals and plants (Orth, 2002), as well as in the plant symbiont *Rhizobium* (Bartsev *et al*., 2003; Ausmees *et al*., 2004), that may function to block a MAPK cascade, so that host signalling responses can be modulated upon infection. Several reports referring to the resemblance between *Rhizobium*–legume infection and pathogenic organism–plant interactions have been published (Long and Staskawicz, 1993; Baron and Zambryski, 1995). It has been suggested that for the early events

(seconds, minutes) one general defence signal transduction chain exists (Carden and Felle, 2003). The MAPK cascade might be a part of this transduction system involved in plant–pathogen infection as well as in plant–*Rhizobium* interaction.

Samaj *et al*. (2002) have implicated SIMK in root hair tip growth in relation to the dynamics of the actin cytoskeleton. This is of great interest in legume infection. In this regard, it has been found that the maximal enhancement of MBP phosphorylation by SIMK and SAMK root immunoprecipitates of seedlings after 10 min inoculation with *B.* sp. (*Lupinus*), takes place in the region of the tip with emerging hairs or preceding hair emergence. Although lupin symbiosis does not follow the conventional patterns of infection of other legumes (curled root hairs and infection threads are only occasionally visible), lupin infection leading to nodulation has been described as occurring in the area of epidermal cells which either lack root hairs or have very young root hairs at the time of inoculation (Tang *et al*., 1992). Lupin root hairs are surface colonized by the bacteria that penetrate at the junction between the root hair base and an adjacent epidermal cell (Gonzalez-Sama *et al*., 2004). The present data indicate that the distribution of MBP phosphorylation is correlated with the sites of infection in the legume root. SIMK and SAMK activation could mediate the early events of rhizobial infection in response to a bacterial signal molecule.

The inhibition of SIMK and SAMK by the specific inhibitors, MAPK inhibitors SB 202190 and SB 203580, showed that these two lupin root MAPKs are functional enzymes that are activated by inoculation and suggest that MAPKs might mediate the infection processes by rhizobia. To investigate whether the effect of mammalian MAPK inhibitors observed *in vitro* could affect the physiological process of nodulation of lupin roots, germinated seedlings were pretreated with SB 202190, a MAPK inhibitor, and UO 126, a MAPKK inhibitor before inoculation with *B.* sp. (*Lupinus*). The three main effects produced by the MAPK inhibitors on the pattern of nodulation were: (i) a decrease in the number and weight of nodules in the upper zone of the main root (zone 1), (ii) an increase in the nodule number and nodule weight in younger sites of the main root (zone 2); and (iii) a larger distance from the base of the main root to the location of nodules, indicating a link to the process of autoregulation of nodulation. It is known that the plant controls the location and spacing of nodules within a defined zone along the root (Schultze and Kondorosi, 1998). Nodule number in legumes is autoregulated and the nodules already existing inhibit the formation of further ones (Caetano-Anolles and Gresshof, 1991; van Brussel *et al*., 2002). The present data suggest that inhibition of MAPKs blocks some early events in the susceptible root zone to rhizobial infection, delaying nodulation. The capability of host cells to be infected is a transient property and infection might be restored when the inhibitor is metabolized and new root cells have grown. Overall, the present results indicate a role for MAPKs in the infection and nodulation of *L. albus* by *B.* sp. (*Lupinus*) and further studies are warranted to clarify the roles of the various MAPK isoforms in plant–rhizobium symbiosis.

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