Activation of a putative MAP kinase in pollen is stimulated by the self-incompatibility (SI) response

Jason J. Rudd[1], Kim Osman, F. Christopher H. Franklin, Vernonica E. Franklin-Tong*

School of Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Received 30 May 2003; accepted 4 June 2003

First published online 25 June 2003

Edited by Ulf-Ingo Flügge

Abstract Mitogen-activated protein kinases (MAPKs) operate downstream of receptor–ligand interactions, playing a pivotal role in responses to extracellular signals. The self-incompatibility (SI) response in *Papaver rhoeas* L. triggers a Ca^{2+}-dependent signalling cascade resulting in inhibition of incompatible pollen. We have investigated the possible involvement of MAPKs in SI. We report the enhanced activation of a 56 kDa protein kinase (p56) in SI-induced pollen and provide evidence that p56 has MAPK activity. This provides an important advance in our understanding of the SI response. We believe this is the first direct biochemical demonstration of activation of a MAPK during SI.

Key words: Self-incompatibility; Protein phosphorylation; Mitogen-activated protein kinase; Pollen

1. Introduction

In flowering plants, self-incompatibility (SI), which is encoded by the *S*-locus, is the single most important mechanism employed to prevent self-pollination. In *Papaver rhoeas* the stigmatic *S*-locus components are S proteins [1–3] which act as *S*-specific signalling ligands [4]. They are proposed to interact with the pollen *S*-locus receptor, triggering a signalling cascade in incompatible pollen, resulting in the rapid arrest of pollen tube growth. The SI response triggers a transient increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) [5,6], which involves influx of extracellular Ca^{2+} [7]. SI also stimulates rapid reorganisation of the F-actin cytoskeleton and F-actin depolymerisation [8,9]. These rapid events are probably directly involved in pollen tube tip growth inhibition. However, there is also evidence of SI-specific events subsequent to inhibition, with SI appearing to trigger a programmed cell death (PCD) signalling pathway [10,11].

Alterations to the phosphorylation state of proteins via activation of protein kinases is one of the most important molecular mechanisms whereby cells respond to extracellular signals. Evidence implicates roles for protein kinases in pollen tube growth [12,13] and SI in *Brassica* [14] and *Papaver* [15,16]. Mitogen-activated protein kinases (MAPKs) are activated by phosphorylation of a conserved TXY motif, and have been well characterised in yeast and mammals [17]. In plants, there is good evidence for MAPKs being involved in stress responses [18,19]. We have investigated a possible role for MAPKs in the SI response, and report the specific activation of a putative MAPK in pollen of *P. rhoeas* undergoing the SI response. This provides a significant advance in our knowledge of signalling components triggered by the SI response.

2. Materials and methods

2.1. Plant material and pollen treatments

Pollen from plants of *P. rhoeas* was stored at −20°C. Pollen was hydrated and grown as previously described in germination medium (GM) [15] for 1 h prior to challenge with S proteins or drugs. Recombinant stigmatic S proteins (25 μg/ml) [20] were added to pollen grown in vitro for 1 h. For La^{3+}-treated samples, 500 μM La^{3+} was added 30 min prior to S protein challenge. For mastoparan-treated pollen, 25 μM mastoparan was added to pollen grown in vitro for 1 h. Control samples comprised addition of GM. Addition of S proteins or drugs was taken as time = 0. Three interactions were used to demonstrate S specificity: an incompatible reaction using S_1e and S_8e recombinant proteins with S_1S_8 pollen; a compatible interaction between the same S proteins and S_4S_5 pollen; heat-denatured (80°C, 1 h) S_1e and S_8e with S_1S_8 pollen. Pollen tubes were examined using a Nikon Eclipse T300 microscope.

Pollen samples were 'stopped' by adding either 5× sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer [21] containing 5 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate or 5× immunoprecipitation (IP) buffer (100 mM Tris–HCl pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 5 mM Na_3VO_4, 25 mM β-glycerophosphate, 2.5% (v/v) NP-40 and 5% (v/v) protease inhibitor cocktail (Sigma)) and snap-freezing in liquid N_2. For in vivo pollinations, pollen was brushed onto mature, emasculated stigmas and left for between 0 and 90 min. Stigmas were dipped into 5× sample buffer, the pollen-covered stigmatic papillae scraped off and snap-frozen in liquid N_2. Controls comprised unpollinated stigma scrapings. Extracts from samples were obtained by sonication (30 s, 10 Hz) and centrifugation (6000 rpm, 5 min, 4°C). Supernatants were used for myelin basic protein (MBP) in-gel protein kinase assays and for immunoblotting.

2.2. MBP in-gel MAPK assay

In-gel MBP kinase assays were performed essentially as described in [22]. Pollen extracts containing 20 μg protein per lane were separated on SDS–PAGE gels containing 0.5 mg/ml MBP (Sigma, UK). The upper reservoir running buffer contained MBP (0.1 mg/ml). Duplicate

*Corresponding author. Fax: (44)-121-414 5925.
E-mail address: v.e.franklin-tong@bham.ac.uk (V.E. Franklin-Tong).

[1] Present address: Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle/Saale, Germany.

Abbreviations: SI, self-incompatibility; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; $[Ca^{2+}]_i$, cytosolic free Calcium; PCD, programmed cell death; IP, immunoprecipitation

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00710-5

gels without MBP assessed autophosphorylation. Gels were washed in 50 mM Tris–HCl pH 8 containing 20% v/v isopropanol (30 min), incubated in buffer A (50 mM Tris–HCl pH 8, 5 mM β-mercaptoethanol) for 1 h, transferred to buffer A containing 6 M guanidine hydrochloride for 1 h, and then incubated in buffer A containing 0.04% Tween 40 for >16 h at 4°C. Protein kinase activity was assessed by incubating the gel in 50 mM HEPES pH 7.6, 15 mM $MgCl_2$, 0.1 mM EGTA, 2 mM dithiothreitol, for 1 h at 20°C in the presence of 150 μCi [^{32}P]γATP (NEN, UK). Unincorporated [^{32}P]γATP was removed by washing with 5% trichloroacetic acid, 1% sodium pyrophosphate. Gels were dried and autoradiographed. Phosphorimaging (Molecular Dynamics) was used for quantitation of protein kinase activities.

For treatments including the kinase inhibitor, apigenin, the gels were incubated in the presence or absence of 100 μM apigenin during the ^{32}P-labelling kinase step. The constitutively active catalytic subunit of mammalian protein kinase A (PKA) acted as a control for inhibitor specificity.

2.3. Immunoprecipitation with anti-phosphotyrosine antiserum

Four per cent (v/v) anti-phosphotyrosine antiserum (mouse monoclonal PY20, Transduction Labs) was added to pollen protein extracts (100 μg soluble protein). Samples were incubated (90 min, 4°C) with agitation, 35 μl of protein A-agarose was added and incubation continued for a further 1 h, 4°C. The resultant immunoprecipitates were collected by centrifugation and extensively washed in IP buffer, then resuspended by boiling in SDS–PAGE sample buffer and analysed using the MBP in-gel kinase assay.

2.4. Immunoblotting

Pollen extracts were separated using SDS–PAGE and blotted onto Hybond-C extra membrane. Membranes were blocked in Tris-buffered saline (TBS, 20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk powder, for 1–4 h at 20°C, then incubated with a phospho-MAPK-specific antibody, Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) antibody (Cell Signaling Technology) according to the manufacturer's instructions. After washing in TBS, blots were incubated with horseradish peroxidase-conjugated secondary antibody (Sigma; 1:10 000 dilution) for 1 h at 20°C, rewashed and visualised using ECL™ Detection Reagent (Amersham Pharmacia Biotech).

3. Results

3.1. Activation of a 56 kDa pollen MBP kinase during the SI response

In *P. rhoeas* pollen growing in vitro, a low level of a 56 kDa MBP kinase activity was identified, and named p56. This basal level of MAPK activity was not detected in dry pollen (data not shown) and its activity was substantially stimulated in SI-induced incompatible pollen (Fig. 1). This MBP kinase activity was independent of Ca^{2+} as it phosphorylated MBP in-gel in the presence of 0.1 mM EGTA. Comparison of p56 activity during normal pollen tube growth revealed that the level of p56 activity was not significantly different at $t = 0$ and $t = 60$ min ($P = 0.29$). At 5 min after an incompatible SI response p56 activity represented a 2.2–2.9-fold increase (Fig. 1a,d) over the activity detected at $t = 0$ ($n = 4$). By 10 min p56 activity had peaked (3.2–3.7-fold greater than its basal activity; Fig. 1d), and levels remained elevated for ~30 min. The controls demonstrate that the increase in p56 activity was specific to an incompatible SI response, since they displayed no appreciable increase in activity compared to the incompatible response (Figs. 1b–d). We also examined p56 activity in pollen from stigmas pollinated in vivo (data not shown). No detectable p56 activity was found in unpollinated stigmas. Five minutes after an incompatible pollination, a 2.4-fold increase over the level of p56 activity at $t = 0$ was detected, while the compatible pollination showed no detectable difference.

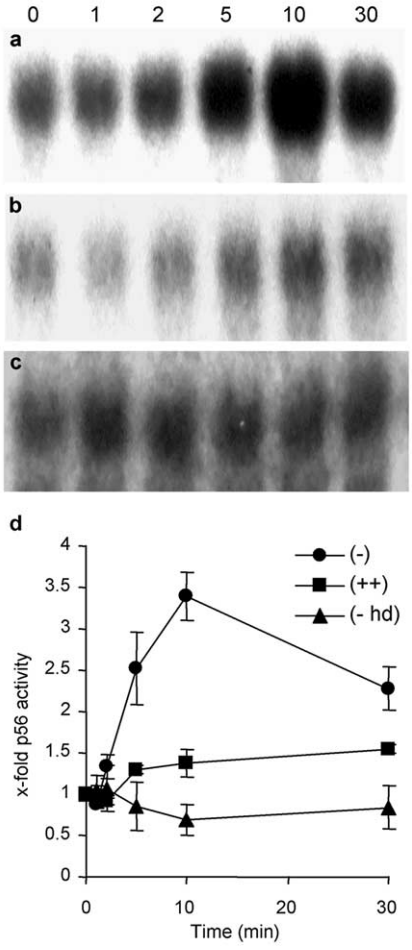

Fig. 1. Enhanced activation of p56 is stimulated by the SI response. MBP in-gel kinase assays were performed on pollen extracts undergoing incompatible (a), compatible (b), or heat-denatured incompatible (c) SI reactions. Times at which samples were taken are indicated above each lane. Increased activity of p56 was detected in the incompatible SI responses, peaking at 10 min. Compatible (++) and heat-denatured incompatible (−hd) controls did not show this activation. d: Plots of quantitation of relative mean p56 activity for each treatment ± S.D. ($n = 4$).

3.2. p56 activity is immunoprecipitated with anti-phosphotyrosine antibody

A useful way of identifying MAPKs is to use immunoprecipitation with an anti-phosphotyrosine antiserum, as a key diagnostic feature of MAPKs is their activation via phosphorylation on tyrosine and threonine residues. Fig. 2 shows that the activated p56 was immunoprecipitated from pollen extracts using anti-phosphotyrosine antiserum. Although there is a hint of p56 activity in the controls, this was expected from the basal levels of phosphorylation, and the MBP kinase activity immunoprecipitated from incompatible pollen was significantly greater (four- to seven-fold higher) than that in the controls. These data provide evidence that the activation of p56 during the SI response correlates with its phosphorylation on tyrosine residues.

3.3. p56 activity is detected by antibodies recognising activated MAPKs

A phospho-MAPK-specific antibody which recognises phosphorylated threonine (T) and tyrosine (Y) residues in

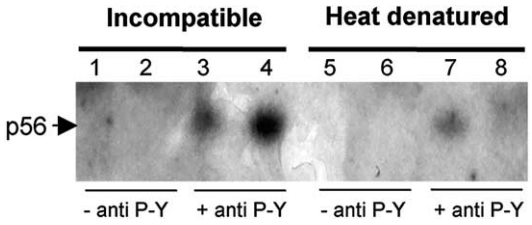

Fig. 2. p56 activity is immunoprecipitated with anti-phosphotyrosine antiserum. Immunoprecipitation assays were performed on pollen challenged for 10 min with either active or heat-denatured S proteins in duplicate. Anti-phosphotyrosine antiserum (+anti P-Y; lanes 3, 4, 7, 8) was used as the primary antibody, followed by protein A-agarose. The primary antiserum was left out of some assays to test for specificity (−anti P-Y; lanes 1, 2, 5, 6). Immunoprecipitates were analysed by the MBP in-gel protein kinase assay. The arrow indicates the position of activated p56, which is detectable in lanes 3 and 4 (incompatible challenge).

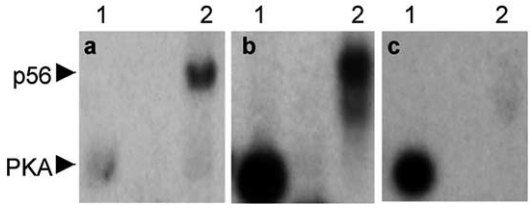

Fig. 4. Activated p56 is sensitive to the MAPK inhibitor apigenin. In-gel kinase assays were performed on extracts from 10 min. SI-induced pollen, containing activated p56 (lane 2 in each panel); the recombinant catalytic sub-unit of PKA (lane 1 in each panel) acted as a control. The phosphorylated p56 MAPK and PKA is indicated by the arrows. a: Protein kinase activity in the absence of MBP, indicating autophosphorylation. PKA is weakly phosphorylated, and p56 slightly more so. b: Protein kinase activity in the presence of MBP. Both p56 and PKA phosphorylate the MBP substrate. c: Protein kinase activity in the presence of MBP and 100 μM apigenin. p56 activity (lane 2) is abolished by the inclusion of apigenin, while PKA activity is not.

the MAPK TXY motif has been used in plant cells to identify activated MAPKs [23]. This antibody cross-reacted with a pollen protein of an identical size and activation profile to the p56 identified by the MBP in-gel kinase assays (Fig. 3). Pollen extracts from incompatible SI inductions exhibited this MAPK activity (Fig. 3a), while those from compatible combinations showed no increase above basal level (Fig. 3b). Although there is a hint of an increase at 30 min, this was detected in other treatments at 30 min (see also Figs. 1 and 5). This provides further evidence that p56 has MAPK activity.

3.4. Activated p56 is sensitive to the MAPK inhibitor apigenin

In mammalian cells, apigenin has been shown to act directly upon activated MAPKs [24] and has been used to provide evidence for MAPK activity in plant cells [25]. We therefore tested the ability of p56 to phosphorylate MBP in the presence of apigenin. Both the activated p56 protein kinase and the PKA control were autophosphorylated in the absence of MBP (Fig. 4a). With MBP included as a substrate, it was phosphorylated by both protein kinases (Fig. 4b). However, incubation with 100 μM apigenin inhibited MBP phosphorylation by p56 (Fig. 4c). Apigenin also has a dramatic inhibitory effect on the autophosphorylation of p56 (compare Fig. 4c with 4a). The susceptibility of the p56 activity to inhibition by apigenin provides support for it being a MAPK.

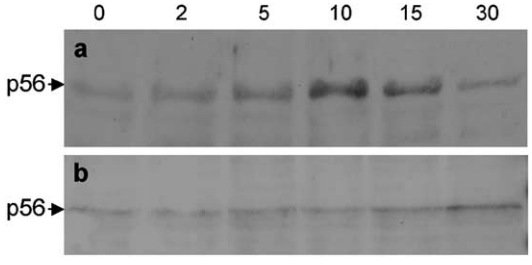

Fig. 3. Cross-reactivity with TEY antibody confirms a 56 kDa MAPK activity in incompatible pollen. Pollen extracts undergoing (a) incompatible and (b) compatible SI reactions were sampled at the time intervals indicated (min) and immunoblotted with Phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) antibody, which detects activated MAPKs. A 56 kDa protein exhibits increased activation of a MAPK activity during an incompatible SI response, peaking at 10 min after challenge.

3.5. p56 activity is stimulated by mastoparan

As the key SI-induced signal in pollen is an increase in $[Ca^{2+}]_i$ [5,6], we tested the involvement of Ca^{2+} signalling in the activation of p56, using mastoparan [26], which also induces many of the SI markers [9,10]. This treatment activated the p56 activity above basal levels within 1 min, reaching a plateau by 2 min (Fig. 5) (~2.1- and 2.6-fold above that observed at $t=0$; $n=3$) and was maintained for at least 30 min. These data imply increases in $[Ca^{2+}]_i$ upstream of p56 enhance its activation.

3.6. p56 activity requires influx of extracellular Ca^{2+}

The SI response in *Papaver* pollen triggers increases in $[Ca^{2+}]_i$ [4–6]. This involves Ca^{2+} influx, and 500 μM La^{3+} will completely inhibit the SI-induced influx [7]. To establish a link between the SI response and the enhanced p56 activation, we examined whether La^{3+} blocks a signalling cascade upstream of the p56 activity by pre-treating pollen with 500 μM La^{3+} and then treating with S proteins for 10 min. The level of p56 activity in these pollen tubes was less than

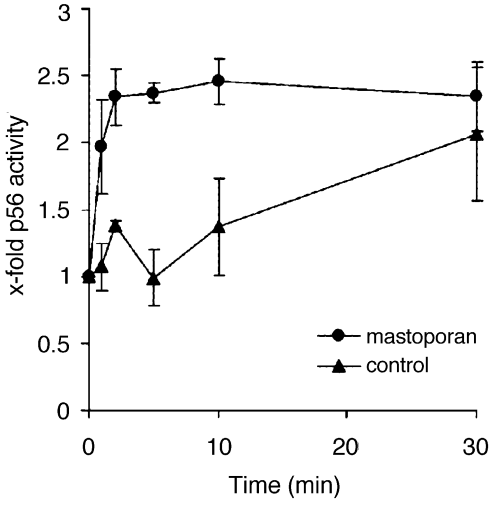

Fig. 5. p56 activity is stimulated by mastoparan (25 μM). The plot shows mean p56 activity for three independent experiments ± S.D. ($n=3$). Treatment of pollen tubes with mastoparan induced a rapid (< 1 min) and sustained activation of p56.

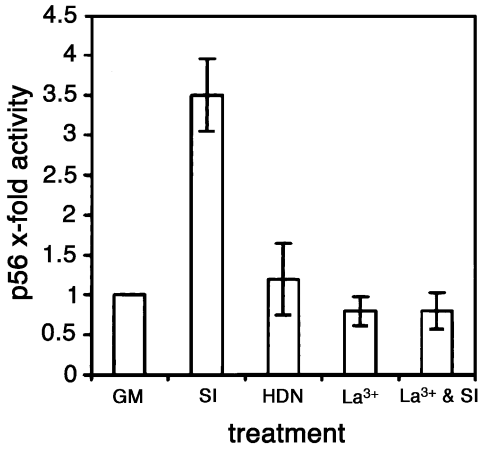

Fig. 6. Lanthanum blocks p56 activity. p56 activity at 10 min after S protein addition is shown. 500 μM La^{3+}, which inhibits Ca^{2+} influx, completely inhibits the stimulation of p56 activity. There is approximately the same level of p56 activity in pollen tubes pretreated with La^{3+} and subsequently treated with S proteins to induce SI, as in unstimulated pollen (GM).

that of the untreated controls (Fig. 6; 0.8-fold, S.E.M. ±0.23, $n=3$). In comparison, the level of p56 activity in samples in which S proteins were added in the absence of La^{3+} was 3.5-fold (S.E.M. ±0.45, $n=3$) and in those treated with heat-denatured S proteins 1.2-fold (S.E.M. ±0.45, $n=3$). These data demonstrate that La^{3+} inhibits the SI-stimulated p56 activity and suggest that Ca^{2+} influx is required upstream of p56 MAPK activation. This provides a link between SI induction and p56 activation and lends further support to the idea that the p56 MAPK is activated downstream of a Ca^{2+}-mediated signal. Our observations concur with data that link increases in $[Ca^{2+}]_i$ and Ca^{2+} influx with the subsequent activation of MAPKs in animal [27] and plant cells [25,28–30].

4. Discussion

4.1. SI-specific enhanced activation of a pollen MBP kinase

Protein phosphorylation is one of the most important molecular mechanisms whereby biological responses in cells are produced as a consequence of extracellular signals. We have identified a 56 kDa protein kinase activity, named p56, which exhibits a basal level of MBP kinase activity in growing pollen tubes and is preferentially increased in incompatible pollen undergoing the SI response in *P. rhoeas*. Our data provide convincing evidence that the enhanced activation of p56 is stimulated by the SI response.

Members of the MAPK family exhibit several characteristic biochemical features. Here we have provided evidence that p56 exhibits some of the key characteristics of plant MAPKs. These include the ability to phosphorylate MBP in the absence of Ca^{2+} ions, and evidence that activated p56 is phosphorylated on a tyrosine residue(s) in vivo. The cross-reactivity of a phospho-MAPK-specific antibody with p56 lends considerable support to the idea that p56 is a MAPK. Furthermore, the ability of apigenin to inhibit both the autophosphorylation and MBP phosphorylation of the p56 protein kinase supports this. Together, the data presented provide strong evidence that p56 represents a MAPK whose activity is stimulated by the SI response in incompatible pollen. Since a MAPK has not previously been implicated in modulating either SI or pollen tube growth, this represents a significant advance in our knowledge about the signalling cascades triggered during SI.

4.2. Is p56 activation involved in regulation of pollen tube tip growth?

As SI stimulates p56 activity, it suggests that enhanced activity of this MAPK signals to targets involved in the SI response. Since p56 SI stimulated enhanced activation peaks after inhibition of pollen tube growth, this suggests that its function is separate from signals leading to inhibition of tip growth. We believe it is probably involved in downstream events to ensure irreversible inhibition of pollen tube growth, as although pollen inhibition occurs within 1–2 min, incompatible pollen remains functionally viable for at least 40–60 min [9,10]. In support of this, several SI-specifically induced events are detected after tip growth has ceased [8–11]. It is likely that p56 may signal to one or more of these events to ensure that growth does not resume. Although links between MAPK activation and changes to the actin cytoskeleton are established in animal cells, evidence for this in plant cells is only just emerging [31,32]. However, a functional role for activation of MAPKs in the induction of PCD is better established. Not only have MAPKs been demonstrated to be activated during PCD, but activation of a salicylic acid-induced protein kinase is sufficient to induce hypersensitive response (HR)-like PCD in tobacco leaves [33–35]. Furthermore, activation of *Arabidopsis* MAPKs by two *Arabidopsis* MAPKKs results in HR-like cell death [36]. Future studies will investigate if these links exist in the SI system.

Although we do not know whether p56 has any physiological significance in normally growing pollen tubes, the basal levels of p56 kinase activity suggest that it may be required for pollen tube growth. This idea is substantiated by preliminary data that indicate that the MAPK inhibitor apigenin and MAPKK inhibitors PD98059 and U0126 inhibit *Papaver* pollen germination and tip growth (G. Wright and V.E. Franklin-Tong, unpublished data). Although data indicate that MAPK signalling cascades play a role in tip growth in yeast [37,38], it is only recently that evidence that MAPKs play a functional role in tip growth in plants has been obtained [31].

In conclusion, we have described the first direct biochemical demonstration of an enhanced activity of a MAPK in incompatible pollen during the SI-specific rejection response. Although at present we do not know the exact role played by the increase in p56 MAPK activity, it seems likely that it is involved in signalling to processes involved in modulation of pollen tube growth.

Acknowledgements: This work was funded by the Biotechnology and Biological Research Council.

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