Involvement of mitogen-activated protein kinase activation in the signal-transduction pathways of the soya bean oxidative burst

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The oxidative burst constitutes one of the most rapid defence responses characterized in the Plant Kingdom. We have observed that four distinct elicitors of the soya bean oxidative burst activate kinases of masses ≈ 44 kDa and ≈ 47 kDa. Evidence that these kinases regulate production of reactive oxygen species include: (i) their rapid activation by oxidative burst elicitors, (ii) their tight temporal correlation between activation/deactivation of the kinases and activation/deactivation of the oxidative burst, (iii) the identical pharmacological profile of kinase activation and oxidant production for 13 commonly used inhibitors, and (iv) the autologous activation of both kinases and oxidant production by calyculin A and cantharidin, two phosphatase inhibitors. Immunological and biochemical studies reveal that the activated 44 kDa and 47 kDa kinases are mitogen-activated protein (MAP) kinase family members. The kinases prefer myelin basic protein as a substrate, and they phosphorylate primarily on threonine residues. The kinases are themselves phosphorylated on tyrosine residues, and this phosphorylation is required for activity. Finally, both kinases are recognized by an antibody against activated MAP kinase immediately after (but not before) cell stimulation by elicitors. Based on these and other observations, a preliminary sequence of signalling steps linking elicitor stimulation, kinase activation and Ca^{2+} entry, to initiation of oxidant production, is proposed.

Key words: disease resistance in plants, MAP kinase signal transduction, oxidative burst in plants.

INTRODUCTION

The oxidative burst constitutes one of the most rapid responses of a plant cell to pathogen attack, reaching its highest magnitude within minutes of pathogen recognition. During this response, reactive oxygen species are generated, which in turn can promote lignification and cross-linking of the cell wall [1,2], transcription of defence response genes [3,4], the hypersensitive response [3], phytoalexin biosynthesis [5–7] and direct pathogen cytotoxicity [8,9]. In cultured cells, the oxidative burst can be induced by isolated elicitors derived from pathogenic fungi or bacteria, or from the host plant itself. The oxidative burst can also be stimulated abiotically by mechanical pressure or hypo-osmotic stress [10], the pesticide fenthion [11] or a variety of phosphatase inhibitors [3,12].

Because of its rapid onset, ease of assay and similarity to an analogous defence response in neutrophils [13,14], the oxidative burst has been exploited as a model system for studying signal-transduction pathways in plants. As the quantity of data has increased, two relevant conclusions have become apparent. First, the initial signalling intermediates induced by oxidative burst elicitors are frequently very different. Thus, the pathway initiated by oligogalacturonides appears to require G-proteins [15] and phospholipase C [16], but not phospholipase A [17], whereas an elicitor from the cell wall of *Verticillium dahliae* utilizes phospholipase A but not phospholipase C [16,17]. Even the initial sites of action may differ, as some elicitors appear to bind to sites on the plant cell surface [5,18–20] whereas an avrPto elicitor may have to penetrate the plant cell to interact with its receptor, Pto [21]. Secondly, despite the above differences in initial signalling events, most of the oxidative burst pathways probably converge to a common downstream pathway that involves both an influx of Ca^{2+} [22,23] and the activation of one or more kinases. Furthermore, a flavin-dependent oxidase that is sensitive to diphenylene iodonium appears to constitute the final component in many elicitor-stimulated burst pathways [23–26].

Evidence for kinase involvement in the oxidative burst has come from both radiolabelling and pharmacological studies. Elicitation of plant cells treated with [^{32}P]phosphate leads to labelling of a number of proteins [27–29], suggesting that kinases are somehow activated during the process. Assuming an analogy with signalling pathways of the neutrophil oxidative burst, these novel phosphoproteins could conceivably be other kinases, members of the oxidase complex itself, or proteins not involved in the burst. More compelling data for kinase participation have come from pharmacological studies, where serine/threonine kinase inhibitors, such as K-252a and staurosporine, block oxidative burst stimulation in a dose-dependent manner [3,27, 30,31]. Because addition of these same inhibitors also causes rapid termination of a previously initiated burst, it can be further suggested that continuous phosphorylation is essential for maintenance of burst activity. Interestingly, protein phosphatase inhibitors such as calyculin A, cantharidin and okadaic acid can autologously activate the burst, even in the absence of elicitors [12,32]. These results suggest that the kinases that induce the burst would normally maintain the oxidase in a constitutively active state if their substrates were not continuously dephosphorylated by a more dominant phosphatase. Taken together, a picture emerges that the on/off switch for the oxidative burst is likely to be controlled by a delicate balance between constitutive kinases and phosphatases.

Although there is significant evidence implicating kinases and phosphatases in signalling the oxidative burst, there remains substantial controversy regarding the identities of the specific

Abbreviations used: MAP, mitogen-activated protein; MBP, myelin basic protein; OGA, oligogalacturonic acid; SIPK, salicyllc acid-induced protein kinase; WIPK, wounding-induced protein kinase; MEK, MAP kinase/ERK kinase; TCA, trichloroacetic acid.

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enzymes involved. The first kinase demonstrated to participate in the oxidative burst was the resistance gene product Pto. Thus, tomato cell cultures transformed with Pto kinase displayed a prolonged two-phase production of the oxidative burst in response to bacteria expressing the *avrPto* avirulence gene, whereas control cells lacking the Pto kinase expressed only the transient first phase of the burst [11]. However, even the cells that lacked Pto kinase were able to generate substantial quantities of oxidants in response to non-race-specific elicitors, suggesting that the Pto kinase communicates the burst signal only when activated by *avrPto*, leaving the task to other kinases to respond to non-host-specific pathogens.

We have explored the signal-transduction pathways of several elicitors of the oxidative burst in soya bean suspension cell cultures. As mentioned above, the elicitors we have examined are believed to initiate the signalling cascade using distinct signalling components, even though they subsequently appear to converge to a common pathway. In this report, we examine whether a common mitogen-activated protein (MAP) kinase intermediate might be shared by the signalling pathways leading to the soya bean oxidative burst. We provide several lines of evidence that a pair of MAP kinases do indeed participate in signalling the oxidative burst. We also partially localize these kinases relative to previously identified signalling intermediates in the oxidative burst pathway.

EXPERIMENTAL

Materials

Anti-phosphotyrosine antibodies and antibodies conjugated to agarose beads were purchased from Santa Cruz Biotechnology, while anti-active MAP kinase (pTEpY) antibodies were from Promega. Myelin basic protein (MBP) was obtained from Gibco BRL or Sigma, while [γ-^{32}P]ATP was purchased from ICN. All other chemicals and supplies were from major providers.

Plant cell culture and oxidative burst assay

Cell suspension cultures of soya bean (*Glycine max* Merr., cv Kent) were maintained in W-38 medium as described previously [15]. Briefly, 6 cm^3 of filtered cells were transferred into100 ml of fresh W-38 medium every 10 days. All assays were conducted 16 h after transfer, when H_2O_2 generation was completely dependent upon elicitor addition, as described in [15]. The elicitors used were an oligogalacturonic acid (OGA) fraction with a degree of polymerization of $\approx$ 7–12 [33], a crude extract from the pathogenic fungus, *V. dahliae* [34], and direct 1:1 dilution with distilled water [10].

Protein sample preparation

For all of the following assays, unless otherwise specified, 1.5 ml of soya bean suspension cultured cells were transferred to a quartz cuvette and monitored for oxidative burst activity after elicitor and/or modulator addition. At the desired time points (as described in the Figure legends), the cells were filtered and frozen in liquid nitrogen and ground with a mortar and pestle. The finely ground powder was solubilized in 0.5 ml of 10 mM Tris (pH 7.5), 10 mM NaF, 20 μg/ml each of PMSF, 12.5 μg/ml leupeptin and 1 μg/ml pepstatin. The samples were centrifuged for 15 min at 15000 ***g*** and the supernatant was assayed for protein content by the bicinchoninic (BCA) method (Pierce).

In some studies a partially purified kinase fraction was used. A crude sample was prepared as described above, except 100 ml of cell culture was stimulated, filtered, and ground in liquid nitrogen. After centrifugation for 15 min at 15000 ***g***, the sample was loaded on to a 5 cm × 40 cm DEAE Fast Flow column (Pharmacia) that was pre-equilibrated with 10 mM Tris (pH 7.5), 10 mM NaF, and 20 μg/ml PMSF (buffer A). The column was washed with 125 mM NaCl in buffer A, then eluted with a gradient from 125 to 350 mM NaCl in buffer A. Fractions were collected and assayed for kinase activity, which eluted at around 225 mM NaCl.

In vitro kinase assay

For evaluation of kinase activation by various elicitors and for determination of the impact of various modulators on kinase activity, the following procedure was used. Protein fractions were prepared as described above, aliquoted to 2.5 μg of protein/sample, and brought to a total volume of 27 μl with the grinding buffer. To initiate the kinase reaction, 3 μl of a solution containing 1 mg/ml MBP, 100 mM $MgCl_2$, 100 μM ATP and 3.7×10^4 Bq/μl of [γ-^{32}]ATP (final concns. of 100 μg/ml, 10 μM, 10 μM ATP 3.7×10^3 Bq/μl, respectively) was added to each sample. The phosphorylation reaction was allowed to proceed for 30 min at 23 °C and was then stopped by addition of 30 μl of boiling 2 × SDS sample buffer containing 100 mM dithiothreitol. A 45 μl portion of each sample was analysed by SDS/PAGE on a 12.5 % Laemmli polyacrylamide gel, stained with Coomassie Blue, and destained. Phosphorylation of the substrate was analysed by autoradiography and quantified with a phosphoimager.

In-gel kinase assay

A variation of the methods of Sessa et al. [35] and Kameshita and Fujisawa [36] was used for the in-gel assay. Briefly, a 10 % Laemmli polyacrylamide gel containing 0.33 mg/ml of MBP was allowed to polymerize overnight. The lanes were loaded with protein as noted in each Figure legend. The gels were then run at 100 V for 2.5 h. To remove the SDS, the gels were washed three times for 20 min each with 20 % propan-2-ol in 50 mM Tris (pH 7.5). The gels were then washed with 50 mM Tris (pH 7.5)/5 mM 2-mercaptoethanol for 30 min to remove the propan-2-ol. The proteins were denatured by incubating for 15 min each in 6, 3, 1.5 and 0.75 M urea in 50 mM Tris (pH 7.5)/5 mM 2-mercaptoethanol. The proteins were renatured for 24 h at 4 °C in six changes of 40 mM Hepes (pH 7.4)/5 mM 2-mercapto-ethanol/0.05 % Tween 20. The gels were preincubated in 25 ml of 40 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA and 25 μM ATP for 1 h. The kinase reaction was initiated by the addition of 9.25×10^8 Bq of [γ-^{32}P]ATP and allowed to proceed for 14 h. The reaction was terminated by removal of the kinase buffer and by multiple washes with 5 % trichloroacetic acid (TCA) and 1 % pyrophosphate. After staining and destaining, kinase activation was identified by autoradiography and quantified with PhosphoImager.

Phospho amino acid analysis

Phospho amino acid analysis was conducted as described by van der Geer et al. [37]. Briefly, the MBP band from an *in vitro* kinase assay conducted with the partially purified kinase was excised from the gel and minced with a razor blade. The protein was eluted from the gel and concentrated by TCA precipitation. The protein was then hydrolysed with acid, the residue was washed twice with distilled water and freeze-dried.

Phospho amino acids were analysed by two-dimensional TLC. Phosphoserine, phosphothreonine and phosphotyrosine standards were spotted at the origin to facilitate identification of the corresponding [^{32}P]phospho amino acids. After elution at pH 1.9, the plate was rotated 90° and electrophoresed in pH 3.5 buffer.

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The phospho amino acid standards were revealed with ninhydrin. The plate was marked in the corners with a ^{14}C-labelled dye to aid alignment and exposed to film for 2 days.

Inhibitor studies

Soya bean cells (1.5 ml) were incubated for 2 min with the final concentration of inhibitor, as indicated in the Figure legends, then elicited with 2.5 μg of OGA. The samples were then processed as described in the oxidative-burst assay and in-gel kinase assay.

Immunoblotting

A crude protein extract (20 μg) was separated by 10% SDS/PAGE. Proteins were transferred to nitrocellulose at 100 V for 2.5 h. For anti-phosphotyrosine immunoblotting, blocking was performed in 3% BSA/0.05% Tween 20 in TBS overnight. The blot was incubated with a 1:1000 dilution of mouse IgG_{2b} PY-99 (Santa Cruz Biotechnology) in blocking buffer for 12 h at room temperature, followed by three washes of 10 min each with 0.3% BSA/0.05% Tween 20 in TBS. For anti- (MAP kinase) immunoblotting, anti-(active MAP kinase) antibody (pTEpY, Promega) was used according to the manufacturer's instructions. A secondary probe of a 1:4000 dilution of horseradish peroxidase-linked goat anti-mouse antibody in wash buffer was incubated for 1 h, then washed as described earlier. The blot was developed using chemiluminescence as specified by the manufacturer (Amersham.)

Immune complex kinase assays

Protein extract (2.5 μg) was incubated on a rotating shaker at 4 °C with 20 μl of a 50% slurry of prewashed mouse anti-phosphotyrosine IgG_{2b} agarose beads (Santa Cruz Biotechnology), or as a control, with the same quantity of non-specific mouse IgG_{2b} agarose beads (Sigma). After 1 h, the slurry was centrifuged briefly to settle the beads, and the supernatant was removed. The beads were washed three times with 20 mM Tris (pH 7.5)/100 mM NaCl/0.05% Triton X-100, then once in the same buffer with 1 M NaCl, then twice in the buffer alone, and finally in the kinase assay buffer, 10 mM Tris (pH 7.5)/10 mM NaF/0.2 mg/ml PMSF. For the kinase assay, 25 μl of kinase assay buffer was added to the beads, and the sample was processed as described in the *in vitro* kinase assay section.

Phosphatase inactivation studies

To evaluate the necessity of tyrosine phosphorylation for kinase activity, the kinase was treated with human cytosolic low-molecular-mass protein tyrosine phosphatase, a gift from Professor R. E. VanEtten, Purdue University, West Lafayette, IN, U.S.A. Partially purified kinase (3.75 μg) was incubated with 0.5 unit of phosphatase for 30 min. A cocktail of inhibitors, yielding final concentrations of 1 mM Na_3VO_4, 0.5 mM *p*-nitrophenyl phosphate and 100 mM Hepes (pH 7.6) was added, and kinase activity was monitored by the *in vitro* kinase assay. To ensure that the phosphatase did not act upon the phosphorylated substrate, controls were conducted where both phosphatase and inhibitors were added immediately before the kinase reaction.

RESULTS

Several different elicitors of the oxidative burst activate both a 44 and 47 kDa kinase

To identify kinase(s) that might participate in regulating the oxidative burst, crude extracts from suspension-cultured soya bean cells that were previously elicited with OGA, *V. dahliae* extract, or 50% dilution in distilled water (which causes an osmotically induced burst [10]) were tested for protein kinase activity towards common exogenous substrates in an *in vitro* kinase assay. Neither dephosphorylated casein nor a polymer of glutamate and tyrosine were appreciably phosphorylated by kinases in the elicited extracts (Figure 1), suggesting that neither casein nor tyrosine kinases are measurably activated by the burst elicitors. In contrast, histone IIIs, a good substrate for protein kinase C family members, was significantly phosphorylated by the plant extracts. However, the active kinase was also observed in extracts from untreated cells, suggesting that it is not responsible for initiating or modulating the burst. Importantly, extracts from cells treated with OGA, *V. dahliae* extract and osmotic stress also displayed substantial kinase activity towards MBP; however, in this case, little activity was measured in untreated cell extracts (Figure 1). These data suggest that an MBP kinase could participate in initiating the soya bean oxidative burst. This hypothesis is strengthened by the fact that the OGA fraction employed here induces no other known defence response in cultured soya bean cells, and that only those OGA oligomer fractions that induced the oxidative burst also activated the kinases (J. R. Merida, J. Kim, S. G. Cessna and P. S. Low, unpublished data).

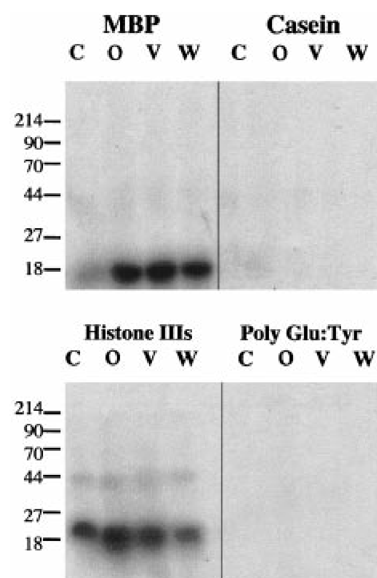

Figure 1 Evaluation of the substrate specificity of kinases stimulated by oxidative burst elicitors

Soya bean cell suspension cultures (1.5 ml) were treated with 2.5 μg of OGA (O), 50 μl of *V. dahliae* extract (V) or 50% dilution with distilled water (W). Untreated cells were processed similarly for control (C). At the onset of the burst, the cells were filtered, frozen in liquid N_2, and processed as described in the *in vitro* kinase assay section, with either MBP (upper left), dephosphorylated casein (upper right), histone IIIs (lower left) or polyglutamate tyrosine (lower right) as a substrate.

To determine the molecular masses of the activated kinases, in-gel kinase assays were performed on the same stimulated extracts. For these assays, the plant extracts were separated by SDS/PAGE, renatured in the polyacrylamide gel, and then allowed to phosphorylate MBP that was co-polymerized in the gel. Importantly, reconstitution of kinase activity was not achieved if Tris buffer was employed or denaturant was omitted during the renaturation procedure [36,38]. However, strong kinase activity was recovered using the reconstitution method of

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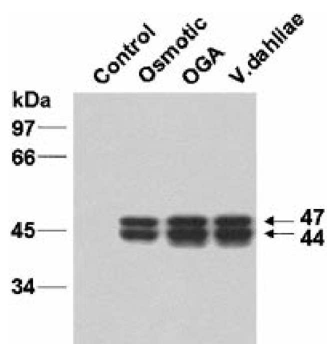

Figure 2 Three distinct elicitors of the oxidative burst activate two kinases with molecular masses of 44 and 47 kDa

Each lane contains 10 μg of protein from 1.5 ml of suspension-cultured soya bean cells untreated (control) or treated with 50% dilution (osmotic), 2.5 μg of OGA, or 50 μl of *V. dahliae* extract. The protein was separated on an SDS/polyacrylamide gel containing 0.33 mg/ml MBP. After protein renaturation, kinase reactions were performed in the gel as described in the Experimental section, and analysed by autoradiography. Numbers at the left indicate the molecular masses of the prestained marked proteins, numbers on the right indicate calculated molecular masses of active kinase bands.

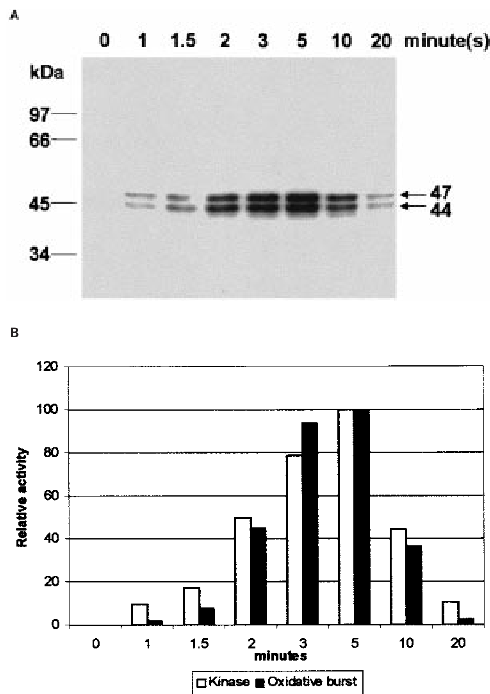

Figure 3 OGA-elicited time course of kinase activation/inactivation and H_2O_2 biosynthesis

Cells were treated with 2.5 μg of OGA for the indicated times, then assayed for (**A**) kinase activation by the in-gel kinase assay, or (**B**) rate of H_2O_2 production (solid bars) via the decrease in pyranine fluorescence. Data are normalized to the maximal rate of H_2O_2 production. Incorporation of phosphate into MBP is also displayed (white bars), as quantified from (**A**) via phosphoimaging. Results are from a single experiment and are representative of four replicates.

Sessa et al. [35], which utilizes Hepes instead of Tris buffer, with either urea or guanidine hydrochloride as the denaturant. Using this method, $\simeq$ 44 and $\simeq$ 47 kDa kinase bands were observed in extracts from all cells stimulated with elicitors, but not in extracts from unelicited cells (Figure 2). These data suggest that OGA, *V. dahliae* elicitor and osmotic stress activate a similar or identical pair of kinases in suspension-cultured soya bean cells.

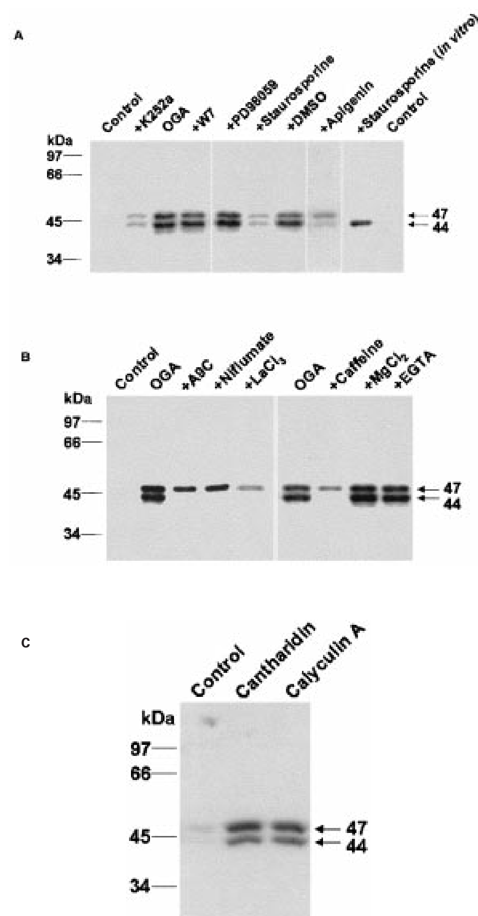

Figure 4 Effect of various inhibitors on the OGA-activated kinases

(**A**) Effect of kinase inhibitors on in-gel kinase activity. Cells (1.5 ml) were incubated for 2 min with the modulator indicated and then stimulated with 2.5 μg of OGA. Three minutes after OGA addition, cells were processed for the in-gel kinase assay. Conditions tested include: no inhibitor and no OGA (control), no modulator (OGA), 3 μM k252a, 100 μM W-7, 250 μM PD98059, 25 μM staurosporine, 18.5 μl of DMSO (solvent control for k252a and staurosporine), or 700 μM apigenin. In the second to last column, 25 μM staurosporine was added during the final phosphorylation step of the in-gel kinase assay. Numbers on the left represent the migration of prestained molecular-mass markers, while the arrows on the right indicate the molecular masses of the activated kinase bands. (**B**) Effect of calcium flux inhibitors on kinase activation. Cells (1.5 ml) were incubated for 2 min with the modulator indicated and then stimulated with 2.5 μg of OGA. After 3 min, the cells were processed for the in-gel kinase assay. Conditions tested include: no inhibitor and no OGA (control), no modulator (OGA), 250 μM anthracene-9-carboxylic acid (A9C), 250 μM niflumate, 5 mM $LaCl_3$, 45 mM caffeine, 5 mM $MgCl_2$ or 3 mM EGTA. (**C**) Kinase activation by oxidative burst-stimulating phosphatase inhibitors. Cells were treated with either no stimulus (control), 100 μM cantharidin for 30 min or 0.2 μM calyculin A for 15 min, then processed via the in-gel kinase assay.

Since kinase inhibitors can rapidly terminate the burst, even after it has achieved its maximum intensity, the kinase responsible for regulating the oxidative burst must remain active for the entire duration of the burst. Therefore, one characteristic of a burst regulatory kinase might be that its time course of activation and deactivation would correlate with the rise and fall of the oxidative burst. To test this possibility, elicitor-stimulated cells were quickly frozen at various time points following elicitation, and the rate of H_2O_2 biosynthesis and extent of kinase activation were compared at this time of quenching. As shown in Figure 3,

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Table 1 Correlation between various pharmacological modulators of the oxidative burst and activation of the soya bean 44 kDa and 47 kDa MAP kinases

			In vivo effect on kinases‡	
Inhibitor*	Putative activity	Effect on oxidative burst†	44 kDa	47 kDa
PD98059	MEK inhibitor	No effect	No effect	No effect
Staurosporine	Kinase inhibitor	Suppression	Suppression	Suppression
K252a	Kinase inhibitor	Suppression	Suppression	Suppression
Apigenin	MAP kinase inhibitor	Suppression	Suppression	Partial suppression
W7	Calmodulin inhibitor	No effect	No effect	No effect
EGTA	Ca^{2+} chelator	No effect	No effect	No effect
La^{3+}	Ca^{2+} antagonist	Suppression	Suppression	Partial suppression
Mg^{2+}	Ca^{2+} competitor	No effect	No effect	No effect
Caffeine	Ca^{2+} store depletor	Suppression	Suppression	Partial suppression
Niflumate	Anion-channel blocker	Suppression	Suppression	No effect
Anthracene-9-carboxylate	Anion-channel blocker	Suppression	Suppression	No effect
Cantharidin	Phosphatase inhibitor	Activation	Activation	Activation
Calyculin A	Phosphatase inhibitor	Activation	Activation	Activation

* Inhibitors were incubated with the cell culture for 2 min prior to OGA addition. For concentrations, see Figure legends for Figures 4(A) and 4(B)
† Determined by comparing the rate of oxidant production to solvent-treated cells.
‡ Evaluated using the in-gel kinase assay.

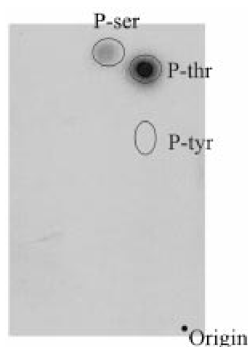

Figure 5 Phospho amino acid analysis of MBP phosphorylated by the partially purified OGA-activated kinase

MBP was phosphorylated in the presence of [γ-^{32}P]ATP with semi-purified OGA-activated kinase in an *in vitro* kinase assay. MBP was extracted from the SDS/PAGE gel, subjected to acid hydrolysis, and analysed by two-dimensional high voltage thin-layer electrophoresis. The circles indicate the positions of phospho amino acid standards, as visualized by ninhydrin staining. The phosphorylated amino acid in MBP were detected by autoradiography.

OGA-stimulated kinase activity was clearly evident by 1 min post-elicitation, reached a maximum by 3–5 min post-elicitation, and returned to basal levels by 20 min post-stimulation. Significantly, H_2O_2 production was also first measurable by 1 min post-elicitation, achieved maximal strength by 3–5 min post-elicitation, and returned to basal level by $\simeq$ 20 min post-stimulation (Figure 3). Furthermore, in various replicates of this experiment, conducted on soya bean suspensions with widely varying rates of elicitor stimulation, a tight correlation was invariably observed between the kinetics of kinase activation and the kinetics of oxidant biosynthesis, with kinase activation occurring either concurrently or slightly prior to oxidant production. As in Figure 3, the activated kinases had molecular masses of $\simeq$ 44 kDa and $\simeq$ 47 kDa, and the two kinases rose and fell in activity synchronously. Because consumption of the elicited H_2O_2 with catalase, or prevention of its formation with diphenylene iodonium, did not suppress kinase activity (results not shown), kinase activation cannot be a consequence of the oxidative burst. These data therefore argue strongly that the kinases revealed in Figure 3 participate in some manner in regulation of the soya bean cell oxidative burst.

A further line of evidence supporting a regulatory role for the above 44 kDa and 47 kDa kinases in promoting/maintaining the soya bean oxidative burst derives from a large body of pharmacological data. As shown in Figure 4(A) several, but not all, protein kinase inhibitors block the OGA-stimulated 44 kDa and 47 kDa kinase activation. Thus staurosporine, apigenin and K252a (Ser/Thr kinase inhibitors) prevent the appearance of the above kinase bands in in-gel kinase assays, whereas PD98059 [a MAP kinase kinase (MEK) inhibitor] and W-7 (a Ca^{2+}/calmodulin-dependent protein kinase inhibitor) do not. Other protein kinase inhibitors that also exert no effect on activation of the 44 kDa and 47 kDa kinases include W-13, forskolin and genistein (result not shown). Curiously, staurosporine treatment of the two kinases after their separation in the polyacrylamide gel results in inhibition of only the 47 kDa kinase (Figure 4A). This suggests that blockade of the 44 kDa kinase seen *in vivo* does not derive from its direct sensitivity to staurosporine, but rather from an inhibition of an upstream kinase, conceivably the 47 kDa kinase. Finally, inhibitors of Ca^{2+} release from intracellular stores (e.g. anthracene-9-carboxylate, La^{3+}, niflumate and caffeine [39] also block activation of the 44 kDa kinase, whereas inhibitors of extracellular Ca^{2+} entry (e.g. EGTA, Mg^{2+}) have no effect (Figure 4B).

To learn whether a relationship might exist between the above inhibition of elicitor-induced kinase activation and induction of the oxidative burst, the same set of kinase and Ca^{2+} flux inhibitors was examined for its effect on OGA-stimulated H_2O_2 biosynthesis. As revealed in Table 1 every reagent that prevented activation of the 44 kDa kinase also blocked activation of the burst. Conversely, any inhibitor that failed to prevent activation of at least the 44 kDa kinase also failed to alter the burst. Taken together, this very similar pharmacological profile for regulation of the 44 kDa kinase and modulation of the oxidative burst confirms the contention that the two processes are interrelated.

Finally, a number of different laboratories have reported that protein phosphatase inhibitors can autologously induce the oxidative burst, i.e. in the absence of any other stimulation [3,32].

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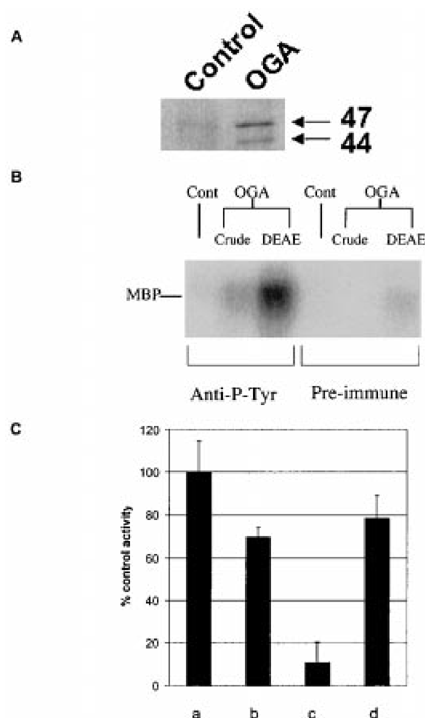

Figure 6 Requirement of phosphorylation of the 44 and 47 kDa OGA-activated kinases on tyrosine for kinase activity

(**A**) Anti-phosphotyrosine immunoblot of control cells and OGA-treated cells. Crude protein extract (20 μg) from unstimulated (control) or OGA-treated cells were separated by SDS/PAGE on a 10% gel and transferred to nitrocellulose. The blot was processed as described in the Experimental section. Arrows on the right indicate the molecular masses of 44 and 47 kDa respectively. (**B**) Kinase activity can be immunoprecipitated with anti-phosphotyrosine antibodies. Crude extract (2.5 μg) from unstimulated cells (control), OGA-treated cells (OGA, crude) or a semi-purified active fraction from OGA-stimulated cells (DEAE) were incubated with agarose beads conjugated to anti-phosphotyrosine IgG_{2b} (Anti-P-Tyr) or non-specific IgG_{2b}. The beads were washed thoroughly and then processed via the *in vitro* kinase assay. The position of MBP in the gel is indicated. (**C**) Tyrosine phosphatase treatment inactivates the OGA-activated kinase. Partially purified OGA-activated kinase was treated for 30 min with human cytosolic protein tyrosine phosphatase either in the absence or presence of an inhibitor cocktail, then measured for activity with the *in vitro* kinase assay. (a) Control; (b) protein tyrosine phosphatase + inhibitors; (c) protein tyrosine phosphatase (inhibitors added immediately before kinase assay); and (d) inhibitors added immediately before kinase reaction *in vitro*.

We have observed the same phenomenon in cultured soya bean cells [12]. While the site of phosphatase inhibitor action has never been established, if the inhibitors were to function upstream of the 44 kDa and/or 47 kDa kinases, then inhibitor treatment might be expected to activate the two kinases. To explore this possibility, soya bean cells were treated with calyculin A or cantharidin, i.e. phosphatase inhibitors that induce the soya bean oxidative burst, and then assayed for kinase activity when oxidant production was maximal. As displayed in Figure 4(C), both the 44 kDa and 47 kDa kinases were indeed stimulated by inhibitor treatment. These observations thus corroborate the hypothesis that the 44 kDa and/or 47 kDa kinases participate in signalling the oxidative burst.

Partial characterization of the 44 kDa and 47 kDa kinases

As an early step in the characterization of any unknown kinase, it is instructive to ascertain the identities of the preferred protein and amino acid substrates of the kinase. Figure 1 demonstrated that the elicitor-stimulated 44 kDa and 47 kDa kinases prefer

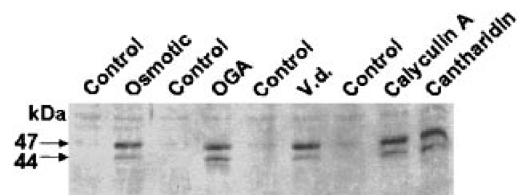

Figure 7 Recognition of 44 and 47 kDa bands in an immunoblot with anti-(active MAP kinase) antibodies

Cells were treated with no elicitor (control), 50% osmotic stress, 2.5 μg of OGA, 50 μl of *V. dahliae* extract, 0.2 μM calyculin A or 100 μM cantharidin. At maximal oxidative burst activity time points cell extracts were prepared, and 20 μg of protein extract were separated by SDS/PAGE on a 10% gel and transferred to nitrocellulose. The blot was processed as described in the Experimental section with anti-(active MAP kinase) antibodies. Arrows on the left indicate the molecular masses of 44 and 47 kDa respectively.

MBP over casein, poly(Glu-Tyr) or histone IIIs as substrate. Figure 5 reveals that the primary amino acid selected by the same pair of elicitor-activated kinases is threonine, with much lower activity towards serine. Importantly, no phosphotyrosine could be detected in the two-dimensional separation. Because both 44 kDa and 47 kDa kinases phosphorylate MBP with equal intensity (Figures 2–4), it can be concluded that threonine must be the primary amino acid substrate of both kinases. This concurs with the phosphorylation pattern observed with other plant MAP kinases, such as SIPK [38] and ATMPK4 [40].

Recognizing that the molecular masses, preferred protein substrate, and primary phospho amino acid products are all characteristic of MAP kinases [41], we decided to investigate further whether the 44 kDa and 47 kDa kinases might be MAP kinases. One common hallmark of MAP kinases is their requirement for tyrosine phosphorylation for full activity [41]. To learn whether the two elicitor-activated kinases might become tyrosine phosphorylated during activation, extracts from unstimulated (control) and OGA-elicited soya bean cells were electrophoretically separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine IgG. As seen in Figure 6(A) elicited (but not control) extracts of soya bean cells contain immunoreactive bands at 44 kDa and 47 kDa, the same molecular masses as the elicitor-activated kinases.

In order to evaluate further whether tyrosine phosphorylation is essential for kinase activity, extracts from elicited soya bean cells were immunoprecipitated with anti-phosphotyrosine or non-specific (control) antibodies and examined for kinase activity towards MBP. As noted in Figure 6(B) kinase activity was readily pelleted by the anti-phosphotyrosine, but not control, IgG. Furthermore, treatment of the kinase with a phosphotyrosine-specific phosphatase reduced kinase activity to only $\sim 10\%$ of the untreated control, and this reduction was largely prevented by pre-administration of phosphatase inhibitors (Figure 6C). Taken together, these data suggest that the 44 kDa and 47 kDa elicitor-activated kinases are both phosphorylated on tyrosine residues during elicitor stimulation and that kinase activation requires this tyrosine phosphorylation.

Finally, to confirm the identities of the two kinases as MAP kinases, an antibody that recognizes the proximal phosphothreonine and phosphotyrosine residues of activated MAP kinases was used to immunoblot the extracts of both elicited and unstimulated cells. As shown in Figure 7, treatment with each of the three unrelated oxidative-burst stimuli enabled recognition of the two kinases by the anti-activated MAP kinase antibody. In

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contrast, treatment of the same cells with a buffer control generated no activated MAP kinases. These data confirm that the 44 kDa and 47 kDa elicitor-stimulated kinases are indeed MAP kinases.

DISCUSSION

We have presented evidence that distinct stimuli of the soya bean oxidative burst (OGA, *V. dahliae* elicitor, osmotic stress and phosphatase inhibitors) activate a pair of threonine/serine protein kinases of masses ≈ 44 kDa and ≈ 47 kDa. Data demonstrating that these kinases are, in fact, MAP kinases include: (i) their preference for MBP as a substrate, (ii) their apparent molecular masses, (iii) their selectivity for phosphorylating threonine over serine and tyrosine residues, (iv) the requirement of tyrosine phosphorylation for their activity, and (v) their recognition by anti-activated MAP kinase antibodies.

Several lines of evidence also suggest that the 44 kDa and 47 kDa MAP kinases are integral to the oxidative burst signal-transduction pathway. First, four distinct oxidative burst stimuli (OGA, *V. dahliae* elicitor, osmotic stress and phosphatase inhibitors) were found to activate the aforementioned kinases. Secondly, the kinetics of kinase activation and deactivation closely paralleled the kinetics of the rise and fall of the oxidative burst, even when changes in responsiveness to burst elicitors were experienced. Thirdly, kinase inhibitors that prevented stimulation of the two MAP kinases also blocked the oxidative burst, and kinase inhibitors that were ineffective at suppressing MAP kinase activation were similarly ineffective in modulating the burst. Fourthly, Ca^{2+} flux inhibitors that suppressed release of intracellular Ca^{2+}, but not entry of extracellular Ca^{2+}, blocked both activation of the 44 kDa kinase and induction of oxidant biosynthesis. Finally, phosphatase inhibitors that have long been recognized to activate the burst also induced both 44 kDa and 47 kDa MAP kinases. While unequivocal evidence for participation of these two MAP kinases in signalling the oxidative burst will require their cloning and manipulation *in vivo*, the current data nevertheless strongly implicate their involvement in this defence pathway.

A large number of different MAP kinases have now been identified in plants [47]. Although salicylic acid-induced protein kinase (SIPK) [38] (≈ 48 kDa) and wound-induced protein kinase (WIPK) [44] (~ 46 kDa), two well-characterized tobacco MAP kinases, have been shown to be involved in a number of other elicitor-induced signal-transduction pathways [25,42–46], it is unclear whether the soya bean kinases described here represent functional homologues of WIPK and SIPK. Elicitor induction of WIPK in tobacco cells requires gene transcription and translation, thereby delaying achievement of maximal activity until ≈ 4 h post-stimulation [42]. In contrast, the two kinases described above reached maximal activity by ≈ 4 min post-elicitation (Figure 3). Although SIPK does not require transcription for activation, it still displays a lower rate of induction (maximal activity 15–240 min post-elicitation) and inactivation (retention of measurable activity for at least 8 h after elicitor addition) than the MAP kinases characterized above [43]. These kinetics are clearly insufficient to mediate the activation and inactivation of the oxidative burst. Further differences between the soya bean kinases and SIPK and WIPK are revealed by inhibitor sensitivity. PD98059 inhibits SIPK and WIPK activation, but does not inhibit the oxidative burst, whereas certain activators of the burst (e.g. cantharidin) do not stimulate SIPK and WIPK [25]. Since the opposite behaviour is manifested by the 44 and 47 kDa soya bean kinases (Table 1), it seems possible that SIPK and WIPK operate in distinct disease/wounding resistance pathways.

Other MAP kinases have been identified in plants, but they appear to be distinct from the kinases described here. Although an *Avr9*-encoded peptide is capable of activating both a pair of MAP kinases (~ 46 and 48 kDa) and the oxidative burst in tobacco plants expressing the *Cf-9* resistance gene, inhibitor sensitivity is different for the burst and the two MAP kinases [25] (*Avr9* is an avirulence gene from *Clodosporium fuluum*). Since similar results have also been obtained by Lebrun-Garcia et al. [48] using cryptogein as an elicitor of the tobacco cell oxidative burst, it can be suggested that oxidant production may not be dependent on activation of these MAP kinases in tobacco cells.

A closer correlation between kinase activation and the oxidative burst has been observed by Cazale et al. [45], who examined hypo-osmotically stimulated tobacco cells for both MAP kinase activation and burst stimulation. These researchers report that cantharidin activates both a ≈ 50 and ≈ 46 kDa MAP kinase over the same time course that the phosphatase inhibitor stimulates the oxidative burst. They also demonstrated that PD98059 exhibits no effect on kinase activation or oxidative burst induction, and that apigenin inhibits both kinases when added directly to the last step of the in-gel kinase assay *in vitro*. However, they report that apigenin exhibits no influence on the two MAP kinases when administered *in vivo*, even though oxidative burst activity is suppressed under the same conditions. In contrast, the *in vivo* effects of apigenin in the soya bean-elicitor system show inhibition of both the kinases as well as the oxidative burst. Further differences from the results of Cazale et al. [45] lie in the effect of anion-channel blockers and strength of kinase activation.

Although similarities in some pharmacological properties do exist, it also seems unlikely that the elicitor-stimulated MAP kinases characterized by Lebrun-Garcia et al. [48] in tobacco cells represent functional analogues of the soybean kinases described in our study. Activation of the 46 kDa and 50 kDa MAP kinases characterized by Lebrun-Garcia et al. [48] was strongly inhibited by addition of EGTA,whereas the kinases examined in our studies were unaffected by chelation of external Ca^{2+}. Furthermore, neither of the tobacco MAP kinases reported by Lebrun-Garcia et al. was induced by calyculin A, whereas both calyculin A and a functionally similar phosphatase inhibitor, cantharidin, induced the kinases and the burst in our studies. While differences in plant species and experimental conditions could conceivably explain these disparities, the involvement of functionally distinct members of the MAP kinase family may also account for the data. In *Arabidopsis*, for example, at least nine MAP kinase family members have been identified to date.

Much of the data in this report suggest a possible sequence of signalling steps in the MAP kinase section of the oxidative burst pathway. The fact that staurosporine blocks activation of both MAP kinases when added to whole cells, but inhibits only the 47 kDa kinase when added to the isolated kinases (Figure 4A), suggests that the 47 kDa kinase or some other staurosporine-sensitive kinase must lie upstream of the 44 kDa kinase. The further observation that anion-channel blockers (anthracene-9-carboxylate, niflumic acid) and Ca^{2+} influx inhibitors (i.e. La^{3+}, caffeine [39]) prevent stimulation of only the 44 kDa kinase (Table 1) requires that the 44 kDa kinase, but not the 47 kDa kinase, occur downstream of anion transport and Ca^{2+} entry into the cytoplasm. This result also places the 47 kDa kinase either upstream of the anion/Ca^{2+} fluxes and 44 kDa kinase activation, or it suggests the 47 kDa kinase lies in a distinct pathway. Evidence for the former interpretation is that (i) staurosporine also blocks both the 47 kDa kinase and Ca^{2+} entry into elicited cells [39], and (ii) both kinases are activated and deactivated in tightly coupled synchrony (Figure 3). Finally, since niflumic acid

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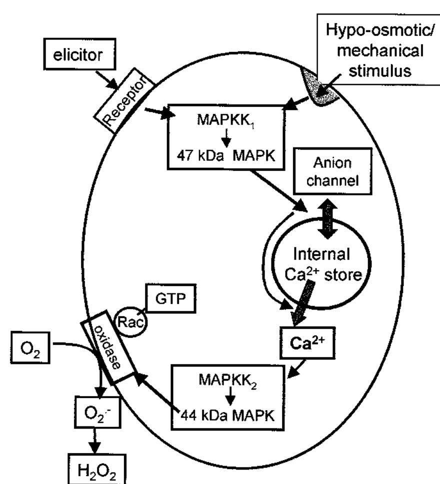

Figure 8 Schematic diagram of the signal-transduction pathway leading to the oxidative burst

and anthracene-9-carboxylate also prevent Ca^{2+} entry into the cytoplasm (S. G. Cessna and P. S. Low, unpublished work), and since tyrosine phosphorylation, presumably by an MEK [40], is shown to be essential for kinase activation, a plausible sequence of steps connecting these many signalling intermediates is:

elicitor stimulation

→ unique steps in each distinct elicitor-stimulated pathway

→ MEK activation → 47 kDa MAP kinase activation

→ anion flux → Ca^{2+} release from internal stores

→ 44 kDa MAP kinase activation

→ oxidant biosynthesis (Figure 8).

While additional intermediates are undoubtedly required along this stretch of the pathway, and although other schemes might also be consistent with the data, this sequence at least constitutes an initial hypothesis worthy of additional testing. At the very least, two MAP kinases activated by several elicitors of the oxidative burst, and distinguishable primarily in their sensitivities to staurosporine and anion/Ca^{2+} flux inhibitors, are important in the regulation of the soya bean oxidative burst.

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