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Wounding activates a 47 kDa MAP kinase in *Catharanthus roseus* (L.) G. Don.

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Catharanthus roseus (L.) G. Don. is an important medicinal plant (Fam. Apocynaceae) known for its alkaloids that are accumulated in response to various biotic and abiotic stresses including wounding. Mitogen activated protein (MAP) kinases are important components of cellular signaling system transducing these stress stimuli into intracellular responses by phosphorylation of downstream transcription factors, regulators or inhibitors leading to expression of stress responsive genes. Here, we report the activation of a 47 kDa MAP Kinase in *C. roseus* in response to wounding. The immunoprecipitation coupled with in-gel kinase assay revealed tyrosine phosphorylation of the protein. Partial purification of the MAP kinase was also attempted. We observed a size variation for the kinase when purified using different schemes.

Keywords: Madagascar periwinkle, MAP Kinase, Wounding

Plants are often exposed to various biotic and abiotic stresses during their growth and development. In order to acclimatize to these hostile environmental conditions, plants develop various adaptive strategies[1] which result from adjustments in physiological and biochemical processes[2-4]. Elicitation by pathogens or other agencies leads to transcriptional activation of several defence genes responsible for synthesis of defence compounds and secondary metabolites[5] which enhance the fitness of plants under stress conditions. However, perception of stress stimuli and their transmission to defence gene activation involves participation of an array of intermediate signaling components. Some of the signalling components common to the most stress signal transduction pathways are calcium ions, inositol phosphates, G-proteins, cyclic nucleotides, protein kinases and protein phosphatases[6]. Protein phosphorylation/ dephosphorylation which plays an important role in modulating defence gene activation is mediated by protein kinases and phosphatases. Various types of protein kinases identified in living organisms have been classified according to the presence of several conserved domains and the particular motifs mediating the phosphorylation. Typical eukaryotic protein kinases phosphorylate tyrosine, serine and/or threonine residues.

The mitogen activated protein (MAP) kinases, a class of serine/threonine protein kinase, are important components of a signaling module that transduce extracellular stimuli into intracellular responses among eukaryotic organisms. A typical MAP Kinase cascade consists of a MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK) and MAP kinase (MAPK). MAPKKKs which are serine /threonine kinases, activate downstream MAPKKs by phosphorylation at S/T-X_{3-5}-S/T motif. The activated MAPKKs phosphorylate downstream MAPKs by dual phosphorylation of threonine and tyrosine residues in the conserved T-X-Y motif[7]. However, recently MPK5, rice MAP Kinase was shown to get phosphorylated and activated by CPK-18, a Calcium dependent protein kinase (CDPK)[8]. MAP Kinases, which are at bottom of this cascade, are able to phosphorylate a range of substrates including other kinases and/or transcription factors. In plants, MAPK mediated signaling has been reported in several abiotic stresses like cold, salt, touch, wounding, heat, UV, osmotic shock, heavy metals, etc.[7].

Catharanthus roseus (L.) G.Don (Fam. Apocynaceae), commonly called Madagascar periwinkle or Rosy periwinkle, is a pharmaceutically important plant known to synthesise more than 130 alkaloids[9-13]. Several biotic and abiotic stresses such

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as pathogen infection and UV light are known to stimulate secondary metabolite accumulation in plants[14]. In tobacco roots, physical injury leads to jasmonate-mediated increase of nicotine biosynthesis[14]. Wounding and methyl jasmonate has been reported to induce accumulation of ajmalicine in *C. roseus* seedlings[16]. Through the use of protein kinase inhibitors, that role of protein kinases in elicitor induced jasmonic acid accumulation was deciphered in *C. roseus*[17]. In transformed hairy roots of *C. roseus*, a MAP kinase like activity, whose levels are altered by exposure to low temperature or hypo-osmotic shock, has been reported[18]. Activation of mylein basic protein (MBP) kinase and CDPK in *C. roseus* has also been reported in suspension cultures exposed to UV-B irradiation[19]. Immunoprecipitation with antiphospho-tyrosine antibody followed by in-gel kinase assay detected a 49 kDa MBP kinase activity which was inhibited by MAPK cascade inhibitors, thereby suggesting the involvement of MAP kinases in UV-B induced catharanthine accumulation in *C. roseus* suspension cultures. Earlier, a 43 kDa MAP kinase has been isolated and characterised in *C. roseus*[20]. Here, we report activation of a 47 kDa MAP kinase upon wounding in *C. roseus* and its partial purification. Size variation was observed for the kinase when purified by different schemes.

Materials and Methods

Plant material and treatments

Plants of *C. roseus* var. Nirmal[21] growing in greenhouse at 28ºC were subjected to wounding treatment. Intact leaves were damaged up to 40% of their leaf lamina using a surgical blade and harvested at indicated time intervals post incubation. The harvested samples were snap frozen in liquid nitrogen and stored at −80°C.

Protein extraction, immunoprecipitation, in-gel kinase and *in vitro* kinase assays

Protein extraction, immunoprecipitation, in-gel kinase *&* *in vitro* Kinase assays were performed as described previously[20]. Briefly, frozen leaf tissue was ground in liquid nitrogen and homogenized in 1 mL of ice-cold extraction buffer [100 mM HEPES -KOH (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerol phosphate, 1 mM PMSF, 10% glycerol and 7.5% PVPP]. Extracts were centrifuged, clear supernatant recovered, and protein quantified by Bradford method. For in-gel kinase assay, 20 µg of protein was fractionated on a 10% polyacrylamide gel containing 0.1% SDS and 0.5 mg/mL bovine brain myelin basic protein (MBP) (Sigma Aldrich). After electrophoresis, the SDS from the gel was removed with buffer (25 mM Tris–HCl pH 7.5, 0.5 mM DTT, 5 mM Na_3VO_4, 0.1 mM NaF, 0.5 mg ml^{-1} BSA, 0.1% Triton X 100) followed by renaturation in buffer (25 mM Tris–HCl (pH 7.5), 0.5 mM DTT, 5 mM Na_3VO_4, 0.1 mM NaF) at 4°C overnight. MBP phosphorylation was performed by incubating the gel in 20 mL of reaction buffer (25 mM Tris HCl (pH 7.5), 2 mM EGTA,12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 1 µM ATP and 50 µCi of γ^{32}P-ATP (3000 Ci $mmol^{-1}$) for 60 min at room temperature i.e 23 °C. The gel was washed thrice with 5% TCA and 1% Sodium pyrophosphate and dried on filter paper and autoradiographed in phosphorimager (Typhoon, GE health care).

In vitro kinase assay was performed by incubating 5 µg of protein with 5 µg of MBP and 1 µCi of γ-^{32}P-labelled ATP in a 15 µL reaction mixture (25 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 25 µM ATP, 1 mM EGTA and 1 mM DTT) at 30°C for 20 min. The reaction was stopped with 5x SDS loading buffer and the samples were resolved on a 15% polyacrylamide gel. The gel was dried on Whatman 3MM paper and exposed to X-ray film.

For immunoprecipitation, 200 µg of protein was incubated with 1 µg of 4G10 antiphosphotyrosine or antiphosphothreonine monoclonal antibody (Upstate Biotechnology, NY) in 250 µL volume of extraction buffer (without PVPP) with 250 mM NaCl and 0.1% (v/v) Nonidet P40. The assay was shaken at 4°C for 2 h and after the subsequent addition of 30 µL of protein A-Sepharose (GE Healthcare) for overnight, followed by centrifugation (13000 rpm) for 3 min at 4°C. The beads were washed thrice with extraction buffer, and boiled for 5 min in 30 µL of 5x loading buffer (0.625 M Tris–HCl pH 6.8, 5% SDS, 50% glycerol, 0.125% bromophenol blue and DTT). The reaction products were loaded on SDS-PAGE gel containing 0.5 mg/mL of MBP and subjected to in-gel kinase assay.

Purification of wound activated protein kinase

Fifty grams of wounded leaf tissue from *C. roseus* were homogenized in 50 mL of extraction buffer (100 mM HEPES –KOH (pH 7.5), 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 10 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerol phosphate, 1 mM PMSF, 10 % glycerol and 7.5 % PVPP). The homogenate was

centrifuged at 13000 rpm for 20 min at 4ºC. The supernatant was again centrifuged at 40000 rpm for 30 min at 4ºC before loading on a Resource Q (Anion exchange) column using AKTA prime plus Fast Protein Liquid Chromatography (FPLC) system (Amersham). The column was equilibrated with Buffer A containing Tris 25 mM (pH7.5), EGTA 1 mM, β-glycerol phosphate 10 mM, Sodium orthovanadate 0.1 mM, DTT 1 mM, PMSF 1 mM and 5% glycerol. After loading, the column was washed with at least 20 mL of buffer A until A280 reached a baseline. The protein was eluted with a linear 0-500 mM NaCl gradient in buffer A at a flow rate of 1 mL/min. Fractions of 2 mL were collected and active fractions pooled and concentrated by dialysis against buffer A containing 50% PEG 4000.

Rubisco depletion and Phosphoprotein enrichment

Rubisco depletion of protein samples was achieved by elution of samples through SEPPRO column (Sigma-Aldrich) as per manufacturer's instructions while Phosphoprotein enrichment was attempted through use of phosphoprotein purification kit (Qiagen) as per mentioned protocol.

Results

Earlier, we have reported transient activation of mitogen activated protein kinases upon wounding in *C. roseus*[20]. Protein samples from unwounded (control) and wounded *C. roseus* leaf tissue when subjected to in-gel kinase assay using MBP as substrate, revealed distinct MAP Kinase activity (Fig. 1). Since monoclonal antiphosphotyrosine (pY) antibodies are a convenient tool to identify the MAP kinases in plants[18], we used these antibodies to immunoprecipitate MAP kinases from *C. roseus* leaf tissue. Protein samples from unwounded and wounded leaf tissue were incubated with antiphosphotyrosine antibody followed by immunoprecipitation with protein A-agarose. The immunoprecipitated samples were subjected to an in-gel kinase assay using MBP as substrate. A distinct MBP kinase activity was observed in the wounded sample while no activity could be detected from unwounded sample (Fig. 1). Based on mobility in SDS-PAGE, the apparent molecular mass of the kinase was estimated to be approximately 47 kDa. The molecular weight of wound activated protein kinase, its MBP phosphorylating capacity as well as immuno-precipitation with antiphosphotyrosine antibody confirmed MAP kinase activity of the protein.

In an attempt to partially purify the wound activated MAP kinase, *C. roseus* leaf tissue was wounded and harvested for protein extraction within 5 min post-wounding. The protein lysate was subjected to ion exchange column chromatography and elution profile monitored by absorbance at 280 nm (Fig. 2). The fractions were collected and tested for kinase activity in an *in vitro* kinase assay. Five fractions eluted between 32-40 mL of elution volume exhibited MBP phosphorylation (Fig. 3A). Based on the intensity of signal detected in autoradiograph, maximum kinase activity was observed in 36 mL of elution volume corresponding to 386 mM NaCl concentration (Fig. 3B).

Since activated MAP kinases are dually phosphorylated at threonine and tyrosine residues, we used antibodies against phosphothreonine and phosphotyrosine residues to immunoprecipitate these kinases from pooled active fractions. In-gel kinase

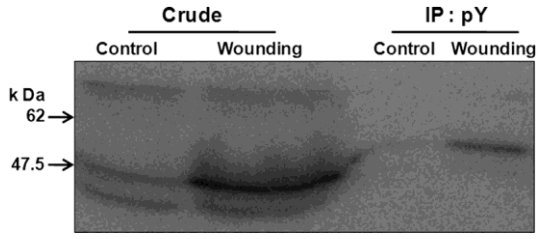

Fig. 1—Immunoprecipitation of wound activated MAP Kinase by antiphosphotyrosine antibody. [Leaves of *C. roseus* plants were wounded and used for protein extraction. Crude protein extracts as well as extracts immunoprecipitated with antiphosphosphotyrosine antibody from control and wounded leaf tissues were tested for their kinase activity in an in-gel kinase assay].

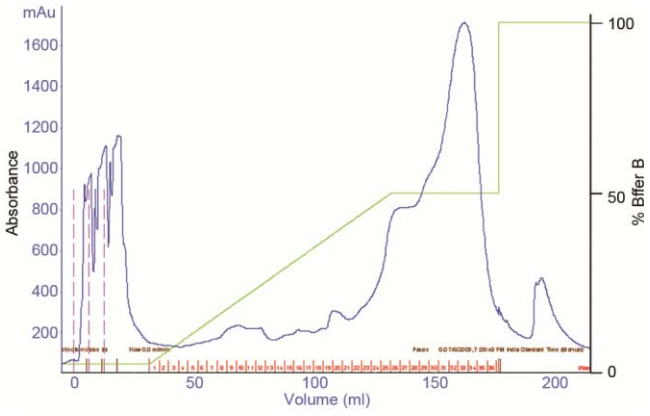

Fig. 2—Ion exchange chromatography of protein extracts from *C. roseus*. [Protein extract from wounded *C. roseus* leaf tissue was injected onto an FPLC Resource Q column. The protein was eluted at a flow rate of 1 mL/min using a gradient of 50% buffer B (25mM Tris-Cl +0.5M NaCl). Elution was monitored by absorbance at 280 nm]

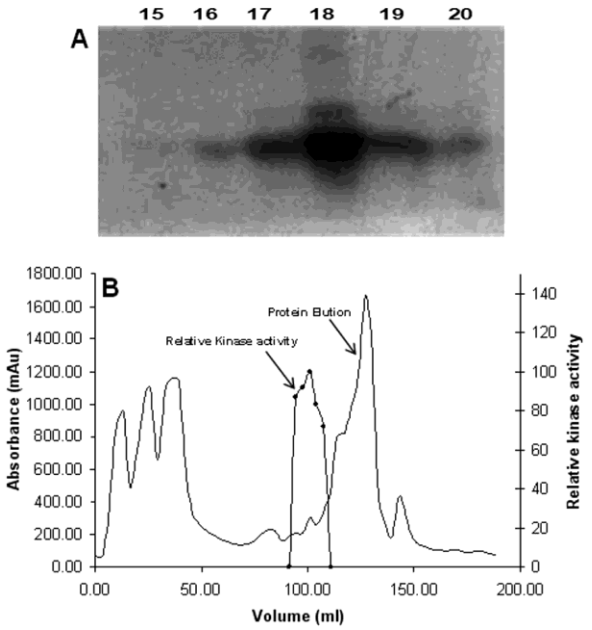

Fig. 3—Elution and MAP kinase phosphorylation profile of protein extracts from wounded *C. roseus* leaf tissue. (A) Selected fractions from the elution volume were assayed in an *in vitro* kinase assay (numbers on the top indicate fraction numbers); and (B) Chromatogram representing the elution profile and relative kinase activity.

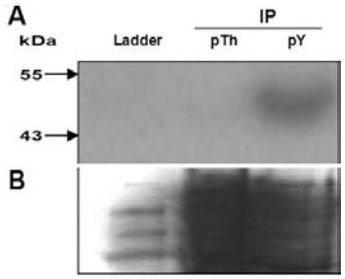

Fig. 4—Immunoprecipitation of kinase active fractions with antiphosphothreonine and antiphosphotyrosine antibodies. (A) The active fractions from Resource Q column were pooled and concentrated by dialysis against buffer A containing 50% PEG. The concentrated protein sample was immunoprecipitated with monoclonal antiphosphothreonine (pTh) and antiphosphotyrosine (pY) antibodies followed by in-gel kinase assay; and (B) The same gel stained with silver stain.

assay revealed a distinct 47 kDa activity in samples immunoprecipitated with antiphosphotyrosine antibody. However, no kinase activity could be detected from antiphosphothreonine (pTh) immuno-precipitated samples (Fig. 4), confirming thereby, that the wound activated MAP kinase is phosphorylated at tyrosine residue. Absence of kinase activity in samples immunoprecipitated with antiphospho-threonine antibody prompted us to confirm the fidelity of antibody. The residual radioactivity was washed off the gel by washing solution (5% TCA, 1% Sodium

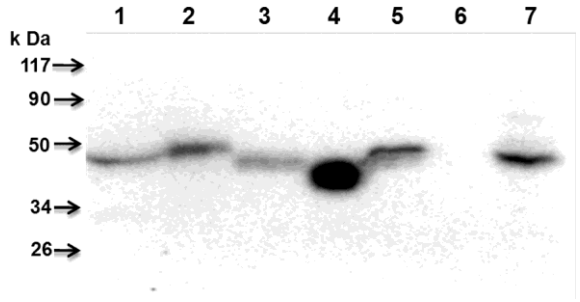

Fig. 5—In-gel kinase assay of wound activated MAP kinase activity from different schemes of purification. [Lane 1, crude sample; lane 2, crude sample eluted from SEPPRO column; lane 3, crude sample eluted from phosphoprotein purification column; lane 4, pooled & concentrated active fractions from Resource Q column depleted of rubisco contamination by elution from SEPPRO column; lane 5, crude sample eluted from SEPPRO column followed by IP with pY antibody; lane 6, crude sample eluted from phosphoprotein purification column followed by IP with pY antibody; lane 7, flow through from phosphoprotein purification column. Protein samples were separated on a 12% polyacrylamide-SDS containing MBP as substrate. MAP kinase phosphorylation was visualized by autoradiography]

pyrophosphate) followed by silver staining of the gel. Silver staining confirmed the immunoprecipitation of other proteins although not the wound activated MAP kinase (Fig. 4).

When the protein band corresponding to 47 kDa kinase activity in samples immunoprecipitated with antiphosphotyrosine antibody (Fig. 4) was sequenced, it revealed profound rubisco contamination. To reduce rubisco contamination, concentrated active fractions were passed through a SEPPRO column which is packed with antirubisco IgY antibodies. Simultaneously, crude protein samples from wounded *C. roseus* leaf tissue were passed through SEPPRO and Phosphoprotein columns and column eluates immuno-precipitated with antiphosphotyrosine antibody. The various column eluates as well as the immuno-precipitated samples were assayed for kinase activity in an in-gel kinase assay. A distinct MAP kinase activity was detected in all the samples except the antiphosphotyrosine immunoprecipitates of phospho-protein column eluate. Although, the flow-through from Phosphoprotein column was kinase active (Fig. 5). Surprisingly, wound activated MAP kinase activity eluated from Resource Q column exhibited a reduction in the size upon rubisco depletion (Fig. 5).

Discussion

MAP kinase activation in response to wounding has been reported in several plants species like rice,

tomato, soybean, cotton, pea and maize[7]. Wounding is reported to induce activation of MAP kinases also in *C. roseus*[20]. As MAP kinases are activated by phosphorylation of threonine and tyrosine residues in TXY motif present between subdomains VII & VIII of kinase catalytic domain[7], monoclonal antibodies against these phosphorylated residues are extensively used to characterise the MAP kinases. Immuno-precipitation followed by in-gel kinase assay detected a distinct kinase activity from wounded tissue. Immuno-precipitation by antiphosphotyrosine antibody and phosphorylation of MBP which is a preferred substrate for MAP kinases, confirmed the MAP kinase activity of wound activated kinase. Our results are substantiated by the fact that myelin basic protein (MBP) phosphorylating capacity of MAP kinases and their immunoprecipitation with antiphosphotyrosine monoclonal antibodies has been widely used to identify MAP kinase activity[22]. Moreover, the fact that the molecular weight of the wound activated kinase falls in the range of MAP kinases (38-55 kDa), further support the identity of wound activate kinase as a MAP Kinase.

As column chromatography has earlier been used to purify MAP kinases from plants[23], the protein samples from wounded *C. roseus* leaf tissue were loaded on Resource Q column, which is a strong anion exchange column. Elution with 0-500 mM NaCl gradient resulted in several fractions which were tested for kinase activity in an *in vitro* kinase assay using MBP as substrate. To further verify the MAP kinase activity of the active fractions, these were pooled, concentrated & immunoprecipitated with antiphosphotyrosine & antiphosphothreonine antibodies and immuno-precipitates analyzed in an in-gel kinase assay. A distinct MAP kinase activity was detected in samples immunoprecipitated with antiphosphotyrosine antibody. However, no kinase activity was observed in protein samples immuno-precipitated with antiphonsphothreonine antibody. Although phosphorylation at both tyrosine and threonine residues is considered essential for activity of MAP kinases, in Arabidopsis, AtMEK1 phosphorylates AtMPK4 at threonine only when recombinant enzymes are used[23]. However, tyrosine phosphorylation which is important for kinase activity results from autophosphorylation activity of AtMPK4[24]. Moreover in rice, MPK5, a MAP kinase has been shown to get activated by phosphorylation of threonine residues only (Thr-14 and Thr-32) outside the TXY motif[7]. Since dual phosphorylation of MAP kinases at threonine and tyrosine residues is considered essential for activity of MAP kinases, lack of threonine phosphorylation observed for the wound activated MAP kinase needs further investigation.

We have earlier reported activation of two different MAP kinases within 15 min of wounding in *C. roseus*[20]. It has been demonstrated in perennial rye grass and *Lolium temulentum* that activation of p46 MAPK occurs within 2 min of wounding while activation of p44, another MAPK requires extended post wounding incubation period of 30 min[25]. In concurrence with these results, we reduced the post-wounding incubation time to 5 min while sampling for purification of 47 kDa MAP kinase.

When the wound activated MAP kinase activity, immunoprecipitated by antiphosphotyrosine (pY) antibody, was sequenced, it revealed abundance of rubisco protein. Identification of plant proteins is often hampered by abundance of rubisco in samples comprising about 30-50% of total leaf protein[26]. Moreover, it is reported to out-compete low abundance proteins[27]. Hence, commercially available SEPPRO column was used to deplete the rubisco contamination of the protein samples. The SEPPRO columns are packed with an immunoaffinity matrix having avian IgY antibodies cross-linked to microbeads which specifically remove rubisco protein from plant extract.

The in-gel kinase assay of immunoprecipitated samples as well as samples eluted from various columns revealed a relatively lower molecular weight kinase activity in concentrated active fractions which had been depleted of rubisco contamination. We may mention here that size variation has also been reported for Cr32 protein in *Cowdria ruminantium* by varying the sample preparation methods[28]. The size variation observed for kinase activity in present case seems to result from loss, during column chromatography, of a part of the protein, non-essential for the kinase activity. It may be worth to mention here that increased sensitivity of lactate dehydrogenase enzyme to proteolysis was reported during copper-iminodiacetic acid (IDA) immobilized metal affinity chromatography (IMAC) even though activity of the enzyme remained high[29].

Enrichment of phosphoproteins by the phosphoprotein purification column was poor and most of the kinase activity was detected in flow-through. The probable reason seems to be presence of vanadate and fluoride ions in protein extraction buffer which are known to hamper binding of phosphoproteins to the

column resin. Most likely, for the same reason, immunokinase assay of phosphoprotein column eluates failed to detect any kinase activity.

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