Chapter 4: Results
4.1 Differential response of MIA pathway and related primary metabolic pathway genes to abiotic stress

Plants produce secondary metabolites in response to different stresses. The use of biotic and abiotic elicitors is a promising tool to improve the yields of products in cell culture systems (Moreno et al., 1995; Verpoorte et al., 1997; El-Sayed and Verpoorte, 2007). Plants respond to herbivory by producing defensive secondary metabolites such as proteinase inhibitors (antidigestive proteins), polyphenol oxidases (anti-nutritive enzymes and toxic compounds (De Luca and St. Pierre, 2000; Kessler and Baldwin, 2002). Alkaloids are toxic compounds which poison generalist herbivores. Treatment of C. roseus seedlings with methyl jasmonate (MeJA) resulted in doubling of alkaloid accumulation (Aerts et al 1994). The effect of UV-B irradiation on expression of MIA biosynthetic genes, Tdc and Str and the catharanthine production has been reported previously in C. roseus leaves (Hirata et al., 1993; Ouwerkerk and Memelink, 1999).

In our study we used three treatments namely, wounding, methyl jasmonate and ultraviolet (UV) rays of wavelength 253 nm to study their effect on expression of MIA pathway genes and accumulation of alkaloids. C. roseus plants growing in greenhouse were subjected to wounding, UV light (2 min) and MeJA (100 μM). The leaves harvested at indicated time intervals post treatment. The MIA pathway genes used for the transcript analysis were tryptophan decarboxylase (Tdc), strictosidine synthase (Str), desacetoxyvindoline 4-hydroxylase (D4h) and acetyl-CoA:4-O-deacetylvindoline 4-O-acetyltransferase (Dat) (Pease refer to Fig. 2.1 for details). C. roseus Actin (CrActin) was used as an internal control.

Semi quantitative RT PCR analysis of the genes under stress revealed that all the four genes of MIA pathway studied, showed induction in their expression (Fig. 4.1). Tdc showed fast induction after 1h of wounding which decreased at 3 and 6h. While MeJa and UV treatment showed gradual accumulation of Tdc transcript up to 6h. Str was induced very strongly by wounding and UV treatment at 3 and 6h while MeJa resulted in slight accumualtion of transcript at 6h. D4h showed strong induction at 6h under all the three stresses studied. Induction of Dat under wounding stress was observed at 3h which increased at 6h, while under UV treatment the induction in gene expression could be observed from 1h which increased with time. MeJa treatment also resulted in
the induction of Dat gene at 6h. Regulation of actin gene from C. roseus was taken as internal control.

Expression of genes of primary pathways related to Monoterpenoid Indole Alkaloid production was also studied under same stress. The genes studied were Anthranilate synthase (As), Deoxyxylulose phosphate reductase (Dxr) and Geraniol 10 hydroxylase (G10h) (refer to Fig. 2.1 for details). Ribosomal gene, Rps9 was used as internal control. The expression of all these genes was studied in response to wounding, UV treatment and Methyl jasmonate (Fig. 4.2). Wounding and methyl jasmonate caused a slight increase in the transcript of Dxr. However, UV treatment caused a progressive decrease in the transcript of Dxr with increased incubation. G10h was induced within 1h of wounding and methyl jasmonate application. However UV treatment caused decrease in the transcript of G10h with time. All the three treatments caused a strong induction of As transcript within 3h. Interestingly under UV treatment, a decline in As transcript was observed, which was almost absent at 6h.
Figure 4.2 Transcriptional regulation of TIA pathway related primary pathway genes by wounding, methyl jasmonate (MeJa) and UV treatment. 6-8 weeks old C. roseus plants growing in greenhouse were subjected to wounding, UV treatment and methyl jasmonate and leaves were harvested at indicated time intervals after treatment. 5 µg of RNA extracted from these tissues was used for cDNA synthesis and amplification of Dxr, G10h and As. Rps9 was used as internal control. The experiment was repeated three times with similar results.

4.2 Differential accumulation of alkaloids in response to various treatments

Since the MIA pathway genes exhibited differential transcript accumulation in response to the stimulation by wounding, UV rays and MeJA application, it was interesting to study the alkaloid accumulation in response to the respective treatments. Leaf tissue from greenhouse grown C. roseus var. Nirmal was subjected to wounding, UV light and Methyl jasmonate and incubated for 24 h, dried and subsequently used for alkaloid extraction as described in materials and methods. Control or unstressed samples were also processed simultaneously for alkaloid extraction. Among all the treatments, UV treatment was able to induce maximum accumulation of total alkaloids, followed by wound and MeJA treatment respectively, compared to the control (Fig. 4.3). However, highest accumulation of Vincristine and Vinblastine was recorded in MeJA treatment followed by UV and wounding, compared to control (Fig. 4.4). With regard to Ajmalicine and serpentine accumulation, UV light was most effective in inducing their accumulation (Fig. 4.4)
Figure 4.3 Differential accumulation of Total alkaloids. *C. roseus* leaf tissues were subjected to wounding, UV light, Methyl jasmonate (MeJA) application and were harvested after 24h for estimation of total alkaloids along with control samples. (n=3).

Figure 4.4. Accumulation of different MIAs in *C. roseus* leaf tissue subjected to wounding, UV light, MeJA treatment and control. 1 gm of dried leaf tissue from each of above mentioned treatments was used for alkaloid extraction and quantification of Ajmalicine (A), Serpentine (B), Vincristine (C) and Vinblastine (D). (n=3).

However, maximum vincristine and vinblastine accumulation was observed in response to methyl jasmonate treatment. Among the four alkaloids studied,
vinblastine was the most abundant one followed by vincristine, serpentine while ajmalicine was present in least quantity.

4.3 Study of protein phosphorylation upon different stress treatments
It has been reported that various forms of environmental stress including high osmolarity, heat shock, acidic stress and oxidative stress, induce the activation of corresponding protein kinases, HOG1 in budding yeast cells (Schuller et al. 1994), MAPKAPkinase-2 (Rous et al. 1994) and SAP kinase/c-Jun kinase (Kyriakis et al. 1994) in mammalian cells. Recently evidence has been accumulating that particular protein kinases in plant cells are also activated in response to various environmental stresses and phytohormones (Shinozaki and Shinozaki, 1997 and Ichimura et al., 2000). Mitogen activated protein kinase (MAPK) activation in response to wounding has been reported earlier (Seo et al., 1995, 1999; Usami et al., 1995; Bogre et al., 1997; Zhang and Klessig, 1998). MAP kinase activation in response to UV irradiation has been reported in higher plants (Stratmann et al., 2000), budding yeast cells and mammalian cells (Engelberg et al., 1994). Also Jasmonic acid has been known to activate MPK6, a MAPK from Arabidopsis (Takahashi et al. 2007). MAP kinase activation in response to cold and drought (Jonak et al., 1996) and during plant pathogen interaction (Ligterink et al., 1997; Zhang et al., 1998; Romeis et al., 1999) has also been reported.

Monoterpenoid indole alkaloid pathway is a stress regulated pathway. Several genes of the pathway have been reported to be upregulated in response to different elicitors. There are earlier reports of the involvement of the protein kinases in the elicitor mediated signal transduction leading to the expression of the MIA pathway genes. Therefore, in order to study the effect of different treatments on the phosphorylation status of the C. roseus proteome, the C. roseus plants were subjected to wounding, UV treatment and Methyl jasmonate application and possible activation of MAP kinase in response to these treatments was studied using myelin basic protein (MBP) as substrate for the activated MAP kinases.

4.3.1 MAPK activation by wounding
To study the effect of wounding on MAPK activity in C. roseus, seedlings of the plants were wounded to 40 % of their leaf blade and harvested at indicated time points post wound treatment. The samples were tested for MAPK activity by an ingel kinase
assay using MBP as substrate. The results indicated that there is activation of MAPKs of two different molecular weights, one around 47 kDa and other 43 kDa within 15 minutes of wound treatment and this activity decreases with increase in the incubation period (Fig. 4.5).

![Figure 4.5](image)

**Figure 4.5 Activation of MAP kinases by wounding in C. roseus.** Leaves of *C. roseus* plants growing in greenhouse were wounded and harvested at indicated time points after treatment. Protein extracts from these samples were tested for their kinase activity in an ingel kinase assay using MBP as substrate.

### 4.3.2 MAPK activation by UV light

To study the MAPK activation in response to UV treatment, the seedlings of *C. roseus* were exposed to UV treatment for 2 minutes and then incubated for indicated time points post UV treatment. The samples from each time point were studied in an ingel kinase assay. Activation of MAPK can be observed 15 min post UV treatment which was maximum at 30 min (Fig. 4.6). Interestingly, activation of multiple kinases was observed upon UV treatments. MAPKs are known to be a multigene family and hence activation of more than one MAPK by a specific stress stimulus can easily be envisaged.

![Figure 4.6](image)

**Figure 4.6 Activation of MAP kinases by UV treatment in C. roseus.** *C. roseus* plants growing in greenhouse were exposed to UV rays for 2 min and brought back to greenhouse. Leaves were harvested at indicated time points after treatment. Protein extracts from these samples were tested for their kinase activity in an ingel kinase assay using MBP as substrate.
4.3.3 MAPK activation by methyl jasmonate (MeJA)

To study the MAP kinase activation in response to Methyl jasmonate application, first a dose response of MeJa application on *C. roseus* leaves was analyzed. Different concentrations of methyl jasmonate, 10 μM, 50 μM, 100 μM, 500 μM and 1000 μM were applied to leaves of *C. roseus* seedlings by painting and were harvested 15 min post painting. The results indicated that 50 μM of Methyl jasmonate was most effective for MAPK activation (Fig. 4.7). This concentration of methyl jasmonate was used to study the MAPK activation in further experiments.

![Figure 4.7 Activation of MAP kinases by different concentrations of methyl jasmonate in *C. roseus*. *C. roseus* plants growing in greenhouse were painted with indicated concentrations of methyl jasmonate and leaves harvested 15 min post treatment. Protein extracts from these samples were tested for their kinase activity in an Ingel kinase assay using MBP as substrate.](image)

To study the time period kinetics of MAPK activation in response to methyl jasmonate application, 50 μM of MeJA was applied to *C. roseus* seedlings and incubated for indicated time periods post treatment. Samples from each time point were analyzed in an Ingel kinase assay. A 15 minute incubation post methyl jasmonate application was found to cause maximum MAPK activation after which it began to decrease (Fig 4.8)

![Figure 4.8 Activation of MAP kinase by methyl jasmonate treatment in *C. roseus*. *C. roseus* plants growing in greenhouse were painted with methyl jasmonate and leaves harvested at indicated time intervals after treatment. Protein extracts from these samples were tested in an Ingel kinase assay using MBP as substrate.](image)
4. 4 Cloning of MAPK from C. roseus

Mitogen activated protein kinase cascade is an important signaling cascade that operates in biotic and abiotic stress signaling in plants (Link et al., 2002b; Brader et al., 2007; Grant and Loake, 2007; Zhang et al., 2007; Link et al., 2002a; Sinha et al., 2002; Teige et al., 2004; Alzwiy and Morris, 2007). Several of the MIA pathway enzymes such as Tdc, Str, D4h and Dat get activated by MeJA. Further, JA biosynthesis and MeJA induced TDC and STR activity is sensitive to protein kinase inhibitor K-252a (Menke et al., 1999a), suggesting the involvement of protein phosphorylation in elicitor induced MIA biosynthesis. Also, our results of MIA pathway gene expression and MAP kinase activation in response to wounding, MeJA application and UV treatment prompted us to search MAP kinases from C. roseus and study their involvement in signaling cascade leading to the induction of MIA pathway gene expression upon elicitation.

With this objective in mind, a search to identify full length MAP kinase from C. roseus was initiated. A cDNA library of C. roseus in λZAP II was screened with a MAPK EST (GenBank accession No. AJ537469) probe. λZAP II cDNA library was generated from C. roseus leaves treated with yeast elicitor. Hybridisation was carried out under stringent conditions at a temperature of 55°C and final washing with 0.1 X SSC and 0.1% SDS. Primary library screening revealed a single positive plaque (Fig 4.9A).

![Figure 4.9 cDNA library screening to identify full length CrMPK3 clones. Positive plagues obtained after primary (A) secondary (B) and tertiary (C) screening of λZAP II library from Yeast elicitor treated C. roseus leaf cDNA and probed with MAPK EST.](image)

The positive plaque obtained after primary screening was picked and diluted in SM buffer before being plated at a titre of 20-50 plaques/plate so as to allow single plaque isolation. These positive plaques were carried to tertiary screening till plaque mixture
was completely overcome. The sequencing of positive plaques obtained after tertiary revealed two MAPKs. One 630 bp fragment showed maximum identity with *Wound Induced Protein Kinase* (WIPK) from *Nicotiana attenuata*. When this fragment was aligned with MAPKs sequences submitted in database from other plant species, it was found to lack in the 5’ end. To get the missing 5’ portion of the full length MAPK, a degenerate oligonucleotide was designed from 5’ end of the plant MAPK sequences showing maximum similarity with the identified 630 bp from *C. roseus*. PCR amplification of *C. roseus* cDNA was carried with 5’ degenerate and 3’ gene specific primers that resulted in an amplicon of about 1.1 Kb. The cloning and sequence analysis of amplicon led to identification of a 1119 bp long cDNA encoding distinct MAPK which was named as *CrMPK3* (Fig. 4.10).

![Figure 4.10](image-url) Amplification of *C. roseus* leaf cDNA with 5’ degenerate and 3’ specific primer for *CrMPK3*

### 4.5 Sequence analysis of full-length *C. roseus* MAP Kinase cDNA, *CrMPK3*

The complete sequence of *CrMPK3* cDNA was found to be 1119 bp long. The translation product of the full-length cDNA contains 372 amino acids with a predicted molecular weight of 43 kDa (Fig. 4.11). At the amino acid sequence level, *CrMPK3* revealed 87% sequence identity with *Solanum tuberosum* MAP kinase (StMAPK), 85% sequence identity with WIPK (Wound induced protein kinase) from *Nicotiana attenuata* and LeMPK3 from *Lycopersicon esculentum*, and 83% sequence identity with AtMPK3 from *Arabidopsis* (http://www.ncbi.nlm.nih.gov/BLAST) (Fig. 4.12). Phylogenetic analysis of *CrMPK3* was performed by multiple alignment of the amino acid sequences of *CrMPK3* and other related MAPKs from *Arabidopsis*, rice and some other plant species (http://www.ebi.ac.uk/clustalw). The dendrogram obtained
from such alignment showed the tree to be rooted with OsMPK6 which is an orthologue to Arabidopsis AtMPK6 (Fig. 4.13). AtMPK6 is linked to defense signal transduction pathways (Asai et al., 2002; Menke et al., 2004). A sphingolipid elicitor from *M. grisea* is known to activate OsMPK6 in cell suspension cultures (Lieberherr et al., 2005). Sequences used for phylogenetic analysis is being mentioned in Table 4.1.

![Figure 4.11 Complete CrMPK3 cDNA sequence and its translation product. The stop codon is indicated by “*”](image-url)

**Figure 4.11** Complete CrMPK3 cDNA sequence and its translation product. The stop codon is indicated by “*”.

63
Figure 4.12 CLUSTALW multiple alignment of amino acid sequence of CrMPK3 with other plant MAPKs. Amino acid sequence comparison of CrMPK3, *Lycopersicon esculentum* MPK3 (Holly et al. 2003), *Solanum tuberosum* MAP kinase (Deguchi et al. 2005), WIPK *Nicotiana attenuate* (Wu et al. 2007) and ATMPK3.
Table 4.1 List of genes whose amino acid sequences were used to study their phylogenetic relationship with CrMPK3.

<table>
<thead>
<tr>
<th>Label</th>
<th>Accession No</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsMPK3</td>
<td>A2XFC8</td>
<td>Song, F. and Goodman, R.M 2002</td>
</tr>
<tr>
<td>OsMPK6</td>
<td>ABO69383</td>
<td>Wang, S. and Yuan, B. 2006</td>
</tr>
<tr>
<td>NtF4</td>
<td>X83880</td>
<td>Wilson et al., 1995</td>
</tr>
<tr>
<td>SIMK</td>
<td>X66469</td>
<td>Jonak et al., 1993</td>
</tr>
<tr>
<td>PsD5</td>
<td>X70703</td>
<td>Stafstrom, J.P. 1993</td>
</tr>
<tr>
<td>ATMPK6</td>
<td>D21842</td>
<td>Mizoguchi et al., 1993</td>
</tr>
<tr>
<td>ZmMPK5</td>
<td>AB016802</td>
<td>Berberich et al., 1999</td>
</tr>
<tr>
<td>WIPK</td>
<td>D61377</td>
<td>Ohashi, Y. 1995</td>
</tr>
<tr>
<td>ATMPK3</td>
<td>D21839</td>
<td>Mizoguchi et al., 1993</td>
</tr>
<tr>
<td>ZmMPK4</td>
<td>AB016801</td>
<td>Berberich et al., 1999</td>
</tr>
<tr>
<td>AsMAP1</td>
<td>X79993</td>
<td>Huttly and Philips, 1995</td>
</tr>
<tr>
<td>LeMPK3</td>
<td>AAP20421/AY261514</td>
<td>Holley and Stratmann, 2003</td>
</tr>
<tr>
<td>StMAPK</td>
<td>BAE44363</td>
<td>Deguchi et al., 2005</td>
</tr>
<tr>
<td>CrMPK3</td>
<td>EF156758</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Bioinformatic analysis of CrMPK3 amino acid sequence revealed an approximate molecular weight of 43 kDa. The analysis of deduced amino acid sequences revealed that the protein contains all the 11 subdomains (Fig. 4.14) that are conserved among all the MAPK families (Hirt, 1997) and that it contains phosphorylation motif TEY between 7th and 8th subdomains (http://www.elm.eu). Prosite results also confirmed the presence of MAP kinase signature i.e F-X(10)-R-E-X(72,86)-R-D-X-K-X(9)-[CS] in CrMPK3 between 75-178 residues. The Motif Scan search showed that it has a Myb DNA binding domain (112-170) and DUF 1977 (233-245). The amino acid sequence of CrMPK3 is conspicuous by the absence of a signal peptide. The proteins without a signal peptide are unlikely to be exposed to N-glycosylation. However, potential glycosylation motifs detected by NetNGlyC 1.0 server are 137NLSE and 214NSSD. Cello v2.5 results (http://cello.life.nctu.edu.tw) show that it is a cytoplasmic protein with a reliability value of 3.928.
Figure 4.13 Phylogenetic relationship between CrMPK3 and MAP kinases from other plant species. The amino acid sequence of the proteins shown in this diagram is listed in Genbank Database. Under the following accessions, OsMPK3 (A2XFC8), OsMPK6 (AB069383), NiF4 (X83880), SIMK (X66469), PsD5 (X70703), ATMPK6 (D21842), ZmMPK5 (AB016802), WIPK (D61377), ATMPK3 (D21839), ZmMPK4 (AB016801), AsMAP1 (X79993), LeMPK3 (AAP20421), StMAPK (BAE44363).

Figure 4.14 Amino acid sequence of CrMPK3. Roman numerals indicate the different motifs of the protein. The TEY motif, typical phosphorylation motif of MAP kinases, between VIIth and VIIIth subdomains is underlined.
4. 6 Expression of CrMPK3 in Bacterial system

To demonstrate that CrMPK3 encodes a functional MAPK, CrMPK3 was expressed as a GST fusion protein in *E. coli* bacterial system. The open reading frame of CrMPK3 was cloned into pGEX-4T2 expression vector (Fig. 4.15A). The resulting construct was introduced in *E. coli* strain BL-21 (DE3) and the transformants grown in liquid medium containing 100 mg/ml of ampicillin. Fusion protein was induced by adding 1 mM of IPTG in the overnight grown liquid culture of the bacteria and incubating it at 37°C for four hour. A thick protein band (around 66 kDa) visible in the extreme left lane corresponds to the expressed protein (Fig 4.15B). However, no such band is observed in the lane containing crude protein extracted from culture of transformants in which no IPTG was added. Transformants containing only vector (pGEX-4T2) showed the induction of GST by the addition of 1 mM IPTG although no GST induction was observed when no IPTG was used.

![Figure 4.15 Bacterial expression of GST fused CrMPK3 protein.](image)

(A) Vector Map of pGEX 4T2 used for expression of CrMPK3 in *E. coli*. (B) Induction of GST-CrMPK3 recombinant protein in *E. coli*. Lane 1- Protein Molecular weight Marker, Lane 2 and 3 show uninduced and induced GST respectively while lane 4 and 5 show uninduced and induced recombinant protein respectively. (C) Purification of recombinant protein. Lane 1- Protein Molecular weight marker. Lanes 2, 3 and 4 show first, second and third elutions respectively.
Thus, CrMPK3 protein fused with GST could be successfully expressed in heterologous system. The bacterially expressed glutathione S-transferase (GST)-CrMAPK3 fusion protein was affinity purified using a GST tag (Fig. 4.15C).

4. 7 In vitro phosphorylation study of CrMPK3

To establish the kinase activity of CrMPK3, purified GST-CrMPK3 recombinant protein was analyzed in an in vitro kinase assay. To rule out the kinase activity due to presence of GST tag, purified GST protein was also used as a negative control in the experiment. Both GST and GST-CrMPK3 proteins were incubated with or without MBP in presence of radio labeled $[^{\gamma-32P}]$ ATP for 20 min and loaded on to the SDS PAGE.

CrMPK3 exhibited autophosphorylation as well as ability to phosphorylate MBP (Fig 4.16) indicating that bacterially expressed CrMPK3 is an active MAP kinase. GST alone or with MBP failed to show any phosphorylation. This clearly shows that phosphorylation is because of CrMPK3 and not due to presence of GST tag.

![Figure 4.16 In vitro kinase activity of recombinant CrMPK3. Bacterially expressed CrMPK3 protein was tested for kinase activity in the presence and absence of MBP in in-vitro kinase assay.](image)

To further validate the phosphorylating activity of the fusion protein, different dilutions (1 $\mu$g, 0.1 $\mu$g, 0.01 $\mu$g nd 0.001 $\mu$g) of GST-CrMPK3 fusion protein were subjected to an ingel kinase assay. As is obvious from the results, a decrease in the concentration of the fusion protein is accompanied with a corresponding decrease in the MBP phosphorylation (Fig. 4.17).
To increase the sensitivity of the assay, we generated a kinase inactive version of bacterially expressed CrMPK3 and tested this protein for its activity in an ingel as well as in vitro kinase assay using MBP as an artificial substrate. The recombinant protein showed autophosphorylation as well as phosphorylation of MBP. On the other hand kinase inactive form in which ATP binding site was modified by changing lysine residue at 69th position to arginine residue by site directed mutagenesis resulted into kinase inactive form, GST-CrMPK3\textsuperscript{K69R} was unable either to autophosphorylate or phosphorylate MBP (Fig. 4.18).

**Figure 4.17** An ingel kinase assay of the recombinant CrMPK3 using serial dilution of the protein 1 μg (lane 1), 0.1 μg (lane 2), 0.01 μg (lane 3) and 0.001 μg (lane 4) using MBP as substrate.

**Figure 4.18** In vitro kinase activity of recombinant CrMPK3 and its Kinase inactive version CrMPK3\textsuperscript{K69R}. 
4. 8 Abiotic stresses induce the expression of CrMPK3

The phylogenetic analysis of CrMPK3 revealed its close proximity to WIPK, AtMPK3, OsMPK3 and StMAPK (StMPK1). Since WIPK and its subgroup members exhibit increases in both their enzymatic activity and mRNA levels in response to various stimuli (Seo et al. 1995; Jonak et al. 1996; Bogre et al. 1997; Ligterink et al. 1997; Zhang and Klessig 1998a, 1998b). WIPK is activated within 15 min of wounding (Seo et al., 1999). Desikan et al. reported AtMPK3 induction in response to ROS and UV-B. StMPK1 is also induced in response to wounding (Blanco et al., 2006). Xiong and Yang (2003) reported a significant reduction in abiotic stress tolerance in OsMPK3 silenced rice plants. So in order to investigate whether *CrMPK3* gene is also induced by abiotic stresses, 2-months-old *in vitro* grown *C. roseus* plants were subjected to different stress conditions like wounding and UV treatment and methyl jasmonate application. The change in the transcript level of *CrMPK3* in response to these stresses was analyzed by northern blot analysis. A significant increase in the level of *CrMPK3* transcript was noted within 30 minutes of these treatments (Fig. 4.19). In case of wounding and MeJA treatments, the transcript level started to decline after 30 min of treatment. However, UV treatment was able to maintain high levels of the *CrMPK3* transcript even upto 2 hours post treatment before showing decline in the transcript abundance. The accumulation of CrMPK3 transcripts in response to wounding, MeJA and UV treatment suggests a role for CrMPK3 in stress mediated responses.

![Figure 4.19 Expression analysis of CrMPK3 transcript during different treatments. *C. roseus* leaves were subjected to wounding, UV and methyl jasmonate application and harvested at indicated time intervals post treatment. 20 µg of RNA from each sample was loaded on gel. Radiolabeled CrMPK3 cDNA was used as probe for northern hybridization. The lowermost panel shows the methylene blue stained rRNA for equal loading and RNA quality.](image-url)
4. 9 CrMPK3 is activated by wounding and UV treatment

As we already observed activation of MAP kinases in response to wounding, UV rays and MeJa application in *C. roseus* leaves, we extended this finding to analyze whether CrMPK3 specially gets activated by these stresses or not. *C. roseus* plants were subjected to wounding, UV treatment and methyl jasmonate application and leaf tissues were harvested at different time points. To confirm that CrMPK3 is activated by wounding and UV treatment, the protein extracts from wounding and UV treated *C. roseus* leaf tissues were immunoprecipitated with antiphosphotyrosine antibody 4G10. This monoclonal antibody 4G10 specifically identifies phosphorylated tyrosine residue and MAP kinases are known to be activated upon phosphorylation at tyrosine residues. The immunoprecipitates were subjected to western blot analysis with anti-CrMPK3 antibody. Antibody against CrMPK3 was developed by injecting heterologously overexpressed and purified GST-CrMPK3 protein in rabbit. A distinct activation of 43 kDa MAP kinase was recognized by anti-CrMPK3 antibody in wounding and UV treated tissues compared with control. However, no MAPK activation was observed in immunoprecipitates of MeJA treated tissues (Fig. 4.20). This shows that CrMPK3 is activated in response to wounding and UV treatment and that some other member of MAPK is involved in MeJA signaling pathway.

![Figure 4.20 Activation of CrMPK3 by wounding and UV treatment.](image)

*C. roseus* plants growing in greenhouse were subjected to wounding, UV and Methyl jasmonate treatment. Protein extracts from control and treated samples were immunoprecipitated with antiphosphotyrosine tyrosine anti-body and immunoblot analysis with anti-CrMPK3 antibody was carried out.
4.10 Subcellular localization of CrMPK3

The nuclear translocation of MAP kinases in response to external stimuli is already reported (Ligterink et al. 1999). Most of the targets reported so far for MAP kinases are transcription factors. Therefore, MAP kinase needs to migrate inside nucleus upon activation in order to activate downstream components. With this curiosity, we studied the localization of CrMPK3 in the cells of C. roseus. For the purpose, CrMPK3 coding region was fused in frame to the coding region of N- terminal side of GUS and GFP under the control of the 35S promoter of cauliflower mosaic virus (CaMV) in the binary vector pCAMBIA 1303 (Fig. 4.21A).

![Diagram of T-DNA region of the pCambia 1303 binary vector.](image)

Figure 4.21 Subcellular localization of CrMPK3-mGFP Fusion Protein in Catharanthus roseus as viewed by Confocal Laser Scanning Microscopy. (A) T-DNA region of the pCambia 1303 binary vector used to study the subcellular localization of CrMPK3. (B) Subcellular localization of CrMPK3-GFP fusion protein in intact C. roseus leaf disc, wounded leaf discs after 5 min. and 10 min. of incubation. Cells were visualized 5 days after Agrobacterium infection.

The resulting binary construct was used for the transient transformation of C. roseus leaf discs. The transformed leaf discs were analyzed under confocal microscope after five days of transformation. The fusion protein was found to be completely localized to cell wall. When the same leaf discs were wounded by cutting the section into pieces and observed after 5 minutes of wounding, fusion protein showed movement from cell wall towards cell interior. However, when the same leaf discs were observed after 10 minutes of wounding, the fusion protein was completely localized into nucleus (Fig 4.21B). The observation clearly indicated that activated CrMPK3 migrates
towards nucleus upon wounding. The nuclear migration of CrMPK3 in response to wounding suggests that CrMPK3 activates transcription factors that regulate wound responses.

4. 11 CrMPK3 overexpression increases MIA pathway gene expression

As a distinct co-regulation was observed between expression of CrMPK3 and that of MIA pathway genes during abiotic stresses, we were interested to study the effect of CrMPK3 overexpression on MIA pathway gene expression. For this purpose, CrMPK3 was cloned in plant binary vector pCambia 1303 under the control of 35S CaMV promoter (Fig. 4.22A). Agrobacterium tumefaciens strain 3101 was transformed with the binary construct and the positive colonies were checked by colony PCR. The positive colony having CrMPK3 in correct frame was used in transient transformation of Catharanthus roseus. C. roseus leaves were transiently transformed with CrMPK3 overexpression construct by vacuum infiltration and studied for MIA pathway gene expression by semi quantitative RT-PCR analysis. As a control, C. roseus leaves were also transiently transformed with empty vector pCambia 1303. 72 hrs post transient transformation, the samples were harvested and used for RNA extraction. Semi-quantitative RT-PCR analysis revealed a distinct upregulation in the expression of D4h, Dat and Tdc in the leaves transiently expressing CrMPK3 compared to the empty vector control (Fig 4.22B). The expression of ZCT1, a repressor of MIA pathway genes was reduced in CrMPK3 overexpressing leaves, while there was no effect on a the positive regulator of the MIA pathway genes, ORCA3. The increased expression of Tdc, D4h and Dat in part can be explained due to reduction in the transcript of ZCT1. However no effect on the transcript of ORCA3 due to overexpression of CrMPK3 and increase in the transcript accumulation of Tdc, D4h and Dat is difficult to correlate with present information. Ribosomal gene, Rps9 was taken as internal control and was found to be similar in both the transient transgenics used.
Figure 4.22 Transient expression of CrMPK3 in C. roseus leaves by vacuum infiltration. (A) T-DNA region of the pCambia 1303 binary vector used for overexpression of CrMPK3. (B) Semiquantitative RT-PCR analysis of CrMPK3 and TIA pathway genes in C. roseus leaves transiently transformed with empty vector and binary plasmid containing CrMPK3. RpS9 was used as an internal control.

4.12 Functional Characterisation of CrMPK3 in budding yeast

There are several reports regarding use of yeast mutants to study the plant abiotic stress tolerance determinants (Sakamoto et al, 2004; Moretti et al, 2006). CrMPK3 transcripts as well as enzyme activity was upregulated in response to wounding and UV stress indicating its role in stress tolerance. Further, yeast and plant stress tolerance mechanisms share several similar pathways. It was therefore, worthwhile to study the function of CrMPK3 gene in yeast. With this objective, the reading frame of CrMPK3 was cloned in yeast vector pYES2.1-V5/His-TOPO and expressed under a galactose inducible promoter in protease deficient budding yeast S. cerevisiae strain.
BCY 213 (Fig 4.23A, B). Cloning of CrMPK3 in pYES2 vector was confirmed by restriction digestion of the plasmid prepared from \textit{S. cerevisiae} (Fig. 4.23C).

Figure 4.23 Cloning of CrMPK3 in pYES2.1/V5 His-TOPO expression vector. (A) Map of pYES 2.1 expression vector (B) CrMPK3 cloned in pYES2.1. (C) Restriction digestion of pYES 2.1-CrMPK3 plasmid with Xho I and BamHI to release the insert.

As a control, BCY213 strain of \textit{S. cerevisiae} was transformed with vector alone. The transformants were selected by growth on synthetic dropout (SD) medium lacking Uracil. Cultures from both the transformants, empty vector as well as CrMPK3 transformed colonies were subjected to stress treatments like heat shock (42°C), cold shock (4°C) and UV treatment (45 min exposure). In contrast to empty vector transformed control, CrMPK3 transformed colonies survived well in heat shock treatment (Fig 4. 24A). This indicates that CrMPK3 enhances the survival rate of yeast during heat shock treatment. Similar effect was also observed when the yeast transformants were exposed to UV rays. CrMPK3 transformed colonies showed better survival than empty vector control during UV exposure (Fig. 4.24B). However, in response to cold shock there was not much difference in the two transformants although CrMPK3 transformants were marginally better that empty vector control (Fig 4.24C).
Since the yeast strain BCY213 transformed with CrMPK3 showed enhanced tolerance to heat shock as compared to the vector alone transformants, the MAP kinase activation in response to heat in planta was analyzed. For this purpose, *C. roseus* plants were subjected to heat treatment at 42 °C and samples harvested at different time intervals. Protein extracts from the samples harvested at different time intervals were subjected to ingel kinase assay. A distinct fast and transient activation of MAPK was observed within 10 min of heat treatment that decreased gradually (Fig. 4.25). The yeast complementation study revealed that CrMPK3 is also involved in heat mediated signaling in *C. roseus*.

**Figure 4.24 Stress tolerance of yeast cells transformed with CrMPK3**  
(A) Yeast strain BCY213 harbouring only vector (pYES2.1) or CrMPK3 was incubated at 42 °C for 30 min. before spotting them onto SD/-Ura/-lue medium were shown after incubation at 30°C for 2 d or 4 d respectively. (B) The same strain, as in A, was exposed to UV for 45 min or (C) 4 °C for 1 hr. before spotting onto SD/-Ura/-lue medium.

**Figure 4.25 Activation of MAP kinases by heat treatment in *C. roseus***  
*C. roseus* plants were subjected to heat treatment and leaves harvested at indicated time intervals after treatment. Protein extracts from these samples were tested for their kinase activity in an ingel kinase assay.
4.13. Biochemical separation of wound induced mitogen activated protein kinase in *C. roseus*

One of the several environmental stresses plants are often subjected to is wounding, which may be caused mechanically, by pathogen or herbivore attack. To overcome this stress, plants have developed defence systems which involve release of pathogen response (PR) proteins or proteinase inhibitors (PIs) (Jonak et al., 1999). There are several reports that implicate MAP kinases in wound induced signaling that leads to activation of defence response genes. In tobacco, wounding (cutting) activates a 46 kDa kinase (Usami et al., 1995). In alfalfa also, a specific 46 kDa protein kinase is activated by wounding (Bogre et al., 1997). Moreover, systemin, a 18 amino acid peptide that confers systemic wound response in tomato, also activates 48 kDa MBP kinase that is tyrosine phosphorylated upon wounding (Stratmann and Ryan, 1997).

In the present work, we also report the activation of a 47 kDa MAP kinase upon wounding in *C. roseus*. In Catharanthus, wounding is also reported to induce the accumulation of alkaloids (Vazquez Flota et al., 2004). Henceforth, an attempt was made to characterize wound activated MAP kinase using biochemical approach so as to establish its probable role in wound activated signaling leading to the accumulation of MIAs.

4.13.1 Activation of wound induced protein kinase

It was observed that wounding induces activation of MAP kinase in *C. roseus* leaves. An attempt was made to biochemically enrich the activated protein kinase and to uncover its identity. For the purpose, *C. roseus* leaves were wounded as described previously, and leaf samples were collected after 15, 30 and 60 minutes after wounding. Analysis of the proteins isolated from the wounded samples by ingel kinase assay revealed activation of two MBP phosphorylating kinases with molecular weight around 43 and 47 kDa (Fig.4.26A).
In order to further characterize these kinases, an immunoprecipitation assay using antiphosphotyrosine antibody 4G10 was performed with protein extract of wounded C. roseus leaf tissue. The immunoprecipitates were subjected to an in gel kinase assay using MBP as an artificial substrate. Immunoprecipitate from wounded leaf tissue showed MBP phosphorylation around 47 kDa while no MBP phosphorylation was observed from unwounded control sample (Fig. 4.26B). The data indicated that the activated kinase is indeed a member of MAPK family as antiphosphotyrosine antibody, 4G10 specifically identifies phosphorylated tyrosine residue. MAPKs are known to get activated upon phosphorylation at tyrosine residue by upstream MAPKK.

4.13.2 Purification of Wound Activated MAP Kinase

Wounding of Catharanthus leaves activates 47 kDa MBP phosphorylating kinase. For further identification and characterization of wound induced protein kinase, biochemical separation and partial purification was carried out using column chromatographic methods. The protein lysate from wounded C. roseus leaf tissue was subjected to anion exchange chromatography after a high speed centrifugation. The isolated protein was subjected to ultra centrifugation at 40K followed by separation on a strong anion exchange, Resource Q column. The column was equilibrated with 30 ml of Buffer A [Tris (pH7.5) 25mM, EGTA 1mM, beta Glycerol phosphate 10 mM, Sodium orthovanadate 0.1 mM, DTT 1 mM, PMSF 1 mM and 5% glycerol] at a flow rate of 3 ml/min and protein sample was injected in to the column at the rate of 1 ml/min up to 20 ml with buffer A. The column was washed to remove the unbound protein with buffer A at a flow rate of 3 ml/min. The bound protein was eluted from the column with a linear gradient of 0 to 500 mM NaCl at the flow rate of 1 ml/min in 50 ml volume and were collected in 2 ml fractions. The eluted protein profile was monitored at 280 nm and depicted as typical chromatogram (Fig. 4.27).
Figure 4.27 Elution profile of protein extracted from wounded *C. roseus* leaf tissue from anion exchange fast protein column chromatography using Resource Q Column. Protein was eluted using a gradient of 50% buffer B (25mM Tris-Cl +0.5M NaCl) at a flow rate of 1 ml/min. Elution was monitored by absorbance at 280 nm.

The eluted fractions were analyzed for MBP phosphorylating activity by invitro kinase assay. As the results indicated the eluted fractions between 32 to 40 ml of elution volume showed MBP phosphorylating activity. The highest activity peak was observed at 36th ml of elution volume in 386 mM NaCl concentration (Fig. 4.28A). Most of the protein bound to the column was eluted at concentration higher than 500 mM NaCl without showing any MBP phosphorylating protein kinase activity (Fig 4.28B).
Figure 4.28 Elution and MBP phosphorylating activity profile of protein extracted from wounded *C. roseus* leaf tissue by anion exchange fast protein column chromatography. (A) Selected fractions from the elution volume were assayed in an in vitro kinase assay with MBP as substrate (numbers on the top indicates fraction numbers). (B) Chromatogram representing the elution profile and relative kinase activity.

The active fractions with MBP phosphorylating activity were pooled, concentrated by dialysis and analysed for activity by in-gel kinase assay. The identity of this MBP phosphorylating kinase was further confirmed by in-gel kinase assay where an MBP phosphorylation was observed around 47 kDa (Fig. 4.29).
175 kDa \rightarrow
82 kDa \rightarrow
62 kDa \rightarrow
47.5 kDa \rightarrow
34 kDa \rightarrow
25 kDa \rightarrow

Fig. 4.29 Ingel kinase assay of the protein eluted from ResourceQ anion exchange column. The active fractions were pooled and concentrated by dialysis against buffer A containing 50% PEG. The resulting concentrated protein was used for ingel assay.

In order to visualize the protein, the gel was stained by silver staining, protein band was excised and sequenced by MALDI-TOF. The sequencing results of the tested protein have shown maximum hits with rubisco protein. This might be due to co-purification of the rubisco protein along with the protein of interest. Rubisco, being most abundant protein in laves are known to interfere with purifications of many proteins in plants. To overcome the rubisco contamination, the active fractions obtained from Resource Q column were pooled, concentrated and loaded in a Seppro column for exclusion of rubisco protein. The Seppro Rubisco Spin Columns are based on avian antibody (IgY)-antigen interactions and optimized buffers for sample loading, washing, eluting, and column regeneration. They are specifically designed to remove D-Ribulose 1, 5-Diphosphate Carboxylase (Rubisco) from plant samples. Rubisco is removed by the immobilized specific IgY when filtered biological samples are passed through the column. Selective immuno-depletion of the highly abundant rubisco provides enriched flow-through fractions of low abundance proteins for further study and downstream proteomics analysis. A phosphoprotein purification column (Qaigen) was also used to enrich the phosphorylated proteins. However, the column failed to sufficiently enrich the phosphoproteins. The eluted protein samples from the seppro column (QS) together with control sample (C), wounded crude protein sample (CR), rubisco depleted wounded sample from seppro column (S), protein from a phosphoprotein column (P) and immunoprecipitated protein from seppro elute (IPS) were analysed by ingel kinase assay for the activity of MAPK.
distinct MBP phosphorylating kinase activity band was observed around 47 kDa (Fig. 4.30A). The same gel was subsequently silver stained to visualise the protein after intense washing to remove all the traces of radioactivity (Fig. 4.30B).

![Image of gel bands](image)

**Figure 4.30** Ingel kinase assay results (A) and silver staining (B) of the protein samples from different stages of purification. C-Control protein sample, CR-wounded crude protein sample, S-Seppro column, P-phosphoprotein purification column, QS-. Resource Q sample after Seppro column, IPS Seppro samples after immunoprecipitation with antiphosphotyrosine antibody.

The protein band at the size corresponding to the signal observed during ingel kinase active assay was excised. In-gel tryptic digestion of the excised protein band was carried out followed by LC-MS analysis. The resulting spectrum from LC-MS was used to search for matching proteins in MSDB using Mascot search program. The search yielded to a hypothetical protein from Arabidopsis (gi T06295) with a top score of 30 and patellin-1 from *Cucurbita pepo* (gi Q200V7_CUCPE) with a score of 29. Interestingly, protein kinase from *Medicago trunculata* (gi Q1SSW1_MEDTR) and serine/threonine protein kinase like from *Oryza sativa* (gi Q5ZBU5_ORYSA) (Table 4.2) were also observed in the hits but with poor score. Unfortunately there was no similarity with any of the known MAPK could be observed. One reason may be that the actual abundance of MAPK protein is very low in leaf and though it can be detected in a very sensitive ingel kinase assay the physical identity could not be detected in LC-MS analysis.
Table 4.2 List of the hits matching to peptides obtained from digestion of protein obtained after partial purification and LC-MS analysis.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Locus ID</th>
<th>Name</th>
<th>Organism</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T06295</td>
<td>Hypothetical protein T9E8.120</td>
<td>Arabidopsis thaliana</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Q2Q0V7_CUCPE</td>
<td>Patellin 1</td>
<td>Cucurbita pepo</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>Q1SSW1_MEDTR</td>
<td>Protein kinase</td>
<td>Medicago truncatula</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Q5ZBUS_ORYSA</td>
<td>Serine/threonine protein kinase-like</td>
<td>Oryza sativa</td>
<td>17</td>
</tr>
</tbody>
</table>