Chapter 8
Chapter 8

Agrobacterium mediated transformation and development of resistance to mosaic disease in *A. paeoniifolius*

8.1 Introduction

Considering the importance of *A. paeoniifolius* as a cash crop and the diseases presenting serious constraints to the production of this crop, resistance development becomes a high priority goal. As an alternative to conventional breeding programs, development of virus resistance can be achieved through transgenic strategy (Zhang et al. 2005; Chellappan et al. 2004). As an initial step towards developing resistance in *A. paeoniifolius* through transgenic approach, an efficient transformation protocol has to be established. The agrobacterium mediated transformation is the most utilised and competent one among the various transformation strategies. However the method has to be optimised for a system, the concerned plant or the explant source. Although the method is applied commonly in dicotyledonous plants, *A. paeoniifolius* being a monocotyledonous plant is difficult to get transformed as it is not a natural host for *Agrobacterium*. The efficiency of transformation protocol can easily be verified with reporter genes, the highly exploited one being β-glucuronidase (GUS) reporter system. The *uidA* gene of *E. coli* encoding GUS has been utilised in numerous plant species as a reporter gene to confirm transformation (Jefferson 1987; Wijayanto and McHughen 1999; Basu et al. 2004). Upon integration into genome, GUS leads to blue colouration upon simple biochemical analysis. In the present study, GUS gene has been utilised to study the transformation efficiency of various explants of *A. paeoniifolius*. In order to bring virus resistance, the antiviral RNA silencing mechanism operating naturally in plants can be activated by integrating a viral gene fragment into the genome of a susceptible plant. This can cause degradation of invading target virus, thus protecting the plant from virus infection. In the present study, virus CP based hairpin construct (DsMV-hp) validated in *N. benthamiana*, has been utilised to develop resistance in *A. paeoniifolius*. The overall objective of the concerned study was the study on transformation efficiency of various explants using GUS gene and
subsequent transformation of the most efficient explant source with DsMV-hp construct.

8.2 Materials and Methods

8.2.1 Transformation with GUS gene

8.2.1.1 Transformation explant source

The callus cultures established from corm bud explant (Figs. 8.1 a and 8.1 b) and swollen petiole (Fig. 8.1 c) of *A. paeoniifolius* were used as explants for agrobacterium mediated transformation using GUS gene.

8.2.1.2 Bacterial strain and media

The culture of *A. tumefaciens* strain AGL0 harbouring the plant transformation plasmid pOYE153 having *uidA* gene conferring GUS activity and *nptII* marker was used to study the transformation efficiency of various explants. The bacterial strain was cultured in YEB medium containing MgSO4 (2 mM) and kanamycin (100 mg l\(^{-1}\)). The preparation of media and the antibiotics used in the study are given in Appendix IV and V. The strain was allowed to grow in 2 sets of medium one with acetosyringone (200 µM) and the other without acetosyringone at 28°C.

8.2.1.3 Preparation of Agrobacterium for transformation

The cultures of *A. tumefaciens* carrying the constructs (OD\(_{600}\) = 1.0) were centrifuged at 5000 rpm for 10 min. and the pellet was washed twice in liquid MS followed by suspension in the same volume of liquid MS.

8.2.1.4 Agrobacterium mediated transformation with GUS gene

Friable callus as well as swollen petiole explants were co-cultured in a Petridish containing *A. tumefaciens* culture and shaken for 30 min and 60 min respectively. The explants were dried on a filter paper. The explants were co-cultivated on CIM (modified MS medium supplemented with 0.5 mg l\(^{-1}\) each of BAP, 2,4-D and NAA) with and without acetosyringone. After 4 days of co-
cultivation at 22°C in dark, the explants were washed thrice with sterile distilled water followed by liquid MS medium supplemented with ticarcillin (500 mg l⁻¹) twice. Then the explants were allowed to grow on selection medium (CIM with 500 mg l⁻¹ ticarcillin and 15 mg l⁻¹ geneticin).

8.2.1.5 GUS assay and molecular analysis

GUS assay (Jefferson et al. 1987) was done to confirm the transformation event. GUS buffer was prepared with 50 mM Na₂HPO₄, 1 mM Na₂EDTA, 1 mM Fe³⁺/Fe²⁺CN and 0.1% Triton X-100. The pH was adjusted to 7.0 with NaOH; filter sterilised and kept in amber coloured bottles at 4°C. The stock solution of 25 mg ml⁻¹ 5-bromo-4-chloro-3 indolyl-β-D-glucuronic acid (X-Gluc) was prepared in dimethyl sulfoxide and was stored at -20°C. The GUS assay buffer was prepared freshly by mixing 1 part of X-Gluc with 49 parts of GUS buffer. The explants were incubated overnight in GUS assay buffer at 37°C and were washed with 70% ethanol.

The putative transgenic callus and swollen petiole explants of A. paeoniifolius found free from Agrobacterium through 4 subsequent subcultures in ticarcillin containing selection medium were used for DNA isolation with GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich) following the procedure described in section 7.2.4.4. The DNA was amplified with GUS specific primers (GUS-F 5’-GGGCATTCAGTCTGGATC-3’ and GUS-R 5’-GTGGCAATTTCAGTCTGGATC-3’) by incubating at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min and a final extension of 72°C for 10 min. The reaction mixture for the amplification contained 5 ng μl⁻¹ DNA, 1X PCR buffer, 0.5 mM dNTP mix, 0.5 pmol μl⁻¹ each of forward and reverse primer, 0.05 Units μl⁻¹ Taq DNA Polymerase. The vector plasmid was used as positive control, whereas the DNA isolated from untransformed callus was used as negative control. Amplified PCR products were electrophoresed on 1.0% agarose gel with ethidium bromide and visualized under UV light. Transformation efficiency was determined by dividing the total number of PCR positive explants by the number of explants inoculated and then multiplied by 100.
8.2.2 Transformation with DsMV-hp construct

8.2.2.1 Explants and bacterial strain

Friable embryogenic callus (FEC) cultures (Fig. 8.1 d) were used as explants. *A. tumefaciens* strain LBA4404 carrying DsMV-hp construct was used for resistance development. It was cultured in YEB medium containing MgSO$_4$ (2 mM), kanamycin (100 mg l$^{-1}$), rifampicin (20 mg l$^{-1}$) and acetosyringone (200 µM) at 28°C. The culture was prepared for the transformation as explained in section 8.2.1.3.

![Fig. 8.1: Explant source of *A. paeoniifolius* for transformation](image)

**Fig. 8.1: Explant source of *A. paeoniifolius* for transformation**

a. sprouted corm bud, b. callus, c. swollen petiole, d. friable embryogenic callus
8.2.2.2 Co-cultivation of explants and *in vitro* propagation

A drop of the bacterial culture was placed on the 1 cm\(^2\) size callus and co-cultivated on SR medium (modified MS medium with 5 mg l\(^{-1}\) each of BAP and NAA) augmented with acetosyringone (200 µM) for 4 days in dark at 22°C. 60 such callus mass were used for transformation. The co-cultivated explants were washed thrice with sterile distilled water followed by liquid MS medium supplemented with ticarcillin (500 mg l\(^{-1}\)) twice. Then the explants were allowed to grow on SR medium supplemented with ticarcillin (500 mg l\(^{-1}\)) for 10 days followed by subculture on SR medium with ticarcillin and genetin (15 mg l\(^{-1}\)).

8.2.2.3 Molecular analysis

DNA and RNA was isolated from the microshoots arising from the individual callus mass using GenElute Plant Genomic DNA Miniprep Kit (section 7.2.4.4) and Spectrum Plant Total RNA Kit (section 7.2.4.5) from Sigma Aldrich. PCR with *npt* II loc/ups primers was done to confirm the presence of the transgene. The PCR reaction was done at 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min and a final extension of 5 min. RT-PCR with DHP primers was followed to record the transgene expression in the transformed cultures.

The first strand cDNA was synthesized using M-MLV Reverse transcriptase (Merck Genei). The cDNA was amplified in a reaction mixture of 20 µl containing 2.5 µl of 10X PCR buffer, 1.0 µl of 10 mM dNTPs, 0.25 µM each of DHP primers and DHP-R and 0.05 Units µl\(^{-1}\) Taq DNA Polymerase. The mixture was incubated at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min and a final extension of 10 min at 72°C. NASH was done following the protocol described in section 3.2.2.4. The RNA from putative transgenic lines as well as non-transformed control lines was spotted randomly on nylon membrane. Non-radioactive DsMV-hp construct specific probe (explained in section 7.2.4.6) was used for hybridisation.
8.3 Results and Discussion

8.3.1 GUS assay and molecular analysis

Transformed callus as well as swollen petiole explants was selected based on its survival on antibiotic selection medium. *Agrobacterium* has been successfully utilised here for genetic transformation and has been reported in many monocotyledon species like rice (Hiei et al. 1994), wheat (Cheng et al. 1997; Sahrawat et al. 2003), maize (Frame et al. 2002) and rye (Popelka and Altpeter 2003). GUS assay proved to be effective in confirming the transformation event within a short period after transformation. The GUS assay of transformed explants (swollen petiole and callus) showed positive blue colour irrespective of the type of explants while the untransformed ones did not yield blue colouration (Figs. 8.2 a, 8.2 b and 8.2 c). For effective GUS sector observation and photography, TS View Version 7.3.1.7. was utilised. The swollen petiole explants showed a transformation efficiency of 3 and 33 in the absence and presence of acetosyringone respectively. However on comparison with the petiole explants, the use of callus as explant showed increased efficiency of 13 and 36 in the presence and absence of acetosyringone. Comparative analysis of transformation efficiency as influenced by explant type and acetosyringone is given in Table 8.1. Only a single concentration of acetosyringone (200 µM) that has proved effective in the preliminary transformation experiments has been used in the study. The presence of acetosyringone was found to increase the transformation efficiency by 20 fold. Early reports also support the fact of enhancing effect of acetosyringone on transformation (Sheikholeslam and Weeks 1987; Suma et al. 2008). The callus was found to be more suitable for transformation due to its ease of regeneration as well as high transformation efficiency of 36.67%. Ban et al. (2009) also used callus cultures of *A. konjac* for agrobacterium mediated transformation for soft rot disease resistance development. After 4 weeks of incubation in selection medium, the transformed callus showed good growth while the untransformed underwent browning without any further growth (Fig. 8.3). PCR amplification of transgenic callus with GUS specific primers which survived in the selection medium gave a band at 400 bp while the non-transformed control callus failed to give amplification (Fig. 8.4).
Fig. 8.2: GUS assay of *A. paeoniifolius*

a. untransformed callus, b. transformed callus, c. untransformed and transformed swollen petiole explants
Table 8.1: Effect of explants and acetylsyringone on transformation efficiency in *A. paeonii folius*

<table>
<thead>
<tr>
<th>Explants</th>
<th>Acetylsyringone (µM)</th>
<th>No. of explants inoculated (~0.5cm²)</th>
<th>No. of GUS positive explants</th>
<th>No. of blue sectors per explant</th>
<th>No. of PCR positive explants</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>5</td>
<td>1-10</td>
<td>4</td>
<td>13.33</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>30</td>
<td>14</td>
<td>5-25</td>
<td>11</td>
<td>36.67</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>30</td>
<td>2</td>
<td>1-5</td>
<td>1</td>
<td>03.33</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>30</td>
<td>9</td>
<td>1-15</td>
<td>7</td>
<td>23.33</td>
</tr>
</tbody>
</table>
Fig. 8.3: Survival of transformed callus of *A. paeoniifolius* on selection medium

Fig. 8.4: PCR amplification of GUS encoding gene in transgenic *A. paeoniifolius* explants
Lane 1: 100 bp ladder, lane 2: positive control, lane 3: transformed callus, lane 4: transformed petiole, lane 5: negative control
The screening based on PCR have been successfully utilised in selecting the transformants (Weber and Bodanese-Zanettini 2011). The callus transformed with the GUS gene did not show any signs of regeneration even in a 5 month time. So, the transformation with DsMV-hp construct was done with certain modifications in both the method and the media composition.

8.3.2 In vitro cultures transformed with DsMV-hp construct

The FEC cultures were transformed with DsMV-hp construct via Agrobacterium mediated transformation. The method has been used by Ban et al. (2009) to develop resistance to soft rot disease in A. konjac. The co-cultivated FEC cultures were allowed to grow on SR medium supplemented with 500 mg l⁻¹ ticarcillin for 10 days. The selection antibiotic was not included at this stage and the cultures were given time for the expression of the transgene for antibiotic resistance, thereby the hp construct. The cultures were exposed to gradually increasing level of selection antibiotic in order to promote regeneration at the same time to bypass the occurrence of false positives. After 10 days, the cultures were transferred to selection medium (SR medium supplemented with 500 mg l⁻¹ ticarcillin and 15 mg l⁻¹ geneticin). The calli which survived in the medium was allowed to grow in medium with 25 mg l⁻¹ geneticin. A time of 2 months was given for the survival and/ growth of the survived callus. 49% of the callus cultures survived in the selection medium.

The putative transformed cells in the callus showed growth while the non-transformed ones did not survive in the selection medium (Fig. 8.5). The survived callus showing signs of growth were separated from dead mass and were subcultured on the fresh selection medium. Although the callus showed good proliferation, there was no sign of regeneration even after 3 months of growth. The selection pressure and the recalcitrant nature of the plant (Mukherjee et al. 2001) might be responsible for inhibition in the regeneration. It also cannot be excluded that some siRNAs derived from the transgene might affect the development from somatic embryos to plant. It is also possible that the CP containing transgene and its transcripts might sequester host replicase and/or replication factors that are required for normal plant development (Vanderschuren et al. 2007).
The antibiotic ticarcillin was used in the medium to kill the *Agrobacterium* thereby reducing its adverse effect on the plant growth. The successful elimination of *Agrobacterium* from regeneration media in transformation protocols is important for higher transformant recovery and increased transformation efficiency (Zhao et al. 2001; Zhang et al. 2003). However the presence of ticarcillin along with the selection antibiotic was expected to completely inhibit the regeneration efficiency. So, after 4 subcultures in the medium containing full concentration of ticarcillin, the concentration was reduced to 300 mg l\(^{-1}\). This modification in the antibiotic concentration had a positive effect on the regeneration. However utmost care was given in observing the contamination by *Agrobacterium* if any present in between the callus crevices. Shoot initials developed from the callus in a period of 2 months (Fig. 8.6). A closer view of the shoot initials is shown in Fig. 8.7. This observation supported that the regeneration was inhibited by the increased antibiotic concentration.

Though a 45% regeneration efficiency was recorded, the shoot elongation was inhibited and/ delayed. It took a period of 5 months for the shoots to elongate to 1 cm (Fig. 8.8) as against a normal shoot elongation of 5-6 cm in 3 month period. The response of the transgenic plants in different stages is explained in Table 8.2. The selection pressure applied might be the reason for the delayed shoot elongation in the transgenic plants.

The putative transgenic shoot initials when subjected to PCR with *npt II* primers gave a band at 280 bp (Fig. 8.9) while RT-PCR with DHP primers gave a band at 300 bp, a size slightly higher than the expected 230 bp (Fig. 8.10). Though the analysis with PCR for confirming the transgene integration resulted in the band of expected size, the RT-PCR for transgene expression analysis failed to produce bands of expected size, rather produced a product of slightly higher size. The difference in the band size upon transgene expression could not be substantiated with the viral CP amplification as the FEC used in the study was free from DsMV. Even if it amplifies the virus, in case of unexpected infection, the primers will amplify the product of the same size (230 bp) upon RT-PCR; as the construct was designed based on the conserved region in the CP gene. Thus, the results of band with higher size may be due to some rearrangements or translocations in the
integrated transgene, but the observation of unexpected band size needs further analysis. NASH with construct specific non-radioactive probes gave positive signals of dark spots for transgenic lines while no such spot was observed for un-transformed ones (Fig. 8.11).

**Table 8.2: A. paeoniifolius transformed with DsMV-hp construct**

<table>
<thead>
<tr>
<th>Explants and response</th>
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</tr>
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<tbody>
<tr>
<td>No. of explants (1 cm² callus )</td>
<td>60</td>
</tr>
<tr>
<td>% survival in selection medium</td>
<td>49</td>
</tr>
<tr>
<td>% regeneration in selection medium</td>
<td>45</td>
</tr>
</tbody>
</table>

The transgenic plants expressing the CP gene in an hp construct are expected to confer complete resistance to DsMV infecting the plant. Many examples of resistance in varying degrees mediated by the CP gene sequence in different constructs in different hosts have been described (Beachy et al. 1990; Kawchuk et al. 1990; Fitchen and Beachy 1993; Cuozzo et al. 1988; Zrachya et al. 2006). The inverted repeat construct used in transforming *A. paeoniifolius* is expected to confer complete resistance to the virus. The inverted repeat silencing approach encoding hpRNAs has been utilized in conferring effective resistance to many viruses like *Bean pod mosaic virus* (Reddy et al. 2001), *Sugarcane mosaic virus* (Bai et al. 2008; Gan et al. 2010), *Soybean dwarf virus* (Tougou et al. 2006), *Tobacco mosaic virus* (Hu et al. 2011), *Cucumber mosaic virus* (Hu et al. 2011), *Maize dwarf mosaic virus* (Zhang et al. 2011) and many others. The use of long hpRNA expression constructs of 900 or more nucleotides like that used in this study has been proved more efficient in conferring viral resistance than shorter constructs of less than 500 nucleotides (Zhang et al. 2011; Bruun-Rasmussen et al. 2007).
Fig. 8.5: FEC of *A. paeonifolius* transformed with DsMV-hp construct in selection medium

a. transformed callus showing signs of growth, b. dead untransformed callus
Fig. 8.6: Shoot regeneration from FEC of *A. paeoniifolius* transformed with DsMV-hp construct

Fig. 8.7: A closer view of shoot initials arising from callus of *A. paeoniifolius* transformed with DsMV-hp construct
Fig. 8.8: Elongation of shoot of *A. paeoniifolius* transformed with DsMV-hp in selection medium

Fig. 8.9: PCR of putative transformants of *A. paeoniifolius* with *npt II* primers

Lane 1: 100 bp marker, lane 2: positive control, lanes 3-9: transgenic *A. paeoniifolius*, lane 10: negative control
Fig. 8.10: RT-PCR of putative transformants of *A. paeoniifolius* with DHP primers
Lane 1: 100 bp ladder, lanes 2, 4: false positives, lanes 3, 5: transgenic *A. paeoniifolius*, lane 6: transgenic *N. benthamiana*, lane 7: untransformed callus

Fig. 8.11: NASH of putative transformants of *A. paeoniifolius*
Spots 1, 7: false positives, spot 2: untransformed, spot 3: buffer control, spot 4: transgenic *N. benthamiana*, spots 5, 6, 8-11: transgenic *A. paeoniifolius*, spot 12: positive control
8.4 Conclusion

The present investigation has led to the development of transgenic *A. paeoniiifolius* via *Agrobacterium* mediated transformation. GUS gene proved to be an effective reporter gene in *A. paeoniiifolius*. The transgenic *A. paeoniiifolius* expressing the DsMV-hp construct developed in the study is expected to be completely resistant to DsMV infecting this plant as the resistance provided by the construct against DsMV has been demonstrated in *N. benthamiana*. 