Chapter 6
Transformation with unedited *atp 9* gene
6.1 Results

At the Directorate of Oilseeds Research, gene constructs for the development of male sterile lines in safflower have been developed by using orf H522 (Narasimha et al., 2009) from sunflower and unedited mitochondrial genes, nad 3 and atp 9 (Yamini, 2007). In the present study, the atp 9 gene was used for safflower transformation. The gene construct has TA 29-a tapetum specific promoter followed by cox IV transit peptide for mitochondrial targeting of the gene product, the unedited atp 9 gene with a nos terminator. The plasmid vector used was pCAMBIA 1305.2 which has hygromycin as plant selection marker and the Uid A gene for GUS reporter activity. In the present study, the gene construct was mobilized into Agrobacterium strain LBA 4404 and used for transformation.

6.1.1 Mobilization of the developed construct into Agrobacterium

The gene construct pCAMBIA 1305.2 TCAN (T-TA29 promoter, C-coxIV transit peptide, A-unedited atp 9 gene, N-nos terminator), already developed at the Directorate of Oilseeds Research (Yamini, 2007) was mobilized into Agrobacterium strain LBA 4404. After 2 days of incubation on YEP medium (with antibiotics), 12 colonies were observed. The transformed colonies were confirmed for the presence of the gene construct by colony PCR, PCR of plasmid DNA and restriction digestion analysis.

6.1.1.1 Colony PCR

Colony PCR was done for preliminary confirmation of the clones. Instead of plasmid DNA, the bacterial cells were used in the PCR reaction mix and the PCR was set as per details provided in section 3.18.2. The primer set used was TA 29 forward and nos reverse to check for the presence of the entire fragment. Out of the 12 clones, 9 clones showed the amplified product of the desired size (1187 bp) of TCAN fragment (Figure 6.1).
Figure 6.1 Colony PCR for confirmation of the presence of *uatp 9* by using the TA29 forward and *nos reverse primers*. Lanes 1-12: Colonies obtained after transformation of the pCAMBIA 1305.2 TCAN into *Agrobacterium*, lane M: λ DNA double digest with *EcoR I/Hind III*.

Figure 6.2 Confirmation of LBA 4404 pCAMBIA 1305.2 TCAN in *Agrobacterium* by restriction with *Hind III* and *EcoR I*. Lane M: λ DNA double digest with *EcoR I/Hind III*, lane 1: Unrestricted plasmid DNA, lane 2: Restricted plasmid DNA.
6.1.1.2 Restriction analysis

The plasmid DNA from the obtained clones upon restriction digestion with *Hind* III and *EcoR* I showed the release of the insert (1187 bp) confirming the presence of the gene construct (Figure 6.2).

6.1.1.3 PCR analysis

PCR analysis of the plasmid DNA with TA 29 forward and *nos* reverse primers showed the fragment of desired size (1187 bp) confirming the presence of the insert in the bacterial colonies obtained (Figure not presented).

6.1.2 *Agrobacterium*-mediated transformation with unedited *atp 9* gene

With the developed gene construct harbouring the unedited version of *atp 9* (for induction of male sterility) and the optimized transformation protocol, transformation experiments were carried out. The 8-day-old seedling explants (root and hypocotyl) were subjected to vacuum infiltration with the bacterial (LBA 4404: pCAMBIA 1305.2 TCAN) concentration of 0.5 OD in the presence of 150 µM acotosyringone and cocultivated in dark for 2 days and transferred to selection media.

The first cycle of selection was carried on SV17 (MS+0.2 mg/l TDZ+0.2 mg/l NAA), followed by the second cycle of selection on 0.5 mg/l BAP and the final selection on media with 0.2 mg/l BAP+0.5 mg/l KN. At all the steps, cefotaxime at a concentration of 250 mg/l was used to check bacterial contamination. The selection regime followed was 10-15-15 mg/l hygromycin for both root and hypocotyl explants for the first, second and third cycles of selection, respectively. After first cycle of selection calli formation was observed from the cut ends. Following the second cycle of selection, shoot formation was observed (Table 6.1). The putative transformants obtained after the third cycle of selection are represented in Figure 6.3 A. The
Table 6.1 Transformation using *uatp* 9 gene construct

<table>
<thead>
<tr>
<th>Explant</th>
<th>Total number of explants</th>
<th>Selection I *</th>
<th>Selection II **</th>
<th>Selection III **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>1352</td>
<td>963</td>
<td>743</td>
<td>683</td>
</tr>
<tr>
<td>Hypocotyl</td>
<td>1502</td>
<td>1021</td>
<td>862</td>
<td>598</td>
</tr>
</tbody>
</table>

Data represents the number of putative transformants from 8-day-old seedling explants transformed with bacterial cell density of 0.5 OD$_{600}$ with co-cultivation period of 2 days. Data was recorded after 15 days on each selection. The gene construct used was pCAMBIA 1305.2 TCAN.

* Data represents number of explants callusing following the first cycle of selection.
** Data represents number of explants with shoot regeneration after the second and the third cycles of selection.
Figure 6.3 Transformation with pCAMBIA 1305.2 TCAN (uatp 9) construct. A. Putative transformed shoots after the final cycle of selection, B. Transformants on elongation medium, 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l AgNO₃, C. Transformed shoots showing blue colouration after the second cycle of selection following the GUS assay, D. The floral organs of the transformed shoots showing blue colour after GUS staining, E. The primary transformed shoot in the soil, F. PCR analysis of the putative transformants for the presence of transgene atp 9: Lanes 1-4: Putative transformants; lane M: λ DNA double digest with EcoR I/Hind III
shoots were elongated on MS medium supplemented with 0.5 mg/l KN+1.0 mg/l 2iP+1.0 mg/l AgNO₃ (Figure 6.3 B). The shoots rooted on half-strength MS medium supplemented with 1.0 mg/l NAA, but with low frequency compared to the untransformed shoots. The rooted shoots were acclimatized and transferred to soil (Figure 6.3 E). The primary transformants were confirmed by GUS histochemical assay and PCR analysis.

### 6.1.3 Confirmation of the putative transformants

#### 6.1.3.1 GUS histochemical assay

The presence of the transgene in the putative transformants was confirmed by the blue colouration observed after GUS staining of the obtained shoots. GUS histochemical assay of shoots recovered after the third cycle of selection showed GUS positive shoots with a frequency of 98.0% (Figure 6.3 C, D) confirming the presence of the transgene.

#### 6.1.3.2 PCR analysis

PCR analysis of four primary transformants with TA 29 forward primer and nos reverse primer showed the amplicon size of 1187 bp (Figure 6.3 F) in all the four plants confirming stable integration of the transgene. Further characterization of the transformants is underway.
6.2 Discussion

Safflower is predominantly self-pollinated crop. For exploitation of heterosis and hybrid seed production development of male sterile lines assumes importance. At the Directorate of Oilseeds Research, gene constructs for induction of male sterility in safflower have been developed. These constructs include the orf H522 gene from sunflower which is known to cause male sterility in the sterile Pet 1 cytoplasm and unedited mitochondrial genes from safflower viz., nad 3 and atp 9. Genetic transformation of tobacco with orf H522 gene resulted in male sterility (Narasimha et al., 2009). The constructs were under TA 29 (tapetum specific promoter) for tissue specific expression of the genes. For targeting the polypeptide to mitochondria, a cox IV transit peptide was used. The construct used nos as the terminator sequence. In the present study, the gene construct harbouring atp 9 (unedited) was used for induction of male sterility in safflower. The study was confined to the GUS histochemical assay and PCR confirmation of the primary transformants. Further characterization of the putative transformants is under progress.

Most of the studies for induction of male sterility were aimed at the disruption of the mitochondrial function which reduces the ATP production that severely affects pollen fertility. In the present study, unedited atp 9 gene (from safflower) was intended to express in the tapetal cell layer to induce male sterility. The gene is driven by the T A29 promoter (tapetum specific promoter), which was fused to the cox IV transit peptide (from yeast) to target the resultant peptide into mitochondria. Similar approach for the production of male sterile lines had been adopted in tobacco (Hernould et al., 1998) and Arabidopsis (Gomez-Casati et al., 2002). RNA editing in atp 9 gene has been reported in several plant species such as wheat (Begu et al., 1990), Petunia (Wintz and Hanson, 1991) and tobacco (Hernould et al., 1993). The previous study (Yamini et al., 2008) reported that safflower atp 9 transcript undergoes editing at 12 sites, 10
complete and 2 partial edit sites resulting in shortening of the \( atp \, 9 \) polypeptide by 12 amino acids due to the stop codon created by editing at nucleotide position 223. Similar observation were reported in \( Oenothera \) (Schuster and Brennicke, 1990), sorghum (Salazar et al., 1991) and \( Petunia \) (Wintz and Hanson, 1991).

This is the first attempt at transformation of safflower using an agronomically desirable gene. The previous studies were confined to genetic transformation of safflower using constructs harbouring only the selectable and reporter marker genes (\( Uid \, A \), \( npt \, II \)). Use of the optimized protocol for transformation resulted in the development of transgenics through deployment of the unedited \( atp \, 9 \) gene. All the putative transformants were confirmed to harbour the introduced gene through PCR analysis. Further studies on characterization of the transgenics are under progress.
6.3 Summary

The present study was aimed to transform safflower with an agronomically desirable trait, that induces male sterility (unedited atp 9). The basis of the study is the expression of unedited version of the important mitochondrial gene (atp 9) under tapetum specific promoter, targeting the resultant polypeptide to the mitochondria that impairs the mitochondrial functionality leading to the male sterility.

With the optimized transformation parameters the Agrobacterium-mediated transformation was carried out with the gene construct pCAMBIA 1305.2 TCAN (T-TA 29 tapetum specific promoter, C-cox IV transit peptide, A-atp 9 unedited version, N-nos terminator). The putative transformants obtained were confirmed by GUS histochemical assay and PCR analysis. The GUS assay showed GUS positive shoots with a frequency of 98.0%. The PCR study (TA 29 forward and nos reverse primers) showed the amplicon of expected size (1187 bp) confirming the presence of the transgene. The further characterization of the transformants (RT-PCR Southern analysis, pollen study, progeny analysis) is an ongoing project at the Directorate of Oilseeds Research.