

Gene Transfer into Indian Cultivars of Safflower (*Carthamus tinctorius* L.) using *Agrobacterium tumefaciens*

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Abstract

Direct and callus-mediated shoot regeneration following co-cultivation with *Agrobacterium tumefaciens* was obtained in two Indian cultivars of safflower (*Carthamus tinctorius* L.), A-1 and A-300. The procedure yielded 23 and 34 transformation events per 100 co-cultivated explants with direct and callus-mediated shoot recovery, respectively. The use of *uid* A gene in pKIWI105 that lacks a bacterial ribosome binding site precluded *uid* A expression in residual *Agrobacterium* cells. High levels of GUS activities were detected in selected putative transgenic calli and in shoot regenerants by histochemical assay. Western blot analyses using GUS antiserum and the NPT II expression assays confirmed the expression of marker genes in the putative transformants. Transgene integration was examined by PCR and dot blot hybridization of the transformants. Compared to controls, the efficiency of regeneration was markedly decreased subsequent to co-cultivation. Extended periods of callus-mediated regeneration led to hyperhydricity and vitrification of the shoots. Shoots regenerated from explants directly had, however, a normal appearance. The rooting response of regenerated shoots was poor and remains a continuing obstacle for safflower plant regeneration and transformation.

1. Introduction

Safflower (*Carthamus tinctorius* L.) is an important oil seed crop. The oil is valued for its high degree of polyunsaturation, and for its industrial applications. Several cultivars of safflower under cultivation are adapted to low humidity and low soil water content and can withstand prolonged drought conditions. India is a major producer of safflower and has a vast array of varietal wealth, mainly generated through selection. The primary agronomical problem associated with safflower is the lack of an effective herbicide. Among the diseases affecting Indian safflowers, the most serious are wilt caused by *Fusarium carthami* and leaf spot caused by *Alternaria carthami*. Very little work has been done to create broad-based genetic improvement of safflower. Recourse to *in vitro* techniques and genetic engineering could go a long way towards genetic upgrading including herbicide and disease resistance and oil enrichment. Tissue culture and regeneration protocols have been developed for Indian (Tejovathi and Anwar 1984, George and Rao 1982) and American cultivars (Orlikowska and Dyer 1933) of safflower, and the only report of genetic transformation performed has been in the

American (Ying *et al.* 1992, Orlikowska *et al.* 1995) 'Centennial'. Callus-mediated and direct regeneration of transgenic shoot buds of the popular Indian cultivars, A-1 and A-300 expressing the *uid* A and *npt II* reporter genes following *Agrobacterium tumefaciens* co-cultivation of primary seedling explants will be presented here.

2. Materials and Methods

2.1 Plant material and culture conditions

Seeds of safflower (*Carthamus tinctorius* L.) cultivars A-1 and A-300 were soaked for 30 min in a dilute (0.1%) detergent solution, Labolene (Qualigens Fine Chemicals, Bombay, India) added with the fungicide Bavastin (1%) followed by washes with sterile distilled water. Further sterilization was carried out with 0.1% HgCl₂ solution for 10 min. Traces of the sterilant were removed by several rinses with sterile water. The seeds were then germinated aseptically on 0.7% agar (Hi-media, Bombay) in Petri plates and incubated in the dark. The germinating seeds were transferred to culture tubes containing agar-gelled MS (Murashige and Skoog 1962) medium containing 3% sucrose. Seedlings were grown under white fluorescent light of intensity 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR) with a

14 h / day photoperiod. Two different explants of 10–12 day-old seedlings used were, the proximal half portion of excised cotyledons of cv. A-300 and seedling axes above the level of the hypocotyl *i.e.* epicotyl, with cotyledons and shoot apex removed in the case of A-1. Direct shoot development in shoot segments of cultivar A-1 was obtained in MS medium containing BAP at 0.1 to 5 mg l⁻¹ and NAA at 0.01 to 1 mg l⁻¹. Callus induction in cotyledon explants of cultivar A-300 and shoot bud regeneration from this callus were obtained in media with BAP and NAA combinations. Prior to root induction, shoots of both the cultivars were cultured in half-strength MS medium containing 0.5 mg l⁻¹ TIBA (2, 3, 5-triiodo-benzoic acid). Rooting was attempted on full-strength or half-strength MS salts and vitamins containing 3% sucrose, with or without auxins. The shoots were cultured on media with NAA or IBA at levels between 0.01 and 0.1 mg l⁻¹ for 3 weeks and were later shifted to auxin-free medium. All media were adjusted to pH 5.8 and autoclaved at 1.4 Kg cm⁻² and 121 °C for 20 min. Culture incubation was carried out in 60 ml culture tubes and 100 ml Erlenmeyer flasks fitted with cotton plugs and at a temperature of 26 ± 2 °C under a 14 h/day photoperiod with cool-white fluorescent light of intensity 35 μmol m⁻² s⁻¹ (PAR). Experiments were repeated thrice with 30 replicates each time.

2.2 Bacterial strains and vectors

In preliminary experiments, two different disarmed *Agrobacterium tumefaciens* strains *viz.*, At459 and LBA4404 both harboring the same binary vector pKIWI105 were used for transformation. Since there was no significant difference between the strains in their infectivity and transformation efficiency as determined by the number and extent of GUS-expressing sectors observed per explant 4 days later, only LBA 4404/pKIWI105 was used for further experiments. In each batch five per 30 co-cultivated explants were tested for GUS activity. The vector pKIWI105 contains the *uid* A reporter gene driven by the CaMV 35S promoter and a neomycin phosphotransferase II (*npt* II) gene driven by the nopaline synthase promoter. The reporter gene of pKIWI105 used is a version of *uid* A which lacks the bacterial ribosome binding site and shows negligible expression in *Agrobacterium* but shows good activity in plant cells (Janssen and Gardner 1993). For probe preparation the pUC-GUS121 from *E. coli* strain XL-1 Blue was used. This plasmid carries the *uid* A gene with the CaMV 35S promoter and the nos terminator. Restriction of this plasmid with *Eco*RI and *Bam*HI releases a

2.1kb fragment comprising of the *uid* A coding region along with the nos terminator.

2.3 Transformation, selection and recovery of the transformants

Bacteria were cultured to mid-log phase in agitated (150 rpm) LB medium added with kanamycin (50 μg ml⁻¹) at 28–30 °C. The bacteria were resuspended in Winans' low phosphate AB medium (Winans *et al.* 1988) and incubated at 28–30 °C for 18 h to enhance virulence. The proximal half of the freshly excised cotyledons, and the seedling segments pricked at the cotyledonary node with a fine needle were dunked in the bacterial suspension in MS medium for 10 min, blot dried on sterile filter paper, and co-cultivated on regeneration medium for 2 days. Later the explants were transferred to a liquid medium containing the same hormone combination with cefotaxime added at 500 μg ml⁻¹ and were agitated in the medium for 30 min to control bacterial growth, blot-dried on sterile filter paper and incubated on regeneration / selection media relevant to the type of regeneration desired. These media were added with cefotaxime at 250 μg ml⁻¹. The time interval before kanamycin selection of co-cultivated explants and the level of kanamycin on both untransformed and transformed explants were assessed. Subculture to fresh media was performed every two weeks. The morphogenetic response of the cultures was recorded commencing from 7 days after inoculation. Regeneration efficiency of co-cultivated seedling shoot axes and cotyledon-derived calli in the regeneration / selection medium was determined by recording the number of shoots and shoot buds developed and was compared with regeneration from untransformed explants.

2.4 GUS histochemical analysis and localization

The β-glucuronidase activity was assayed (Jefferson 1987) with samples of putative transformants at intervals between day 4 post-inoculation and shoot regeneration. The activity was checked at the callus stage and in the leaves of the regenerants in the cv. A-300 and in segments of regenerated shoots in case of cv. A-1. Uninfected tissues were similarly assayed to detect any GUS-like activity in the tissues of this plant species.

Tissues, which have tested positive to GUS expression were fixed in formalin-acetic acid-ethanol (FAA), dehydrated and embedded with *n*-butanol series (Johansen 1940). Paraffin sections were cut at 14–16 μm thickness, deparaffinized and mounted in Michrome mountant (Edward Gurr Ltd., London). Micrographs were taken using Kodacolor 100 ASA film for bright field images.

2.5 NPT II expression assay

Neomycin phosphotransferase activity was detected in the total proteins of GUS positive shoots and calli by the non-denaturing PAGE assay of Reiss *et al.* (1984). The phosphorylated kanamycin was detected on the X-ray film after exposing it to the phosphocellulose paper for two days.

2.6 Western blot analysis

The β -glucuronidase protein in the putative transformants was detected by Western blotting using an anti β -glucuronidase antibody (Clontech, Palo Alto, USA). The immunostaining was carried out according to the GUS gene fusion system user manual (Clontech, Palo Alto, USA).

2.7 DNA analysis

For DNA isolation, 2 g of tissue from the transformants and from the non-transformed control plants was used and the procedure described by Dellaporta *et al.* (1983) was followed. The DNA extraction was carried out from those putative transformants that were tested free of residual *Agrobacterium* in their tissues. Plasmid DNA from XL-1 Blue/pUC-GUS121 was prepared (Sambrook *et al.* 1989). PCR was performed not only to determine the presence of the *uid A* transgene (Stummer *et al.* 1995) in the putative transformants but also to determine that these plants are not carrying the residual *Agrobacterium* (Lev'ee *et al.* 1997). In order to amplify the *uid A* gene sequence, PCR was initiated by a hot start at 94 °C for 4 min followed by 32 cycles of 1 min at 94 °C, 2 min at 55 °C and 2 min at 72 °C. PCR conditions to amplify the *vir C* region to check for the presence of residual bacteria were, 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min for 30 cycles. The products were run on a 1% agarose gel. Purified genomic DNA (2–5 μ g) was immobilized onto nylon membrane (Hybond, Amersham, Buckinghamshire, England) for dot blot hybridization. The membrane was hybridized with α -³²P random prime labelled 2.1 kb fragment of the *uid A* gene.

3. Results and discussion

3.1 Transformation, selection, and recovery of transformants

Experiments were performed to empirically determine the regeneration competence of the explants of safflower cultivars and their transformability using the GUS enzyme activity (data not shown). On the basis of the results obtained, shoot segments of cultivar A-1 and cotyledon explants of A-300 were chosen as target tissues for transformation. Callus formation from cotyledon explants occurred in all

the BAP and NAA containing media tried. Callus growth was visible on 70% of explants after two weeks of culture. Callus with shoot-forming capability however, was obtained with media supplemented with BAP at 1 mg l⁻¹ and NAA at 0.1 mg l⁻¹. Shoot bud regeneration occurred in the callus induction medium. Four weeks after the first subculture, buds appeared as green protuberances on light green friable callus (Fig. 1 a). The number of buds on the individual calli varied. Callus from cotyledons co-cultivated with *Agrobacterium* gave a lower number of shoot buds than callus of uninoculated explants. On average, the uninoculated controls produced 8

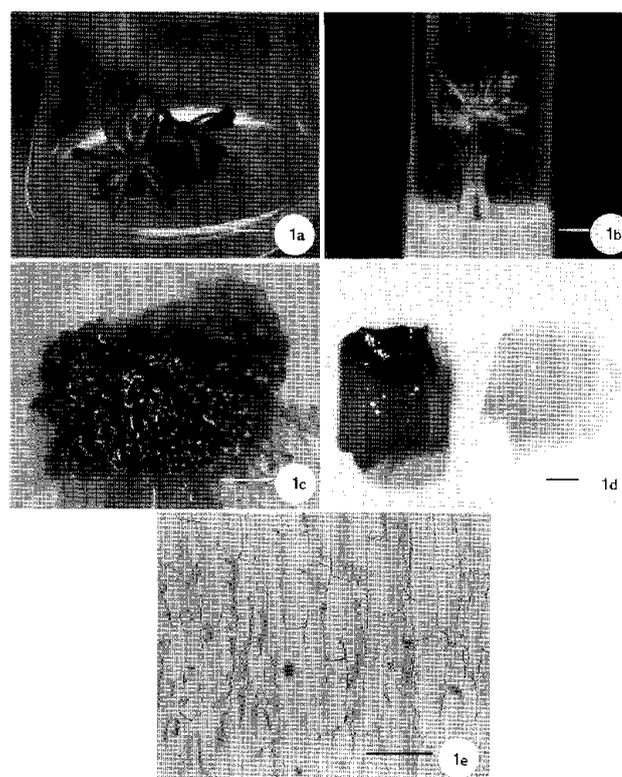


Fig. 1 Induction of putative transgenic shoots and histochemical analysis of *uid A* gene in safflower. (a) Callus-mediated regeneration in safflower cv. A-300 in the presence of kanamycin (100 mg l⁻¹). (bar = 1 cm). (b) Multiple shoots from the apex of the decapitated epicotyl of safflower cv. A-1 in the presence of kanamycin (100 mg l⁻¹). (bar = 0.9 cm). (c) Callus showing GUS expression in safflower cv. A-300. (bar = 0.1 cm). (d) Shoot segments of safflower cv. A-1: one showing GUS expression and the other (shoot of uninfected plant-negative control) lacking GUS expression. (bar = 0.13 cm). (e) Longitudinal section of shoot segment of cv. A-1 showing intracellular localization of GUS expression. (bar = 30 μ m).

Table 1. Recovery of transformed shoots from the *Agrobacterium tumefaciens* co-cultivated explants of safflower cultivars

Explants	No. of explants tested (3 replications) (A)	No. of explants callusing	No. of explants forming buds/shoots (B)	Frequency (%) of transformation (B/Ax100)	No. of shoots/buds per explant \pm S.D
Cotyledons (A-300)	30	21	6	20	23.3 \pm 3.51
	30	24	8	27	
	30	18	7	23	
Seedling axes-apical (A-1)	30	—	10	33	34.3 \pm 2.31
	30		10	33	
	30		11	37	

buds per callus while the transformed callus produced 2–3 buds (Table 1). Shoot bud development on the medium as for callus growth was previously reported in the safflower cultivar ‘Centennial’, although rooting of shoots was unsuccessful (Orlikowska and Dyer 1993).

In cultivar A-1, multiple shoots appeared in the cotyledonary node as well as at the apex of the decapitated epicotyl in about two weeks when the seedling shoot segments were cultured on medium containing 1 mg l^{-1} each of BAP and NAA (Fig. 1 b). Ten to twelve shoots developed on each uninfected shoot explant whereas the number of shoots diminished subsequent to co-cultivation with *Agrobacterium* (Table 1). The cause of this reduction in shoot regeneration, directly on seedling axes and in cotyledon-derived callus may be due to the organogenic potential of this species being strongly influenced by the conditions of transformation or it may be related to a hypersensitive response of safflower explants to *Agrobacterium tumefaciens* infection (Orlikowska *et al.* 1995) It may more likely be due to the inhibitory effects of kanamycin in the selection media (Ying *et al.* 1992).

Of the several rooting media tested, only medium containing NAA at 0.1 mg l^{-1} formed roots occasionally on cotyledon-derived shoots of the cultivar A-300. Axillary shoots of the cultivar A-1 did not respond to the NAA-containing and other root-inducing media used in the study. A similar lack of effective protocols for rooting of Indian and Turkish safflower varieties was reported earlier (George and Rao 1982, Tejovathi and Anwar 1987). Nevertheless, Baker and Dyer (1996) optimized rooting of regenerated shoots for the American cultivar ‘Centennial’ by a 7-day exposure of the regenerated shoots to 10 mg l^{-1} IBA in root induction medium. The regenerated shoots of both safflower cultivars A-300 and A-1 in the present study have shown a high degree of vitrification and/or formed callus at

the base when treated with IBA in the medium. These differences in the rooting behavior observed with auxin treatments could be related to varying endogenous levels of auxin among cultivars. Tejovathi and Anwar (1987) obtained rooting in Indian safflower cultivar ‘Manjira’ with TIBA, an auxin transport inhibitor (Gaspar *et al.* 1996) in the rooting medium. TIBA, used in our study to overcome the apparent auxin sensitivity of the shoots of cultivars A-300 and A-1, did not result in root formation. Nevertheless, these shoots showed elongation when treated with 0.5 mg l^{-1} of TIBA in half-strength MS medium prior to root induction treatment. Further, the symptoms of hyperhydricity and vitrification of shoots disappeared with this treatment. These differences in response may at least partially explain the inhibitory effect of high endogenous auxin levels that might exist in some cultivars. It was observed (Orlikowska and Dyer 1993) that rooting of callus-derived shoots is difficult in safflower. In our study, root induction was possible from callus-derived shoots of cv. A-300.

The bacterial contamination of co-cultivated explants was largely controlled by agitation in liquid regeneration medium containing 500 μ g ml^{-1} of cefotaxime for 30 min. Preliminary dose response experiments using untransformed safflower seedling explants showed that cotyledon-derived callus growth and shoot bud development in the case of shoot axis were inhibited above 50 μ g ml^{-1} kanamycin. Explants gradually turned chlorotic and showed hyperhydricity. Bacterial overgrowth occurred when both kanamycin and cefotaxime were simultaneously introduced into the medium soon after co-cultivation. Therefore, kanamycin at 100 μ g ml^{-1} was added to the medium a week later to select the transformants. Orlikowska *et al.* (1995) also suggested a specific time interval between co-cultivation and transfer to selection medium for a different reason; direct shoot regeneration was

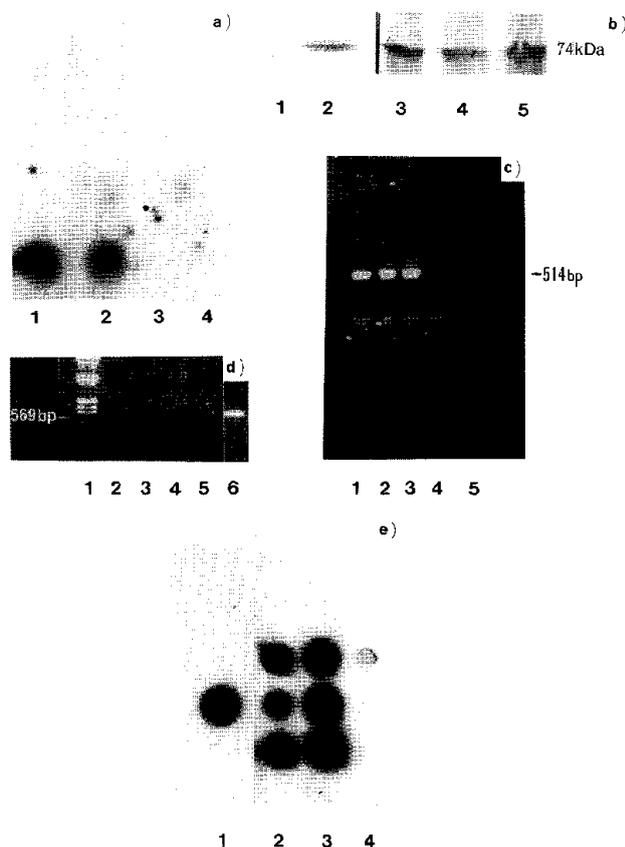


Fig. 2 Molecular analysis of introduced genes in putative transgenic shoots of safflower.

- (a) NPT II enzyme assay. Lane 1: protein extracts from the shoots of safflower cv. A-1. Lane 2: protein extracts from the callus of safflower cv. A-300. Lane 3 & 4: protein extracts from the uninfected safflower cvs. A-1 and A-300 respectively.
- (b) Western blot analysis. Lane 1: protein extracts (50 μ g) from uninfected tissue. Lane 2: Purified GUS protein (20 μ g) (Clonotech). Lanes 3 & 4: protein extracts (50 μ g) from GUS positive shoots of safflower cv. A-1. Lane 5: protein extracts (50 μ g) from GUS positive callus of safflower cv. A-300.
- (c) PCR analysis for the presence of *uid A* gene. PCR of safflower A-1 and A-300 DNA using a 21 mer primer, 5' CTG TAG AAA CCC CAA CCC GTG 3' and 5' CAT TAC GCT GCG ATG GAT CCC 3' which amplifies a 514 bp *uid A* gene fragment. Lane 1: pKIWI105 DNA. Lane 2: DNA from GUS positive shoots of A-1. Lane 3: DNA from GUS positive callus of A-300. Lanes 4 & 5: DNA from uninfected shoots and callus of A-1 and A-300 respectively.
- (d) PCR analysis for the presence of *vir C* gene. PCR products after 569 bp *vir C* gene amplification, using the primers, 5' GCA TGC TTT GAG TTC GAC GAC 3' and 5' TTC GTA CGG GGG TGT GAT GG 3'. Lane 1: Marker. Lane 2: DNA from GUS positive shoots of A-1. Lane 3: DNA from GUS positive callus of A-300. Lane 4 and 5: DNA from uninfected tissues of A-1 and A-300 respectively. Lane 6: pKIWI105 DNA
- (e) Dot blot analysis of uncut safflower genomic DNA. Lane 1: DNA from LBA4404/ pKIWI105. Lane 2: DNA samples from GUS positive shoots of safflower cv. A-1. Lane 3: DNA samples from GUS positive callus of safflower cv. A-300. Lane 4: DNA from uninfected safflower A-1 and A-300 respectively.

obtained only from explants transferred to kanamycin medium one or two days after co-cultivation.

3.2 Analysis of putative transformants

The β -glucuronidase enzyme activity was initially used to monitor transformation efficiency. Similarly, calli from putatively transformed cotyledon explants were assayed. Differences between control and transformed tissues were evident following co-cultivation for two days. Most co-culti-

vated explants expressed intense GUS activity (Figs. 1 c and 1 d), although the blue staining was confined to sectors. Microscopic examination showed that indigo dye precipitates were localized within cells (Fig. 1 e). Further, the *uid A* gene construct of pKIWI105 precluded GUS expression from residual *Agrobacterium tumefaciens* cells. Endogenous GUS activity in the tissues of safflower cultivars was also not observed. Selection to obtain stably transformed calli and regenerants was at-

tempted from batches of explants that showed considerable transient GUS expression. Some of the transformants, calli and shoot buds derived from them exhibited sustained growth on subculture to media which were added with selective levels of kanamycin ($100 \mu\text{g ml}^{-1}$) and expressed GUS activity at different points of time (Table 1). The GUS expressing cells occurred as large extended blue zones and in a few cases blue sectors covered the entire shoot bud. Expression of the *uid A* gene was further assayed by Western blotting using the antiserum specific to the GUS protein (Fig. 2 b). A 74 kDa protein with a molecular mass similar to the GUS protein was detected in the total proteins from the callus of A-300 and the multiple shoots of A-1. No proteins reacted with the GUS antibody when the total proteins from the callus or the multiple shoots of the non-transformed safflower were assayed. Co-transformation of safflower with *npt II* was confirmed when the total proteins of the GUS positive putative transformants showed phosphorylated kanamycin at the expected position on the autoradiogram (Fig. 2 a). In contrast, the control plant tissues showed no NPT II enzyme activity, even after prolonged autoradiographic exposure. PCR amplification of the *uid A* gene yielded a DNA fragment of the expected size (514 bp) in the GUS expressing calli of cultivar A-300 and multiple shoots of cultivar A-1 tested (Fig. 2 c). On the other hand, when PCR was performed to amplify *vir C* gene sequences in the same DNA samples, no amplification was detected indicating that no residual cells of *Agrobacterium* were present (Fig. 2 d). Both *uid A* and *vir C* DNA fragments were not detected in the control plants. Dot blot of uncut genomic DNA of transformants when hybridized with labelled *uid A* probe gave a strong signal indicating the presence of the introduced *uid A* gene (Fig. 2 e). There was no signal where the DNA of uninfected plant was loaded. Taken together, the results show that the transgenes viz., *uid A* and *npt II* are incorporated into the genome of the shoots of safflower cultivars A-300 and A-1. The recovery of transgenic plants however is limited as rooting of shoots was difficult. A single rooted transformant was recovered in the cultivar A-300. The monitoring of transgene inheritance consequently was rendered difficult.

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