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Regeneration and *Agrobacterium* - Mediated Transient Transformation of Button Daisy (*Leucanthemum vulgare*)

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Abstract

Explants of button daisy were screened for their regeneration potential and transient GUS gene expression. Medium containing MS salts minerals and B_5 vitamins supplemented with 0.1 mg/L BA and 0.1 mg/L TDZ showed the best regeneration. Disc florets and receptacles were the most responsive explants in regeneration and transient gene expression respectively. Regenerated plants were successfully rooted and established in the greenhouse conditions. Infection and co-cultivation of explants with *Agrobacterium tumefaciens* containing pCAMBIA 1301 resulted in transient GUS foci. Among the different explants, receptacles showed the highest percentage of transient GUS gene expression. Enzymatic and molecular analyses of transformed calli confirmed the integration of GUS gene.

Key words: disc floret, receptacle, thidiazuron, GUS gene, T-DNA transfer

Introduction

Leucanthemum vulgare, commonly known as 'button daisy' or 'moon penny' belongs to *Chrysanthemum*-complex is an important ornamental-bedding plant included in popular seed mixes for its beauty. It is a perennial, composite flower spanning from 1½ to 2 inches across, consisting of a yellow disc center surrounded by about 15-30 white ray flowers (Howarth and Williams 1968). The flowers are long lasting, and excellent for bouquets. The young leaves and tender parts of these plants are also used in a salad (Roberts 1976). It is also an important medicinal plant used to cure whooping cough, asthma, jaundice and nervous excitability (Grieve 1995).

The essential oil of *L. vulgare* contains sesquiterpene alcohols nerolidol, α-bisabolol, and farnesol and the sesquiterpene hydrocarbon farnesene. The principal constituents of the essential oil, farnesene (38.3%) and α-bisabolol (15.5%) are close in content to commercial products (Sagarieshvili 2002). Both farnesene and α-bisabolol are anti-inflammatory sesquiterpenes. Alpha bisabolol exhibits specific interaction with pepsin activity and has great implication for the treatment of gastric and upper intestinal diseases.

Biotechnological approaches may prove useful for obtaining genotypes with improved flower quality and increased metabolite content. Standardization of an efficient regeneration protocol is an essential requirement for genetic engineering of plants for desirable traits. Regeneration and transformation of many species in the *Chrysanthemum*-complex (*C. morifolium, C. cinerariaefolium* and *Dendranthema grandiflora*) have been reported and reviewed recently (Teixeira da Silva 2003). Surprisingly, in spite of its medicinal and horticultural value, neither regeneration nor transformation of button daisy (*L. vulgare*) has been attempted so far. Hence, an attempt was made in the present study to regenerate and transform this species for the first time.

Materials and methods

Source and preparation of explants

Seedlings of *L. vulgare* cultivar 'Snowland White' were raised in garden pots containing perlite and farm yard manure (1:1) in the greenhouse. Seedlings at different stages

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(15 and 25 d) were uprooted carefully from the pots and unopened head inflorescences excised from the mature plants were washed in running tap water for several times and treated with Clorox (0.75% active chlorine) for 5 min. To facilitate the disinfectant to reach all parts of the head inflorescence, a mild vacuum was applied during Clorox treatment. Following surface sterilization, the explants were washed with sterile distilled water for five times to remove the residues of Clorox. Carefully dissected explants from surface sterilized 15 day old seedlings (cotyledon and hypocotyl), 25 day old seedlings (leaf disc) and unopened heads (disc florets and receptacle) were inoculated on regeneration medium.

Plant regeneration

To standardize the plant growth regulator (PGR) requirement for optimal regeneration, cotyledons were cultured on medium supplemented with different concentrations of PGRs (BA and TDZ) either alone or in combinations. The optimal PGR combination was further used to study the morphogenetic responses of various explants. Basic medium contained MS (Murashige and Skoog 1962) mineral salts, B5 vitamins and 3% (w/v) sucrose was gelled with 7.0 g/L tissue culture grade agar (Phytotechnologies, USA). The pH of all media was adjusted to 5.8 before adding the gelling agent and autoclaving for 15 min at 1.4 kg cm^{-2}. Each disposable culture plate containing 25 ml medium was inoculated with 10-12 explants was incubated at 25℃ under 16 hours photoperiod. Cool-white fluorescent lights providing a quantum flux density of 30 μmol m^{-2} s^{-1} was used for illumination. Explants with or without shoot initials were transferred to basal medium after 25-30 days of culture initiation. Thereafter, explants were subcultured to basal medium once in every 20 days until the shoots elongated. When the shoots reached 3-5 cm height, they were excised and transferred to half strength MS medium containing 0.5 mg/L NAA for rooting.

Hardening and Transplantation

Rooted plants were thoroughly washed in running tap water and transferred to plastic pots filled with moistened perlite. The plants were covered with polythene bags for maintaining the humidity for 5 days. Polythene bags were gradually removed over a period of 5 days and the plants were exposed to the greenhouse conditions for hardening. Acclimated plants were finally transferred to garden pots containing soil and farmyard manure (1:1) and irrigated with tap water.

Bacterial strains and vector

Agrobacterium tumefaciens strain LBA4404 (Invitrogen, USA) transformed with the binary vector pCAMBIA1301 (Cambia, Australia) using $CaCl_2$ method (Tzfira et al 1997) was used for plant transformation. Binary vector pCAMBIA 1301 contains hygromycin phosphotransferase (*hph*) gene conferring resistance to antibiotic hygromycin as plant selection marker and *int-gusA* as reporter gene under the control of 35S promoter (Figure 1).

Infection, co-cultivation and selection

A culture was initiated by inoculating a fresh *Agrobacterium* colony into 5.0 ml liquid YEP medium (10 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl, pH 7.0) and incubated at 28℃ in a rotary shaker at 200 rpm for one day. Subsequently, 0.5 mL of grown bacterial culture was subcultured to 250 mL conical flask containing 100 mL liquid YEP and cultured as mentioned above. All bacterial culture media were supplemented with 25 mg/L rifampicin and 50 mg/L kanamycin for bacterial selection. When the optical density (OD) reached 0.8-1.0 at 660 nm bacteria were spun down using a tabletop centrifuge at 5000 x g and re-suspended in induction medium (1x AB salts (Chilton et al. 1974), 2 mM $NaPO_4$, 50 mM Methyl ethane sulfonate (pH 5.6), 0.5% glucose and 100 μM acetosyringone). This bacterial suspension was kept on a rotary shaker at 50-60 rpm at room temperature for 4 hrs. Explants pre-cultured for two days on regeneration medium were infected with the above bacterial suspension for 30 min at room temperature. Infected explants were blot-dried using sterilized Whatman No 3 filter paper and co-cultivated on regeneration medium containing 200 μM acetosyringone for 3-5 days.

Selection and transgenic callus formation

After co-cultivation, the explants were thoroughly washed with sterilized double distilled water and then with 500 mg/L ticarcillin clavulanate once to remove *Agrobacterium* contamination. The explants were then blot-dried and transferred to selective regeneration medium (Optimal regeneration medium containing 15 mg/L hygromycin B and 400 mg/L ticarcillin clavulanate). Explants were repeatedly subcultured to fresh selection medium once in every 15 days. Resistant calli were separated from the explant and subcultured.

Enzymatic GUS Assay

After co-cultivation, the explants were thoroughly washed with sterile distilled water. Ten explants from each type were

assayed for GUS (Jefferson et al. 1987). Briefly, explants were incubated in GUS solution (20 mM Phosphate buffer, pH 7.5, 5 mM of each K_4FeCN_6 and K_3FeCN_6, 10 mM EDTA and 0.1% Triton X 100) at 37℃ in the dark for overnight. The explants were decolorized in methanol and the number of blue spots was counted under a stereo microscope. T-DNA transfer rate was calculated for a total of thirty explants for each type derived from three repetitions.

Polymerase chain reaction (PCR) analysis

Genomic DNA from the putatively transgenic and control calli were isolated using modified CTAB method (Sambrook et al., 1989). Presence of GUS coding region in the genomic DNA was analyzed by PCR amplification of 0.8 kb fragment in the GUS gene using forward primer sequence 5'-GATCGCGAAAACTGTGGAAT-3' and reverse primer sequence 5'-TGAGCGTCGCAGAACATTAC-3' at annealing temperature of 55℃.

Southern analysis

Integration of *hph* gene in the genome of putative transgenic lines was analyzed by Southern hybridization. Twenty μg of DNA from putative transgenic and control calli were digested with restriction endonuclease X*hoI*. Similarly, 1 μg of plasmid DNA was digested with X*hoI* and used as positive control. Restricted DNA was electrophoresed on 1% (w/v) agarose gel and mobilized onto a positively charged nylon membrane via capillary transfer and fixed by UV cross linking. Pre-hybridization and hybridization of the blot were performed using church buffer at 55℃. The *hph* gene (1.1 kb) released from the vector by X*hoI* digestion labeled with α-^{32}P dCTP (Redivue, Amersham) using 'Rediprime' random primer labeling kit was used as probe. Hybridized blot was washed by agitating in 2X SSC + 0.1% SDS for 15 min at 55℃ and in 1X SSC + 0.1% SDS for 30 min at 60℃. The blot was exposed to Kodak X- Omat film for one day and scanned in a phosphorimager.

Statistical Analysis

The effects of various parameters on regeneration and T-DNA transfer were analyzed statistically. One-way ANOVA with Kruskal-Wallis test was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA.

Results

Regeneration

Efficiency of plant regeneration from cotyledon explants varied with PGR concentrations and combinations (Table 1). Among the various combinations of BA and TDZ tested, medium supplemented with 0.1 mg/L of BA and TDZ showed efficient regeneration from cotyledon. In explant type, disc floret and receptacle showed high regeneration ratio and number of shoots was highest in the culture of disc floret (Table 2). The media containing only BA or TDZ exhibited poor regeneration.

Within ten days of culture, initial explants swelled and subsequently shoot initials were formed. Callusing was noticed in the most of disc floret cultures predominantly at the junction between petal and immature seed (Figure 2A). When subcultured on hormone-free medium, disc florets quickly produced numerous green shoots (Figure 2B). Direct shoot formation was observed from all other types of explants within 30 days of culture. Receptacles showed direct shoot regeneration at the scars made by the excision of disc

Table 1. Effect of combined plant growth regulators on regeneration of plants from 15 day old cotyledon explants of *L. vulgare* cv. 'Snowland White' after 30 days of culture

Plant growth regulator mg/L		Regeneration	
BA	TDZ	Regeneration (%)*	No. of shoots ± SE / explant
0.1		0	0.0 ± 0.0
0.5		0	0.0 ± 0.0
0.1	0.1	42.0	5.3 ± 1.7 a
0.5	0.1	35.0	2.8 ± 1.2 b
0.0	0.1	17.5	1.6 ± 1.2 c
0.0	0.0	0	0.0 ± 0.0

*PGR treatments significantly affected the percentage of plant regeneration (Kruskal-Wallis test, $P < 0.05$)

Table 2. Effect of *L. vulgare* explant types on regeneration of plants on medium containing 0.1 mg/L of BA and TDZ after 45 days

Explant	Regeneration	
	Regeneration (%)*	No. of shoots ± SE / explant
Cotyledon	42.0	5.3 ± 1.7
Leaf disc	17.5	1.6 ± 1.17
Hypocotyl	49.0	8.2 ± 3.85
Disc floret	73.0	41.1 ± 19.4
Receptacle	78.0	9.3 ± 6.2

*The explant type significantly affected the percentage of plant regeneration (Kruskal-Wallis test, $P < 0.05$)

florets (Figure 2C). Induction and development of shoots on the surface of the hypocotyls was noticed after two and four weeks respectively of culture initiation (Figure 2D, E).

Continuous culture of explants on regeneration medium more than forty days resulted in browning of the explants and formation of vitrified shoot-like structures. Hence, all the explants were subcultured to basal medium within 30 days. Basal medium favored the formation of shoot initials and elongation. By the end of second sub culture on basal medium, most of the cultures appeared as clumps of shoots. In subsequent culture periods, each clump was cut into 4-8 pieces and inoculated to increase the contact of the cut end with the medium for favor efficient uptake of nutrients.

Half strength MS medium supplemented with 0.5 mg/L of NAA favored quick formation of healthy roots (Figure 2F). Higher concentrations of NAA induced the formation of callus and lower levels influenced the formation of a weak root system at the cut ends. The acclimatization rate of plants obtained from the optimal rooting medium was 95%. Acclimated plants in the small cups were transferred to garden pots filled with soil and farm yard manure without disturbing the root system and periodically irrigated with water. After 30-35 days of transfer to the greenhouse, the plants produced normal flowers (Figure 2G).

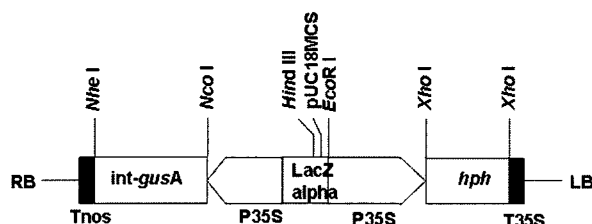

Figure 1. T-DNA portion of pCAMBIA1301 map showing restriction sites. T-DNA region of pCAMBIA1301 (RB- Right border, T- terminator, int-gusA- GUS gene interrupted with eukaryotic intron, P- promoter, *hph*- hygromycin phosphotransferase gene, LB- Left border).

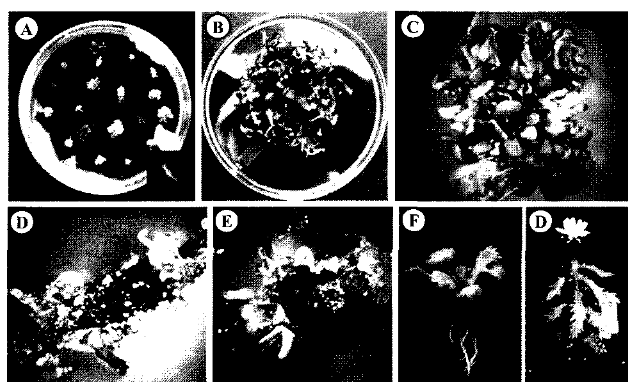

Figure 2. Regeneration of *L. vulgare* from various explants. A, callus induction from disc florets after 25 days on MS medium containing B_5 vitamins supplemented with 0.1 mg/L BA and 0.1 mg/L TDZ; B, regeneration of numerous shoots from disc florets after transferring to basal MS medium; C, multiple shoot regenerated directly from receptacle; D, direct formation of shoot initials from cultured hypocotyls; E, later stage of shoot formation from hypocotyls; F, plants with NAA-induced root system; G, a hardened plant at flowering stage.

Agrobacterium- mediated T-DNA transfer

All the tested explant types were amenable to *Agrobacterium* infection as the co-cultivated explants showed blue spots after GUS assay (Figure 3A, E). The rate of transformation was dependent on explant types (Table 3). Vegetative parts such as cotyledonary leaf, leaf disc and hypocotyl (Figure 3A, B, E) were less susceptible than reproductive organ explants such as disc florets and receptacles (Figure 3C, D). After infection, many of the explants changed to yellow on co-cultivation medium and further selection on medium containing hygromycin lead to browning. Very few hypocotyls showed direct shoot regeneration (Figure 3F). Resistant calli were arising from the surface and cut ends of the explants after two subcultures. The efforts to regenerate plants from the transgenic calli by manipulating PGR, withdrawal of hygromycin and changes in nutrient levels failed. However, withdrawing hygromycin and PGR from the medium influenced faster growth of callus mass. When assayed for GUS, all the tested hygromycin-selected calli turned into characteristic deep blue color (Figure 4A). As expected, the non transformed control calli did not show any blue coloration (Figure 4B). PCR analysis of genomic DNA isolated from transgenic calli using sequence specific primers for GUS gene amplified the expected 0.8 kb fragment (Figure 4C). Restriction digestion of the genomic DNA from transgenic calli with X*hoI*, and subsequent hybridization with specific probe confirmed the presence of *hph* gene in the transgenic calli (Figrue 4D).

Table 3. Rate of T-DNA transfer in various explants of *L. vulgare* after 3 days of *Agrobacterium* infection

Explant type	T-DNA transfer	
	GUS expression (%)*	No of GUS spots ± SE/ explant
Cotyledon	52.0	1.4 ± 0.5
Leaf disc	24.0	1.7 ± 0.5
Hypocotyl	37.5	3.8 ± 1.6
Disc floret	63.5	4.2 ± 1.3
Receptacle	94.5	7.6 ± 2.1

*The explant type significantly affected the rate of T-DNA transfer (Kruskal-Wallis test, $P < 0.05$)

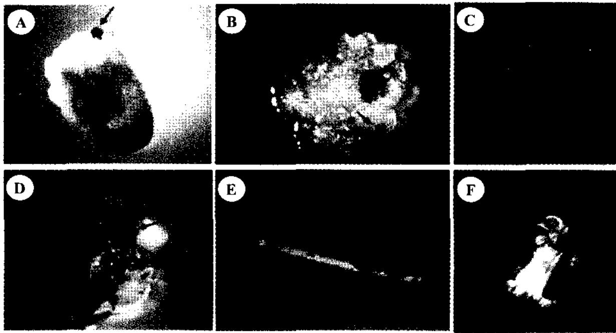

Figure 3. Transient expression of GUS gene in various explants of *L. vulgare* after infection and co-cultivation with *Agrobacterium* strain LBA4404 (GUS spots shown by arrows). A, cotyledonary leaf; B, leaf disc; C, disc floret; D, receptacle; E, hypocotyls; F, direct regeneration of shoots from a hypocotyl on selection medium.

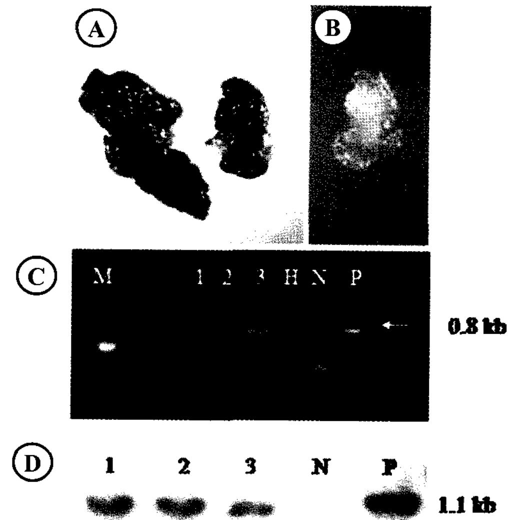

Figure 4. A, Stable GUS gene expression in transgenic callus derived from disc floret explant; B, PCR analysis of DNA isolated from one non-transformed and 3 transgenic T0 calli; C, agarose gel electrophorogram of PCR amplification performed with primers for the GUS gene. Lane M: molecular size marker (1.0 kb ladder), Lanes 1-3: transgenic calli, Lane N: non-transformed control callus, H: water control, P: plasmid pCAMBIA1301; D, Southern blot analysis of DNA isolated from one non-transformed control and three callus lines transformed with *Agrobacterium tumefaciens* LBA 4404 harboring the pCAMBIA1301. Ten μg DNA was digested with *XhoI*. Lanes 1-3: transgenic calli, Lane N: non-transformed control callus, Lane P: *hph* fragment of pCAMBIA1301 released by restriction enzyme *XhoI*.

Discussion

Standardization of micro propagation and gene transfer technology has become routine for many species in the *Chrysanthemum*-complex due to their aesthetic and floricultural value. However, the genetic improvement of button daisy has been limited because of the unavailability of suitable protocol for regeneration and transformation. In the present study, the problem was approached systematically by studying various parameters and the important results alone discussed in detail.

Regeneration of the cultured explants varied with the explant types. Among the various concentrations of PGR either alone or in combination tested, 0.1 mg/L BA plus 0.1 mg/L TDZ resulted in the best regeneration. This optimal PGR combination influenced direct regeneration of adventitious shoots from all the tested explants excluding disc florets. Nevertheless, callus induction along with direct regeneration noticed in few cultures, suggests that the cells of initial explants are heterogeneous. Similar cellular heterogeneity was also noticed in *D. grandiflora* explants (Tanaka et al. 2000).

Irrespective of the explant type, the media containing only BA or TDZ exhibited poorer regeneration than BA-TDZ combinations. The regeneration frequency of cotyledons was 17.5 % with 0.1 mg/L TDZ and 0% with 0.1 mg/L BAP. However, when the two cytokinins were provided together, the percentage of regeneration increased to 42%. This may be the result of synergistic effect of BA and TDZ as reported recently in other species (Khalafalla and Hattori 1999; Murch et al. 2000; Franklin et al. 2004). When different explants were cultured on the optimal regeneration medium, the percentage and frequency of regeneration varied. Thus, the regeneration potential of *L. vulgare* is PGR regimen- and explant type-dependent as observed previously in other species of the *Chrysanthemum*-complex (Teixeira da Silva 2003). Statistical analyses of the data revealed that explant types and PGR combinations significantly affected the percentage of plant regeneration ($P < 0.05$, Figure 5A).

Disc florets and receptacle explants contributed more number of shoots (41 and 9) and higher percentage of regeneration (78 and 73) than other types of explants. Generally, the results of our experiments showed that the floral explants are better responsive than vegetative explants. This observation is also supported by the previous observations from *Chrysanthemum*-complex. In *C. cinerariaefolium* flower head explants exhibited 100% callusing (George et al. 1999) and shoot formation (Hitmi et al. 1999). Similar observations were also recorded for *C. morifolium* ray florets (Chackrabarty et al. 2000; Dwivedi et al. 2000) and *D. grandiflora* floret and petal explants (Mizutani and Tanaka 1994).

Exposure of explants to TDZ-containing medium for more than forty days influenced the formation of malformed, vitrified shoots which later became brown and died. As noticed

in the present study, continuous culture of any explant on a medium containing TDZ more than a specific period resulted in the formation of short vitrified, translucent and unhealthy shoots previously (Magioli et al. 1998; Franklin and Lakshmi Sita 2003). To overcome this problem with TDZ, generally explants are subcultured to hormone-free medium within a period of time since; the most important factor in the induction of regeneration is the length of exposure to TDZ. In *Hypericum perforatum,* also the optimal culture period on TDZ-supplemented medium was 9 days, followed by subculture on basal medium (Murch et al. 2000).

The standardized regeneration procedure was further used to study *Agrobacterium tumefaciens*-mediated transformation. *Agrobacterium*-mediated transformation has been reported in many species of the complex with low transformation efficiencies and high levels of cultivar specificity (Van Wordragen et al. 1992; Boase et al. 1998) reduced the utility of these systems. Susceptibility of the plant cell towards *Agrobacterium* infection is the foremost requirement for the T-DNA transfer. Susceptibility of *Chrysanthemum* to wild type *Agrobacterium* strains has also been reported (Van Wordragen et al. 1992). Result of the GUS assay revealed that the susceptibility to *Agrobacterium* infection varied significantly ($P < 0.05$, Figure 5B) with explant types. Since intron-GUS gene has been used in the present study, we can safely conclude that the blue coloration was due to integration of T-DNA and not due to *Agrobacterium* contamination. As the non transformed explants and callus did not show any blue coloration, there was no background GUS activity. Commonly, background GUS activity would show blue coloration all over the explants and not as spots.

Necrosis of explants after infection may be correlated to the pathogenic effect of *Agrobacterium,* which is a plant pathogen capable of inducing necrosis (Hansen 2000). To overcome browning response, media were supplemented with sodium thiosulfate (Boase et al. 1998) or with phenolic compounds (Olhoft et al. 2001). However, addition of such compounds in the medium did not seize browning in the present investigation suggests that the browning may be due to some other reason.

GUS assay of hygromycin-selected calli suggested that there are no escapes at the callus level. However, the GUS negative plants obtained from the explants via direct organogenesis under similar selection may be escapes. Some authors suggested that the origin of escape shoots may be transiently transgenic cells (Ledger et al. 1991; Fukai et al. 1995). Generally, plant-selecting antibiotics have a negative influence on regeneration of plants from either transformed or non-transformed cells of various species. If this was the case, plants should have arisen from the transgenic calli after removal of selection pressure. Recalcitrance of the transgenic calli even after the withdrawal of selection may be due to the activation of cellular defense mechanism after *Agrobacterium* infection which might have altered the cellular totipotency.

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