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Optimization of transient *GUS* expression of *Agrobacterium*-mediated transformation in *Dierama erectum* Hilliard using sonication and *Agrobacterium*

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ABSTRACT

Efficient *Agrobacterium*-mediated transformation was established via sonication of embryonic shoot apical meristems (ESAMs) of *Dierama erectum* Hilliard. Effects of explant types, co-cultivation time, acetosyringone concentration, *Agrobacterium* concentration and different gene delivery methods were evaluated for higher efficiency of genetic transformation in *D. erectum*. An explant type (ESAMs), concentration of *Agrobacterium* inoculum (OD_{600} of 1.6) and acetosyringone (50 mg L^{-1}), and co-cultivation duration (3 days) were optimized for efficient genetic transformation of *D. erectum*. The transformation efficiency varied with explant types (from 0 to 60%), concentrations of bacteria (10 to 55%) and acetosyringone (50 to 90%) and period of co-cultivation (30 to 70%). The transformation efficiency was best with ESAMs explants compared with callus clusters. The gene delivery method via sonication-assisted *Agrobacterium*-mediated transformation (SAAT) provided higher transformation efficiency (40%) *GUS* expression compared with agrobacterial monolayer and agrobacterial suspension which gave less than 5% transformation efficiency. The putative transgenic plants which histochemically expressed *GUS*, were confirmed further with PCR and 35.3% of the plants were *GUS* positive. Stable integration of the transgene was not demonstrated hence the *GUS* expression observed was regarded as transient. This newly developed transformation system may facilitate improvement of *D. erectum* characteristics and other related geophytes.

1. Introduction

Dierama erectum Hilliard (Iridaceae) is a geophyte grown for medicinal and ornamental purposes. In ornamental geophytes, the timing of transition from vegetative to the flowering phase is critical as it determines the plant's growth cycle (Scortecci et al., 2001). Most geophytes must pass through a long juvenile phase of vegetative development before flowering (Lin et al., 2003). For example, after seed germination, *Dierama* seedlings remain in a juvenile, floral incompetent stage which can last 3 to 4 years. In view of this problem, the development of a basic genetic transformation protocol is recommended. This will lay a foundation for important features such as shortening of juvenility in *D. erectum*, manipulation of the corolla size and colour which will be of immense biotechnological interest and horticultural benefit. The study is not only important for the development of ornamental traits in *Dierama*, but will also afford basic in-depth biological studies, as well as adding more details to the existing knowledge on the genus *Dierama*.

One outstanding feature of this species is the large-sized, magenta-pink flowers, allowing for its development as an ornamental plant. Challenges facing *Agrobacterium*-mediated transformation of monocotyledonous geophytes such as *Dierama* are well known (Koetle et al., 2015). Despite this, many related species have reportedly been successfully transformed via *Agrobacterium*-based systems of gene delivery. For instance, cormels and shoot tips of a close relative *Gladiolus* were transformed (Babu and Chawla, 2000; Kamo et al., 2010). Other examples include *Narcissus tazzeta* (Lu et al., 2007), *Agapanthus praecox* (Suzuki et al., 2001) and *Allium sativum* (Kondo et al., 2000).

The use of a low frequency of ultrasound (up to 60 kHz) for enhancement of genetic transformation has received increased attention. This is a longitudinal pressure wave whose frequency exceeds 20 kHz (Raichel, 2006). Recent developments in sonochemistry have made ultrasound irradiation procedures more useful in a broader range of applications (Rokhina et al., 2009). This low frequency ultrasound acts as an abiotic stress in plants and has many biological effects, hence growth and development of several plants have been stimulated by ultrasound (Teixeira Da Silva and Dobránszki, 2014). The present study investigated the

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; CaMV35S, Cauliflower mosaic virus 35S promoter; CTAB, Cetyltrimethylammonium bromide; ESAMs, Embryonic shoot apical meristems; *GUS*, β-Glucuronidase; IBA, Indole-3-butyric acid; MS, Murashige and Skoog; NAA, 1-Naphthaleneacetic acid; OD, Optical density; PCR, Polymerase chain reaction; SAAT, Sonication-assisted *Agrobacterium*-mediated transformation; SEM, Scanning electron microscope.

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fundamental factors contributing to successful *Agrobacterium*-mediated genetic transformation in *D. erectum*.

2. Materials and methods

2.1. Plant material and explant selection

Mature seeds were collected from a wild population at Mt. Gilboa, KwaZulu-Natal (S 29°15.873, E 30° 29.743 ± 5 m). The seeds were washed in running tap water and dried at room temperature. They were disinfected with 70% (*v*/v) ethanol for 8 min followed by 0.1% (*w*/*v*) mercuric chloride (5 min) and 3 rinses in sterile distilled water. Seeds were germinated aseptically on 1/10th strength Murashige and Skoog (1962) medium (MS) at 25 °C and a photoperiod of 16 h light/8 h dark. The embryonic shoot apical meristems (ESAMs) were derived from 2-day-old seedlings and hypocotyls from 6-day-old seedlings. These were used as explants for subsequent genetic transformation experiments. To obtain callus, full strength MS medium was supplemented with 1-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 0, 0.5, 1.0, 2.0 and 2.5 mg L^{-1}. Hypocotyl explants were isolated from 6-day-old seedlings and inoculated in the callus induction medium. The concentration of 1.0 mg L^{-1} NAA gave the best callus and hence was used to obtain organogenic callus used for transformation.

2.2. Sensitivity test of explants to kanamycin and cefotaxime

The explants of ESAMs were placed on shoot multiplication medium (Koetle et al., 2010) supplemented with 0.23 mg L^{-1} benzyladenine (BA) and various concentrations of kanamycin (0, 25, 50, 100, 200 and 400 mg L^{-1}). The optimal kanamycin concentration (100 mg L^{-1}) that was found to kill all explants was used for selection of putative transformants in the subsequent experiments (Table 1). For determining the concentrations of cefotaxime for transformation experiments ESAMs were inoculated in an overnight *Agrobacterium* suspension and placed on the same shoot multiplication medium supplemented with different cefotaxime concentrations (0, 25, 50, 100, 200 and 400 mg L^{-1}). The optimal cefotaxime concentration that eliminated all the bacteria while maintaining regeneration capacity was 100 mg L^{-1}. This was used in the experiments that followed. Data was collected after 6 weeks.

2.3. Agrobacterium tumefaciens-mediated transformation

Various factors influencing transformation such as explant type (ESAMs, hypocotyls and organogenic callus), co-cultivation time (0, 1, 2, 3, 4 and 5 days), acetosyringone concentration (0, 25, 50, 100 and 200 mg L^{-1}), *Agrobacterium* concentration (OD_{600} of 0.0, 0.2 0.4, 0.8, 1.6 and 2.0), and different methods of gene delivery (agrobacterial monolayer, agrobacterial suspension and sonication-assisted *Agrobacterium*-mediated transformation (SAAT)) were optimized for efficient genetic transformation. For all experiments, the *Agrobacterium* strain LBA4404 harbouring the binary plasmid vector pCAMBIA1301, with the T-DNA region consisting of the *GUS* gene driven by the Cauliflower Mosaic Virus 35S (CaMV35S) promoter, was grown overnight (up to mid-log phase) in Luria-Bertani (LB) medium containing 0.1 mg mL^{-1} kanamycin and 0.15 mg mL^{-1} rifampicin. The bacterium was pelleted at 5000 × *g* for 20 min, washed in antibiotic free LB medium, re-pelleted and re-suspended in fresh LB medium. Explants were co-infected in LB medium containing the *Agrobacterium* for 30 min before transfer to co-cultivation medium (MS + 0.23 mg L^{-1} BA). After a pre-determined co-cultivation period, explants were washed in 350 mg L^{-1} cefotaxime for 10 min and blotted on a sterile filter paper to remove excess bacteria. Explants were then inoculated onto the pre-selection phase medium (MS + 0.23 mg L^{-1} BA + 50 mg L^{-1} cefotaxime). After 7 days of explant recovery, they were washed again in 350 mg L^{-1} cefotaxime and transferred to the selection medium (MS + 0.23 mg L^{-1} BA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin) for selection of putative transformants. Where applicable, the rooting medium supplemented with indole-3-butyric acid (MS + 0.20 mg L^{-1} IBA + 100 mg L^{-1} cefotaxime + 100 mg L^{-1} kanamycin) was used.

2.3.1. Effect of explant type on efficiency of GUS expression

The ESAMs and hypocotyls were excised and used for transformation. The ESAMs were inoculated in pre-determined MS medium supplemented with 1.0 mg L^{-1} NAA to obtain callus. All explants (callus, ESAMs and hypocotyls) were inoculated in an agrobacterial suspension for 20 min and placed on co-cultivation medium (MS + 0.23 mg L^{-1} BA) until the bacteria were visible around the explants (after 3 days). After co-cultivation, explants were washed, transferred to pre-selection and selection media. The efficiency of *GUS* expression was determined in shoots after 4 weeks.

2.3.2. Effect of gene delivery systems on efficiency of GUS expression

The ESAMs were aseptically transferred to 20 mL sterile distilled water and put on a sonicator (Julabo Labotechnik GMBH, West Germany) operating at a maximum frequency of 35 kHz for 0, 10, 20, 30, 40, 50 and 60 s to determine the best duration for wounding of explants without detrimental effects to the tissue. Explants were then placed on MS medium containing 0.23 mg L^{-1} BA. After 10 days, the percentage of surviving regenerating explants was recorded. This preliminary experiment revealed that explants could not be exposed to sonication beyond 30 s since this impeded their regeneration ability (Fig. 1). Therefore, for all experiments involving SAAT, explants were sonicated for 30 s.

Explant tissues were viewed under the scanning electron microscope (SEM) to further investigate the effect of sonication on meristematic cells. To achieve this, ESAMs were suspended in distilled water contained in a 50 mL conical flask and sonicated for 30 s. Control explants were only immersed in distilled water. Explants were then prepared for viewing on SEM (James Cook University, Advanced Analytical Centre, Australia, 2016). Samples were viewed with the SEM (Zeiss Evo/Ls15) fitted to a secondary electron detector compatible with SmartSEM V05.04.02.00 computer software.

To investigate the effects of different systems of gene delivery on *GUS* expression, ESAMs were either inoculated in agrobacterial suspension (overnight culture) for 30 min without sonication, or sonicated in agrobacterial suspension for 30 s or placed on an agrobacterial monolayer (preparation is described below). Plants that survived the selection phase (MS + 0.23 mg L^{-1} BA + 100 mg L^{-1}

Table 1
Effect of antibiotics on embryogenic shoot apical meristem survival of *Dierama erectum*.

Antibiotic concentration (mg L^{-1})		Growth parameters		
Cefotaxime	Kanamycin	No. of regenerating shoots	No. of roots	Length of longest root (mm)
0	–	1.20 ± 0.13^{a}	2.10 ± 0.35^{a}	25.30 ± 1.55^{a}
25	–	1.20 ± 0.13^{a}	1.30 ± 0.15^{b}	25.20 ± 2.35^{a}
50	–	1.20 ± 0.13^{a}	1.00 ± 0.00^{bc}	16.60 ± 1.05^{b}
100	–	1.00 ± 0.00^{ab}	1.00 ± 0.00^{bc}	18.70 ± 1.86^{b}
200	–	0.80 ± 0.13^{b}	0.70 ± 0.21^{cd}	3.30 ± 0.97^{c}
400	–	0.70 ± 0.15^{b}	0.70 ± 0.30^{cd}	0.60 ± 0.31^{c}
–	25	0.80 ± 0.13^{b}	0.60 ± 0.27^{cd}	0.50 ± 0.32^{c}
–	50	0.20 ± 0.13^{c}	0.40 ± 0.16^{de}	0.45 ± 0.29^{c}
–	100	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
–	200	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
–	400	0.00 ± 0.00^{c}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}

In each column, values with different letter(s) indicate significant differences among treatments ($P \leq 0.05$, n = 25) based on Duncan's Multiple Range Test. Data was recorded 8 weeks after experimental set-up.