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Transient Expression of *uid*A Reporter Gene in Regenerable Callus Tissues of *Anthurium andraeanum* Lind. by *Agrobacterium* Mediated Transformation

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ABSTRACT. *A tissue culture scheme for plant regeneration via callus, and a protocol for gene transfer to regenerable callus are described for Anthurium andraeanum variety Avo Nette. Callus was induced from shoot bases and from leaf blades of Anthurium andraeanum on modified MS media containing 0.3 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP. Shoot bases, leaf blades with a single cut at the base, and sectioned leaf blades produced callus at frequencies of 90%, 20% and 8%, respectively. Plant regeneration was obtained at a high frequency in all callus types, irrespective of their origin, in modified MS media containing 0.5 mg l^{-1} BAP. Callus was tested for gene transfer efficiency by 2 Agrobacterium tumefaciens strains, C58 and LB4404, carrying binary vector $P^{CAMBIA1301}$ containing hpt and uidA reporter genes under plant expression signals. Transient expression of uidA gene monitored by GUS histochemical assay was observed only in callus inoculated with Agrobacterium strain LB4404 at a frequency of 3.33%.*

INTRODUCTION

Anthurium andraeanum, a perennial herbaceous plant, is a highly priced ornamental for its attractive, long lasting flowers and for exotic foliage. It is being traditionally propagated by off shoots, nodal cuttings and seeds. The uniformity of flowers is an important character, and hence clonal propagation has become an important practice in *Anthurium* cultivation.

In vitro clonal propagation is widely used for commercial cultivation of ornamental plants. One of the major disadvantage of growing clones in any plant species is the danger of devastation by pest and disease epidemics. The biggest threat for *Anthurium* cultivation worldwide is the disease 'bacterial blight' caused by *Xanthomonas compastris* pv. dicambacia. Lack of resistant genotypes for certain pests and diseases of *Anthurium* led to initiate genetic engineering approaches to bring resistant characteristics from other organisms into *Anthurium*.

The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (De Block *et al.*, 1984; Horsch *et al.*, 1984; Paszkowski *et*

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al., 1984), has been extended to over 150 species in at least 35 families (Dale, 1995; Birch, 1997). Among the different types of gene transfer techniques, the presence of *Agrobacterium* mediated gene delivery is not only for historical and economic reasons, but also for the fact that insertion of transgenes in the host genome, is a very precise technique. Gene transfer to *Anthurium* has been achieved by inoculation with *Agrobacterium* to embryogenic callus etiolated internodes (Chen and Kuehnle, 1996), and roots (Chen *et al.*, 1997).

The choice of explant for co-cultivation with *Agrobacterium* is one of the most important factors in rice transformation (Hiei *et al.*, 1994). Tissue culture is not a theoretical prerequisite for plant transformation, but it is employed in almost all current practical transformation systems to achieve a workable efficiency of gene transfer, selection and regeneration of transformants. Plant regeneration *via* somatic embryogenesis from *Anthurium* is important and facilitate micropropagation and genetic engineering (Kuehnle *et al.*, 1992).

This study was concentrated on developing a plant regeneration system for *Anthurium andraeanum* variety Avo Nette from different explants *via* callus phase. A preliminary attempt for transient expression of a foreign gene (*i.e. uid*A gene) in callus tissues is described. Two *Agrobacterium* stains, a low virulent type (*i.e.* LB4404) and a super virulent type (*i.e.* C58) were tested for their ability to transform *Anthurium*.

MATERIALS AND METHODS

Plant material, callus induction and regeneration

Immature leaf blades and shoot bases were collected from *in vitro* grown *Anthurium* plantlets. Fully expanded tender leaves cut into rectangular pieces of 1–2 cm^{-2}, whole leaf blade with a single cut at the base, and shoot bases of approximately 1 cm long were incubated on callus induction medium (CIM) in the dark at 28°C. Callus developed was subcultured twice and transferred onto shoot induction medium (SIM) and incubated under continuous fluorescent light at 28°C. CIM and SIM media contained half-strength macro nutrients, full-strength micro nutrients and vitamins of MS medium (Murashige and Skoog, 1962), 100 mg l^{-1} myoinisitol, 3% (W/V) sucrose, and 0.7% (W/V) agar, pH 5.8. CIM media contained 0.33 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP, whereas SIM media contained only 0.5 mg l^{-1} BAP.

Bacterial strains

Disarmed *Agrobacterium tumefaciens* strains C58 and LB4404 carrying the binary vector $P^{CAMBIA1301}$ (Picambia, Australia) were used for transformation experiments. Overnight cultures of *Agrobacterium*, prepared by inoculation with a saturated preculture at 1:10 ratio, were induced by adding acetosyringone at a final concentration of 200 µm 1 h before inoculation to explants. The induced bacterial cells were pelleted and resuspended in plant transformation media (PTM) containing salts, vitamins and hormones of CIM, 10% (W/V) sucrose, 1% (W/V) glucose, 5 mM $MgCl_2$ and 200 µm acetosyringone.

Preparation of explants for inoculation

One-month-old callus was plasmolyzed for 60 min prior to inoculation by dipping in a liquid CIM media containing 10% sucrose. Plasmolyzed callus was directly transferred to induced bacterial suspension in PTM, and incubated at room temperature for 20 min. Inoculated callus was transferred onto co-cultivation media, which is similar to PTM but contains only 3% (W/V) sucrose and solidified with 7% (W/V) agar. After 2 days of incubation at room temperature, the calli were subjected to GUS histochemical assay.

Histological staining for GUS enzyme activity

GUS assay (Jafferson, 1986) was performed according to a modified method of Mendal *et al.* (1989). The callus co-cultivated with *Agrobacterium* was incubated overnight at 37°C in darkness in sterile GUS staining solution containing Magenta Gal (Cat. No. B-4657, Sigma Chemical Co., St. Louis, USA). The Magenta coloured loci or sectors were counted under dissecting microscope.

RESULTS AND DISCUSSION

Before attempting transformation experiments, different types of explant of *Anthurium andraeanum* Lind. variety Avo Nette were tested for their ability to produce callus and regenerate plants from them. The highest frequency of callus formation was observed in shoot base explant. Other 2 explant types had very low frequency of callus formation (Table 1). Most important factors affecting callus induction and regeneration are the genotype, physiological status of explant, culture media and incubation conditions. Theoretically, for each type of explant, the best media composition and incubation conditions have to be experimentally found. For this experiment the culture media was adopted from previous reports (Kunisaki, 1980; Kuehnle and Sugii, 1991; Pierik *et al.*, 1974; Geier, 1986) and modified accordingly.

Table 1. Frequency of callus formation from different explants.

Type of Explant	Number of Explants Cultured	Number of Calli-formed Explants	Frequency of Callus Formation
Shoot base	100	90	90%
Whole leaf blade with a single cut	70	25	20%
Sectioned leaf blade	80	10	8%

Least number of callus was produced from sectioned leaf blades. Kuehnle *et al.* (1992) reported that sectioned leaf blades of *Anthurium* have not produced any callus under their experimental conditions. Callus derived from different explants did not differ in their regeneration frequency and morphology of the shoots developed. Nearly 100% of the

callus produced shoot upon transfer onto shoot induction medium (data not shown). It appears that physiological differences of different explants, used for callus induction, do not carry over to the callus they produced. The physiological differences affect when explants of different origin, that are devoted for specialized functions, are forced back to meristematic function and to produce callus. Higher callusing frequency of shoot bases may be attributed to presence of axillary buds, which are of meristematic nature.

Transformation was conducted in 2 independent experiments to test the feasibility of 2 types of callus for gene transfer efficiency by 2 *Agrobacterium* strains (*i.e.* C58 and LB4404). However, *Agrobacterium* strain LB4404 showed 33.3% transformation frequency only in the callus originated in the shoot base (Table 2). It has been observed, in various plant species both under natural and laboratory conditions, that the transformation frequencies by *Agrobacterium* vary according to the genotype, age and physiological status of the explant. Meristematic tissues, young plants and cells undergoing dedifferentiation are the choicest material for *Agrobacterium* mediated transformation (Mahalakshmi and Khurana, 1997). Another reason for choosing callus in our experiments for gene transfer is the higher probability of transformed cells to regenerate via somatic embryogenesis, which then minimize the number of chimeric plants for the transgene. Further, the relative easiness of callus induction, multiplication, maintenance and ability for aseptic handling have made callus the ideal material for gene transfer experiments. However, callus can cause somaclonal variation. Transformation frequency to *Anthurium* by different *Agrobacterium* strains has not yet been documented. In this study, a low virulent strain (LB4404) and a supervirulent strain (C58) of *Agrobacterium* were evaluated for their ability and frequency to transform regenerable *Anthurium* callus. Both strains carry binary vector $p^{CAMBIA1301}$ (Picambia, Australia).

Table 2. Effect of callus type and *Agrobacterium* strain on transformation frequency.

Callus origin	*Agrobacterium* strain	Number of calli co-cultivated	Number of GUS positive calli	Transformation frequency
Shoot base	LB4404	30	1	3.33%
Shoot base	C58	30	0	0%
Shoot base	-	30	0	0%
Leaf blade	LB4404	30	0	0%
Leaf blade	C58	30	0	0%
Leaf blade	-	30	0	0%

Only 1 magenta sector, corresponding to β-glucuronidase activity of transformed *uidA* reporter, was observed on a shoot base derived calli inoculated with *Agrobacterium*, strain LB4404. Lack of GUS activity in negative controls, where calli were not inoculated with *Agrobacterium*, indicates no detectable endogenous β-glucuronidase activity in *Anthurium*.

It is difficult to conclude that those tissues inoculated with *Agrobacterium* and produced no GUS expression are completely incompetent for infection by *Agrobacterium* strains used. Very low frequency of gene delivery may exist due to low competency of target tissue. It could also be due to suboptimal conditions for the infection process to occur. The hidden low frequency of gene transfer could be examined by employing large number of calli for transformation experiment. Also, the efficiency of gene transfer could be improved by manipulating the transformation conditions. Immature embryos of maize of different ages were differentially susceptible to *Agrobacterium* (Mahalakshmi and Khurana, 1997). Results also indicated that there exists a specific window of competence in the explant for gene transfer. Further, the correlation between transient gene expression and stable transformation is not clear (Laparra *et al.*, 1995).

CONCLUSIONS

In this preliminary study, we described a method for *Agrobacterium* - mediated gene delivery to regenerable callus of *Anthurium andraeanum* variety Avo Nette. The results indicate that combination of shoot base derived callus and the low virulent *Agrobacterium* strain LB4404 is the choice for further experiments. Further improvement of transformation frequency could be obtained by manipulating the crucial steps in transformation protocol such as age of the explant, cocultivation and inoculation temperature, media composition and bacterium concentration and growth media.

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