I.1 Transgenic *Anthurium*

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1 Introduction

1.1 Importance of *Anthurium*

Anthurium is the largest and most complex genus in the family Araceae. It consists of about 1000 species (Croat 1992). The distribution of this genus extends from northern Mexico and the Greater Antilles to southern Brazil, northern Argentina, and Paraguay (Croat 1983, 1986). The chromosome number of most investigated species is 2n = 30 (Sheffer and Kamemoto 1976; Sheffer and Croat 1983).

Anthuriums are known as an exotic ornamental crop. The commercial flower is a combination of a colorful modified leaf, termed spathe, subtending bisexual flowers carried in a spiral fashion on a spadix. Leaves are simple with netted venation. The attractive foliage of some species makes anthurium suitable for harvesting the leaves and as a potted flowering plant. Commercial production has focused on two major species: A. *andraeanum* and *A. scherzerianum. A. andraeanum* is grown mostly for cut-flower production, the main production areas being Hawaii, The Netherlands, and some other tropical and subtropical countries. *A. scherzerianum* is sold as a flowering potted plant, with main production areas located in Europe. The 1998 combined Dutch auctions ranked anthurium 12th of all cut-flower sales, with almost 46 million stems sold for over 29.4 million Euro (Online Report 1999). In Hawaii, anthurium is the leading cut flower with a 1998 wholesale value of over $6 million for 10 million stems sold (Hawaii Agricultural Statistics Service 1999).

1.2 Need for Genetic Transformation

Anthurium andraeanum Hort. is generally clonally propagated for cut-flower and potted plant production. New cultivars of this outbreeding crop are developed through sexual hybridization and progeny evaluation and selection.

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Breeding has been very successful in providing the market with many different flower colors and shapes (Kamemoto and Kuehnle 1996; Kuehnle et al. 1996a,b). Development of a new anthurium cultivar usually takes from 8 to 10 years, due to the long life cycle of the plant (3 years from seed to seed).

A major problem in production of anthurium is disease: while resistance to anthracnose (*Colletotrichum gloeosporioides*) has been identified among accessions (Aragaki et al. 1968) and used successfully in breeding, genetic resistance to burrowing nematode and bacterial blight is not currently available. Anthurium decline caused by the burrowing nematode, *Radopholus similis*, can be controlled by application of nematicides (Kamemoto 1988), but these chemicals may have restricted use in the near future. The worldwide spread in the 1980s of bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* has caused major problems. In Hawaii alone, anthurium production declined steadily between 1986 and 1996 from 28 million to almost 11 million stems (Hawaii Agricultural Statistical Service 1998). Most commercial cultivars are susceptible to the blight, and no single economical control measure exists (Nishijima and Fujiyama 1985). In response to these breeding challenges, a program was initiated to develop a genetic transformation method to introduce disease-resistant genes into anthurium (Kuehnle 1989).

2 Transformation

2.1 Previous Work

In a survey of the Araceae, only calla and philodendron were reported to be susceptible to infection by tumorigenic *Agrobacterium tumefaciens* strain B6 (De Cleene 1985). Subsequently, Kuehnle and Sugii (1991) reported tumor formation and nopaline production in *Anthurium andraeanum* Hort. when cocultivated with *A. tumefaciens* strains A281 and C58 in an induction medium containing acetosyringone. Molecular analyses indicated the presence of the nopaline synthase (NOS) gene of T-DNA in the plant tissue. Etiolated shoots proved to be the most susceptible explant. More recently, cocultivation of root cuttings with *A. tumefaciens* resulted in Anuenue plants transgenic for *neo* and *att* (see below) with a transformation efficiency of 1.3% (Chen et al. 1997).

Plant regeneration from tissue cultures is necessary for transformation work. Regeneration of *A. andraeanum* and *A. scherzerianum* has been obtained via a callus stage from cultured embryos and explants of leaf lamina, petiole, spadix, spathe, roots, and etiolated shoots (reviewed by Geier 1990 and Matsumoto and Kuehnle 1997; Chen et al. 1997). The time required for callus formation in leaf explants, depending on the genotype, usually takes from 2 weeks to 3 months. Shoot initiation from the callus takes from 4 weeks to 6 months. In root explants, callus and shoot formation occurred within 2 and 3 months, respectively. Somatic embryogenesis and plant regeneration, requiring 3 to 4 months, have also been reported for *A. andraeanum* Hort. (Kuehnle

et al. 1992). Results from a recent study on histological origin of somatic embryos derived from lamina showed somatic embryos to arise from the mesophyll, either from a proembryonic cell complex or from a single cell. These results are encouraging for regeneration of nonchimeric transformed plants, provided gene transfer is targeted to the mesophyll (Matsumoto et al. 1996). Microcalli, but not plants, are reported from *Anthurium* protoplasts (Kuehnle 1997).

2.2 Methodology

Tissue culture methods, combined with the use of nontumorigenic *Agrobacterium* strain LBA4404, containing the vir-helper plasmid (pAL4404) in strain Ach5 chromosomal background (Hoekema et al. 1983), have resulted in an effective method for genetic engineering of *Anthurium* (Chen and Kuehnle 1996). This method was demonstrated with two cultivars of diverse species background, Rudolph (*A. andraeanum*) and UH1060 (*A. lindenianum, A. kamemotoanum*) detailed in Kuehnle and Chen (1994). It has recently been further refined, as described below. Although the following protocol is intended for use with etiolated shoot explants, intact laminae or root segments from in vitro-grown plantlets can also be used with modifications in cocultivation media (Chen 1993; Chen et al. 1997).

2.2.1 Transformation and Plant Regeneration

1. Pick a single colony of disarmed *A. tumefaciens* strain LBA4404 carrying NPT II (neomycin phosphotransferase II enzyme for kanamycin resistance) in a binary vector system and culture in 5 ml LB medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7) containing 50 µg/ml kanamycin and 25 µg/ml streptomycin, 250 rpm, 28 °C overnight or 2 nights until the bacterial suspension becomes turbid.
2. Prior to cocultivation, add 2 µl of 0.5 M acetosyringone (AS) to overnight-grown bacteria and mix well. AS stock (0.5 M) is prepared by dissolving 0.25 g AS in 2.55 ml dimethyl sulfoxide (DMSO); the stock should be filter-sterilized (0.22 µm) and stored at –20 °C. Dilute the bacteria + AS suspension tenfold with LB in a 10-cm glass Petri dish (final concentration of AS = 20 µM). For control (no bacteria) treatment, retain 4 ml of LB medium in a separate Petri dish.
3. Prepare one or two Magenta GA-7 boxes containing etiolated shoots by culturing callus or intact laminae on standard anthurium shoot proliferation medium (Kunisaki 1980) in complete darkness for several months. Use a new scalpel blade to carefully cut internodes (1 cm) with the help of a pair of forceps. Drop the internode explants immediately into the diluted *Agrobacterium* suspension. Immerse individual explants into the suspension with forceps. Immerse control explants in LB medium similarly. Time in suspension is about 8 min for 40 explants.

4. Closely space all explants of the same treatment side by side in the center of a plastic Petri dish containing about 25 ml of solid H3 medium (Table 1). Seal dishes with Parafilm and incubate at 25 °C in the dark for 2 to 4 days.
5. On the third day, examine the cocultured explants. If bacterial growth is visible around the explants, it is time to transfer explants onto selection/regeneration medium.
6. Pick individual internode explants and blot to remove excess *Agrobacterium* cells. Transfer 20 to 25 explants onto a 10-cm plastic Petri dish with H3 medium containing 500 µg/ml carbenicillin or 250 µg/ml cefotaxime and 50 µg/ml kanamycin.
7. Incubate Petri dishes in the dark at 25 °C for 1 to 2 months. Transfer explants monthly onto fresh selection medium with both antibiotics. Examine at least once a week for any regrowth of *Agrobacterium* and subculture if necessary.
8. In the second month, examine cocultured explants to see if any callus has formed on the cut ends. Explants with callus, should be transferred to fresh H3 medium (Table 1) containing 500 µg/ml carbenicillin or 250 µg/ml cefotaxime and 100 or 50 µg/ml kanamycin, and incubated in weak light (about 4 µE/m^2/s, 16-h light/8-h dark cycle) at 25 °C. It should be cautioned that the antibiotic kanamycin at 50 µg/ml may inhibit callus formation by internodes of some cultivars. Thus, reduction in kanamycin concentration to 25 µg/ml may be advisable at the outset; after callus formation, return to a higher kanamycin concentration (50 µg/ml). Conversely, some genotypes proliferate excessively during callus formation and thus 100 µg/ml kanamycin should be used up to this step.
9. One month after incubation of explants in weak light, pale green callus should be visible. Transfer these callused explants to fresh H3 medium with both antibiotics and incubate under the same conditions.
10. Approximately 9 months following cocultivation, green shoots on the calli are removed with forceps or a scalpel and transferred onto H1 (Table 1) medium (for multiplication of individual lines) containing 50 µg/ml

Table 1. Composition of media used in cocultivation and tissue culture of anthurium

Component	C	H1	H2	H3
Macronutrients	1/2 × MS	1/2 × MS	1/2 × MS	1/2 × MS
Micronutrients	MS	MS	MS	MS
NaFeEDTA	25.7 mg/l	36.7 mg/l	36.7 mg/l	36.7 mg/l
Vitamins	As MS[a]	As 1/2 × MS[b]	As 1/2 × MS[b]	As 1/2 × MS[b]
Sucrose	20 g/l	20 g/l	20 g/l	20 g/l
Glucose	10 g/l	–	–	–
Myo-inositol	100 mg/l	100 mg/l	100 mg/l	100 mg/l
2,4-D	1.5 mg/l	–	–	0.4 mg/l
BA	–	0.2 mg/l	–	0.2 mg/l
Kinetin	0.5 mg/l	–	–	–
Coconut milk	–	–	150 ml/l	

[a] With 0.4 mg thiamine·HCl/l.
[b] With 0.2 mg thiamine·HCl/l.

kanamycin in Magenta GA-7 boxes. Incubate in higher light (32 μE/m^2/s, 16-h light/8-h dark cycle) at 25 °C for 1 to 2 months. Subculture to fresh medium every 1 or 2 months. Discard those shoots with bleached, newly developed leaves. Leaves can be sampled by polymerase chain reaction (PCR) at this stage.

11. During this period, roots emerge from the base of shoots. Transfer putative kanamycin-resistant plantlets or shoots to H2 medium for line maintenance (not multiplication) with 50 μg/ml kanamycin. Antibiotic can be discontinued once a line is verified to be transgenic. Rooted plantlets with at least three leaves, 3 to 5 cm in height, can be transferred ex vitro into 13-cm pots containing 10 to 20 plantlets in a well-draining potting mix for hardening off in the greenhouse. Approximately 2 years are required from stage 4 plantlet until first flower in anthurium.

This protocol was followed to introduce an antibacterial gene (Jaynes et al. 1987) encoding the protein attacin (Hultmark et al. 1980) into anthurium cultivars Rudolph and UH1060 (Kuehnle et al. 1995; Chen and Kuehnle 1996) The attacin gene *att* was placed under control of a double CaMV35S promoter (Kay et al. 1987) and terminated with the NOS-3′ terminal sequence (Bevan et al. 1983). It was subcloned into the *Hind* III site of pBI121 (Clontech Labs, Palo Alto, California) and designated plasmid pCa2Att (Destefano-Beltran 1991), courtesy of J. Jaynes (Demeter Biotechnologies, Research Triangle Park, N.C., USA). This plant gene expression vector also encodes NPT II for antibiotic resistance and GUS as a reporter of transgene expression (Jefferson et al. 1987).

2.2.2 Molecular Analyses

Nucleic acid extraction from in vitro leaf tissues was according to Dellaporta et al. (1983). DNA or RNA isolated from leaves of untreated and kanamycin-resistant anthuriums was used for DNA PCR amplification or reverse transcriptase PCR (RT-PCR) of specific target genes (Chen 1993; Chen and Kuehnle 1996). The primers (0.25 μg each) for the *gus* (Jefferson et al. 1986), *npt*II (Chee et al. 1989), and *att* (Kockum et al. 1984) genes are listed in Table 2. After PCR-amplified DNAs of each reaction were separated by agarose gel electrophoresis and visualized, identity of amplified fragments was also confirmed by Southern blot analysis (Southern 1975) using nonradioactive probes prepared for *att, gus*, and *npt*II (DIG DNA Labeling and Detection Kit, Roche Molecular Biochemicals, Indianapolis, Indiana). For genomic DNA restriction digestion analysis, about 10 μg plant DNA were digested with *Hind* III, separated, blotted, and bands visualized as described above.

For PCR purposes, other DNA extraction protocols are preferable to the Dellaporta method due to their speed, with DNA ready to use within 2 to 3 h. The CTAB protocol from Iqbal et al. (1995), modified to include 1.0% (v/v) β-mercaptoethanol and 1% (w/v) PVP (J.Y. Wang, Taiwan Agric. Res. Inst., unpubl.), or the commercial kits Wizard (Promega) or DNeasy-plant (Qiagen)

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