Induction of Tumors in *Anthurium andraeanum* by *Agrobacterium tumefaciens*

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Abstract. A method was devised for infecting *Anthurium andraeanum* Linden ex André, an economically important ornamental monocot, with *Agrobacterium tumefaciens*. Tumors were obtained on plant stems 7 to 10 weeks after inoculation with oncogenic *A. tumefaciens* strains C58 and A281 cultured previously in an induction medium containing 200 μ M acetosyringone at pH 5.5. A higher percentage of tumors were formed in vitro on etiolated internodes (32%) than on green leaf (2%) or petiole explants (3%) 4 weeks after inoculation with induced C58. All explants treated with nontumorigenic *A. radiobacter* or with induction medium alone failed to produce tumors. Chromatograms showed an accumulation of nopaline in internode explant tumors induced with C58. DNA amplification and hybridization studies showed that the DNA from these tumors, but not from noninoculated anthurium tissue, contained sequences homologous to the nopaline synthase gene of *A. tumefaciens* T-DNA. Chemical names used 3,5-dimethoxy 4-hydroxyacetophenone (acetosyringone).

Improvement of anthuriums, an important flower crop in Hawaii and Holland, through hybridization and selection is a long-term effort requiring 2 to 3 years for plants to mature and produce seed. A reliable gene transfer system for anthuriums could be convenient to breeders interested in improving flower color and bacterial disease resistance.

The soil bacterium *Agrobacterium tumefaciens* can naturally transfer a segment of plasmid DNA, T-DNA, into the genome of a wounded plant (Chilton et al., 1977) and is well established as a vector for plant genetic transformation. Expression of some wild-type T-DNA genes can cause overproduction of auxins and cytokinins in plant cells, leading to the growth of tumors (Braun, 1958; Garfinkel et al., 1981; Ooms et al., 1981). Other T-DNA genes are responsible for the synthesis of opines, such as nopaline, in infected plant cells (Bomhoff et al., 1976). The *vir* gene region of plasmid DNA affects T-DNA transfer and integration; while it is required for plant infection, it is not integrated into the host genome (Horsch et al., 1986). Expression of *vir* genes can be induced by various phenolic compounds, including acetosyringone (Bolton et al., 1986; Stachel et al., 1985).

Agrobacterium-mediated transformation has been most useful with dicotyledonous plants

that, upon wounding, can induce expression of the *vir* genes. Recent studies have supported infection by *Agrobacterium* of monocots found in the Liliaceae, Iridaceae, Gramineae, and Dioscoreaceae (Bytebier et al., 1987; Graves and Goldman, 1986, 1987; Grimsley et al., 1987; Hemalsteens et al., 1984; Raineri et al., 1990; Schafer et al., 1987; Woolston et al., 1988). Of the 13 genera examined within the monocotyledonous family Araceae, only *Calla* and *Philodendron* were reported to be susceptible to infection by *A. tumefaciens* strain B6 (de Cleene, 1985); however, anthurium was not susceptible when inoculated with uninduced *A. tumefaciens* strains C58, 920, A4, CG935, 937, K27, CG108, and 49 (Kuehnle, unpublished data).

In this study, we examined the susceptibility of *Anthurium andraeanum* plants and explants to infection by induced oncogenic strains of *Agrobacterium tumefaciens*.

Agrobacterium biovar 1 strains, from the collections of L. Moore (Oregon State Univ.,

Table 1. Response of *Anthurium andraeanum* selection UH965 explants to *Agrobacterium tumefaciens* strain C58 induced with acetosyringone.

Plant part and treatment	No. explants	
	Total	With tumor (%)
Petiole		
Control (YEP) ^a	40	0 (0)
<i>A. tumefaciens</i> C58	60	2 (3)
Leaf		
Control (YEP) ^a	40	0 (0)
<i>A. tumefaciens</i> C58	60	1 (2)
Etiolated internode		
Control (YEP) ^a	135	0 (0)
<i>A. radiobacter</i> control	94	0 (0)
<i>A. tumefaciens</i> C58	187	60 (32)

^aInduction medium containing 200 μ M acetosyringone.

Corvallis) and A. Alvarez (Univ. of Hawaii, Honolulu), were grown for 24 to 36 h at 50 rpm, 28C, in liquid YEP medium consisting of 1% Bacto-peptone, 1% Bacto-yeast extract, and 0.5% NaCl (An et al., 1988) with 200 μ M acetosyringone (Aldrich Chemical, Milwaukee) at pH 5.5 (YEP induction medium). Two common tumor-inducing strains with a wide host range were tested: C58 harboring the nopaline plasmid pTi-C58 and A281 combining the chromosomal background of C58 with the succinamopine plasmid pTi-Bo542. To ensure that the C58 and A281 cultures were virulent at the time of experimentation, inoculations were performed on young plants of Turkish tobacco (*Nicotiana tabacum* L.) as positive controls.

Anthurium seedlings from four genetically diverse crosses, referred to by cross numbers 926, 932, 935, and 976 hereafter, were grown in flats in a glasshouse with 80% shade and $\approx$ 29C day/23C night with a natural photoperiod. Stems of 8- to 12-month-old juvenile plants of all four crosses were punctured with a sterile dissecting needle dipped in heavy aqueous suspensions of strain C58. Other seedlings of crosses 926 and 976 were treated with strain A281. Stems wounded with a dissecting needle dipped in YEP induction medium served as controls. Wound sites were covered with petroleum jelly to maintain a humid environment favorable for bacterial proliferation and infection. The tumorigenic response was evaluated after 7 to 10 weeks by use of a 0 to 5 scale, where 0 = a smooth swelling of <1 mm in diameter at the wound site and ratings of 1 to 5 = rough, raised growths $\geq$ 1 to 5 mm in diameter, respectively. Sample sizes were n = 13 controls and 14 inoculations for 935 and 932; n = 9 controls and 10 inoculations each for C58- and A281-exposed 926; and n = 27 controls

Fig. 1. Mean response rating of seedlings from four anthurium crosses 7 to 10 weeks after inoculation with *Agrobacterium tumefaciens* strain C58 induced with acetosyringone. Controls were treated with YEP induction medium lacking bacteria. Ratings reflect tumor size and appearance, with large raised tumors receiving higher ratings than small, smooth tumors. Means were compared using the Mann-Whitney test; (NS) nonsignificant, (**) significant at $P < 0.001$.

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Fig. 2. Tumor growth on etiolated internode segments of anthurium selection UH965 7 weeks after inoculation with YEP induction medium lacking bacteria (A) or with *Agrobacterium tumefaciens* strain C58 induced with acetosyringone (B). Scale bar = 1 mm.

and 23 and 27 inoculations for C58- and A281-exposed 976, respectively. As a negative control, inoculations were also made with nonpathogenic *A. radiobacter* using cross 926 ($n = 10$). Data were analyzed using the nonparametric Mann-Whitney and Kruskal-Wallis tests (Sokal and Rohlf, 1981).

Micropropagated plantlets of the Univ. of Hawaii selection UH965, grown in vitro either under a 16-h light/8-h dark photoperiod with $54 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation at $25 \pm 2^\circ\text{C}$ or in the dark at 21°C , provided 0.5-cm^2 leaf pieces and 1-cm-long petiole or etiolated internode explants. UH965 plantlets, established in vitro via axillary bud culture (Kunisaki, 1980), were subculture for 1 year at regular intervals on a modified MS medium (Murashige and Skoog, 1962) composed of half-strength MS macronutrients, full-strength MS micronutrients, MS vitamins with thiamine-HCl increased to $0.4 \text{ mg}\cdot\text{liter}^{-1}$, $25 \text{ mg NaFeEDTA}\cdot\text{liter}^{-1}$, $100 \text{ mg myo-inositol}\cdot\text{liter}^{-1}$, 2% sucrose, $2.2 \mu\text{M N}$ -(phenylmethyl)-1H-purine-6-amine (BA), and solidified with 0.7% Bacto-agar (half-strength MS medium).

Explants were dipped in aqueous bacterial cultures, blotted, and then cocultivated with bacteria on 7-cm-diameter cellulose filter papers supported by 3-day-old subcultures of Su tobacco nurse callus (gift of E.D. Earle, Cornell Univ., Ithaca, N. Y.) grown on MS medium containing $1 \text{ mg (2,4-dichlorophenoxy)acetic acid (2,4-D)}\cdot\text{liter}^{-1}$. After 3 days of culture in the dark at 25 to 28°C , explants were blotted and transferred from the nurse cultures onto half-strength MS medium containing no growth regulators and $500 \text{ mg carbenicillin}\cdot\text{liter}^{-1}$. Control explants were treated similarly using YEP induction medium either lacking bacteria or containing *A. radiobacter*. Cultures were grown in the dark at 21°C for 4 weeks and then evaluated. The number of explants with at least one tumor visible without magnification was recorded. Non-callus swellings occurred occasionally at the

Fig. 3. Diagram of a thin-layer chromatogram showing nopaline and other guanidine compounds accumulated in extracts of tumors from etiolated internodes of anthurium selection UH965 infected by acetosyringone-induced *Agrobacterium tumefaciens* strain C58. Turkish tobacco leaf extracts served as controls. Lane 1, uninfected tobacco, $R_f = 0.41, 0.73$; lane 2, tobacco tumor, $R_f = 0.25, 0.41, 0.73$; lane 3, uninfected anthurium, $R_f = 0.08, 0.40$; lane 4, anthurium tumor, $R_f = 0.08, 0.25, 0.40$; lane 5, nopaline standard, $R_f = 0.26$; and lane 6, arginine standard, $R_f = 0.41$.

wounded edges of explants and were judged not to be tumors. Leaf explants of Turkish tobacco inoculated at the same time as anthurium explants served as positive controls for C58 virulence.

Nopaline accumulation was examined in tissues treated with C58 and in control tissues; free-living C58 served as an additional control. Extracts obtained by the procedure of Hemalsteens et al. (1984) were concentrated by freeze-drying and resuspended in small volumes of sterile water. Nopaline was detected by the procedure of Herrera-Estrella et al. (1988), modified for ascending thin-layer chromatography by using glass plates precoated with $250\text{-}\mu\text{m}$ -thick MN300 cellulose (Analtech, Newark, Del.). Chromatograms were run for 2 h before development and observed under long-wave ultraviolet illumination. The solvent front was marked, the outlines of any visible spots traced, and R_f values calculated.

DNA from anthurium and tobacco tumors and control tissues was extracted for polymerase chain reaction (PCR) and Southern blot (Southern, 1975) analyses using the procedure of Dellaporta et al. (1983), modified by precipitation of the final DNA pellet in $350 \mu\text{l } 7.5 \text{ M ammonium acetate}$, $75 \mu\text{l } 3 \text{ M sodium acetate}$, and $500 \mu\text{l isopropanol}$ with microcentrifugation for 10 min.

A 1.2-kb nopaline synthase (*nos*) gene fragment from the plasmid pMON530 in *Escherichia coli* strain NM294 was used as the probe for transformed tissue DNA. Plasmid DNA was isolated from *E. coli* using an alkaline lysis method (Liszewski et al., 1989; Sambrook et al., 1989). PCR amplification of the *nos* fragment for probe preparation or for plant DNA analysis was carried out according to Sambrook et al. (1989) using two 21 bp primers whose sequences were derived from the first and last seven codons of the

nos coding sequence of the nopaline plasmid pTi-T37 (Depicker et al., 1982). The oligonucleotide primers, GCAATTACCT-TATCCGCAACT and GACCATCTC-GTCCTTATTGAA (5' to 3'), were produced at the Univ. of Hawaii Biotechnology Instrumentation Facility. PCR amplification involved three temperature/time profiles for a total of 30 cycles; a first cycle of $94^\circ\text{C}/4 \text{ min}$, $60^\circ\text{C}/2 \text{ min}$, $72^\circ\text{C}/3 \text{ min}$ followed by $94^\circ\text{C}/1 \text{ min}$, $60^\circ\text{C}/2 \text{ min}$, $72^\circ\text{C}/3 \text{ min}$, with a final cycle of $94^\circ\text{C}/1 \text{ min}$, $60^\circ\text{C}/2 \text{ min}$, $72^\circ\text{C}/11 \text{ min}$. An aliquot of $2 \mu\text{g}$ plant DNA from tumor or control tissue was used for *nos* amplification. The PCR-amplified DNA fragments were purified and quantified according to Sambrook et al. (1989).

Probe DNA was prepared using random-primed incorporation of deoxyuridine triphosphate linked to digoxigenin according to directions for the Genius Nonradioactive DNA Labeling and Detection Kit (Boehringer Mannheim Biochemical, Indianapolis).

Amplified DNA was electrophoresed in 50 ml of 0.8% agarose gel (Fisher Biotech Low EEO Agarose; Fisher Scientific, Fair Lawn, N. J.) using $0.5 \times \text{TBE}$ buffer containing $44.5 \text{ mM Tris-base}$, $44.5 \text{ mM boric acid}$, and 1 mM EDTA . DNA was transferred overnight from the gel onto a Zetabind nylon membrane according to the manufacturer's directions for capillary transfer (CUNO, Meriden, Conn.). The membrane was air-dried for 2 h at 28°C . Prehybridization and hybridization of the membrane was carried out overnight at 68°C in a sealed, plastic hybridization bag following instructions for the Genius Kit. About 100 ng of digoxigenin-labeled *nos* probe, freshly denatured at 94°C for 10 min followed by an ethanol/ice bath, was used in 2.5 ml of hybridization solution. Immunological color detection of probe DNA hybridized to PCR-amplified products was as described for the Genius Kit. In this procedure, the membrane was blocked, reacted with antidigoxigenin alkaline phosphatase conjugate, washed, then reacted with freshly prepared color solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt. The reaction was stopped after 1.5 h, and the wet membrane was photographed.

A second membrane was spotted with denatured genomic DNA from anthurium tumors or control tissues ($30 \mu\text{g}$) and pMON530 plasmid DNA ($0.01 \mu\text{g}$) using a standard dot blot procedure (Davis et al., 1986). After spotting, the membrane was air-dried for 2 h. Southern hybridization was done using digoxigenin-labeled *nos* probe as described above. Immunochemiluminescent detection of hybridized probe using Lumi-Phos 530 (Boehringer Mannheim Biochemical) was done following manufacturer's directions. Using this method, the membrane that had been reacted with antidigoxigenin alkaline phosphatase conjugate was dipped into Lumi-Phos 530 substrate solution, incubated in a sealed hybridization bag for 30 min at 37°C , and exposed to XAR-5 film (Kodak Co., Rochester, N. Y.) for 7 to 10 min. The film was developed for 20 to 30 sec, fixed ac-

Fig. 4. Polymerase chain reaction analysis of DNA from anthurium selection UH965 tumor tissue. A probe specific for *nos* from the T-DNA region of the Ti plasmid hybridizes to a 1.2-kb fragment amplified using primers for pTi-T37 *nos*. Lane 1, Turkish tobacco leaf tissue treated with induced *Agrobacterium tumefaciens* C58 (positive control); lane 2, anthurium etiolated internode tissue treated with induced *A. tumefaciens* C58; lane 3, uninfected UH965 etiolated internode tissue; lane 4, pMON530 carrying pTi-T37 *nos* (positive control). Lane M is Hind III-digested Lambda DNA.

Fig. 5. Dot-blot hybridization of genomic DNA from anthurium selection UH965 tumor tissue to a probe specific for *nos* from the T-DNA region of the Ti plasmid. Dot 1, anthurium etiolated internode tissue treated with induced *Agrobacterium tumefaciens* C58; dot 2, uninfected UH965 etiolated internode tissue; and dot 3, pMON530 carrying pTi-T37 *nos* (positive control).

cording to Kodak X-ray film processing instructions, and photographed.

Small tumors resembling raised scars were produced on plants treated with induced *Agrobacterium* and were significantly larger ($P < 0.001$) in two out of the four anthurium crosses tested than those on control plants inoculated with YEP induction medium (Fig. 1). Variability among crosses was highly significant ($P = 0.0001$), indicating that tumor formation by induced C58 is strongly affected by the plant genetic background. In addition, the response of cross 926 to induced C58 was significantly greater than that produced by *A. radiobacter* ($P = 0.02$), with

a mean response rating of 1.2 compared to 0.4. These results indicate that tumor growth in anthurium is not a general reaction to *Agrobacterium* or to the wounding itself.

Among all the crosses, plants of 976 treated with *A. tumefaciens* produced the largest tumors. Tumorigenesis in C58- and A281-treated plants of cross 976 was highly significant compared to the response of controls, with mean response ratings of 3.3 and 2.6, respectively ($P = 0.0001$). Tumor growth differed significantly between C58 and A281 ($P = 0.05$), with C58 producing the largest growths. In contrast, the response of cross 926 to infection by strain A281 did not differ significantly from the control response ($P = 0.67$). This result supports the observation that the pathogenicity of different *A. tumefaciens* strains varies with the host genotype (Anderson and Moore, 1979).

Agrobacterium tumefaciens C58 induced clearly visible tumors on etiolated internode explants (32%) and about one-tenth as many or fewer on green leaf and petiole explants (Table 1, Fig. 2). Tumors were firm and pale yellow, could be maintained on medium with no growth regulators, and resembled the callus that develops on explants during the early stages of micropropagation on auxin- and cytokinin-rich medium (Pierik, 1976). Leaf and petiole explants treated with *A. radiobacter* or with YEP induction medium failed to produce tumors (Table 1). A few etiolated explants treated with *A. radiobacter* (3%) or YEP induction medium (3%) produced swellings that were initially difficult to distinguish from callus-type tumors but that did not continue to grow. Chromatograms showed an accumulation of nopaline in C58-exposed anthurium internode and tobacco leaf tumors (positive control), but not in noninoculated tissues (Fig. 3); C58 extract did not show nopaline accumulation.

Southern analysis of PCR-amplified DNA showed a 1.2-kb band present in C58-induced anthurium tumors but absent in uninfected control tissue exposed to YEP induction medium (Fig. 4). The positive controls, C58-exposed tobacco leaf tumor and pMON530 plasmid DNAs, also showed a 1.2-kb amplified band with homology to the *nos* probe. From dot-blot hybridization (Fig. 5), it is evident that the *nos* gene is absent in normal anthurium DNA but is present in tumorous anthurium DNA. These data, taken together with the whole-plant observations and the biochemical evidence, demonstrate that the tumorous phenotype seen on C58-treated anthurium explants is due to the presence of T-DNA transferred from *A. tumefaciens*.

These results show that infection of etiolated tissue by acetosyringone-induced *A. tumefaciens* strain C58 is a reliable method for the production of tumors carrying transgenes in anthurium. This work expands the host range of *A. tumefaciens* to include the monocot family Araceae and emphasizes the importance of explant, genotype, and bacterial strain selection in development of such transformation systems. As methods for callus induction and plant regeneration have been

developed for many anthurium cultivars (Geier, 1990; Kuehne and Sugii, 1991) and as transgenic anthurium shoots with a normal vegetative appearance have been recovered from some C58-induced tumors (unpublished data), this work represents a potentially significant advance in the tools for breeding anthurium. Further studies with nontumorigenic C58 strains carrying selectable marker genes in the T-DNA could demonstrate that horticulturally desirable traits can be introduced and expressed in anthurium using *A. tumefaciens* as a vector.

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