Obtaining Transgenic *Anthurium* through *Agrobacterium*-mediated Transformation of Etiolated Internodes

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Abstract. Several procedures were tested in development of a gene transfer protocol for anthurium. Etiolated internode segments of anthurium cultivars 'Rudolph' and 'UH1060' were co-cultured with *Agrobacterium tumefaciens* LBA4404 carrying the chimeric genes *neo*, for antibiotic resistance, and *att* encoding antibacterial attacin. Assays of genomic DNA and RNA from kanamycin-resistant 'Rudolph' and DNA from 'UH1060' plantlets, recovered as soon as 1 year after culture on selection media, indicated the presence of introduced genes, including *neo* and *att*, and transcription of *att*. Western analysis confirmed the expression of attacin protein in calli induced from laminae of regenerated kanamycin-resistant 'Rudolph' plantlets. Use of tobacco nurse cells during co-cultivation of internodes with *Agrobacterium* did not increase recovery of shoots under the regeneration conditions used. Improvements in culture and antibiotic selection conditions during plant development are suggested.

Anthurium hybrids are used worldwide for cut flowers and flowering potted plants. Hawaii produces about one-fifth of the world's export supply with over 10 million stems sold in 1994 (Hawaii Agricultural Statistics Service, 1995). Due to the recent devastation of many Hawaiian anthurium farms by bacterial blight caused by *Xanthomonas campestris* pv. *dieffenbachiae* McCull and Pirone, there is a need for blight resistant cultivars. A transformation method to introduce antibacterial genes into this commercial monocot is desirable to complement current hybridization breeding for disease resistance (Kamemoto et al., 1992). Such a method could allow introgression of other desirable traits, such as novel flower color or nematode resistance.

Feasibility of *Agrobacterium*-mediated gene transfer has been demonstrated in several monocot crops (Conner and Dommissie, 1992). We showed that etiolated internodes of *Anthurium andraeanum* Lind. ex André 'Rudolph' (formerly UH965) were suitable for infection by wild-type *Agrobacterium tumefaciens* (Smith and Townsend) Corm strain C58; plants were not regenerated from tumors (Kuehnle and Sugii, 1991a).

This report describes several procedures tested in development of a transformation protocol for etiolated anthurium internodes by non-tumorigenic *A. tumefaciens* LBA4404 using tissue culture and plant regeneration methods for Hawaiian cultivars (Kuehnle and Sugii, 1991b). The first example of transgenic anthurium plants is presented.

Materials and Methods

Plant materials and media. Etiolated internode explants of anthurium cultivars 'Rudolph' and 'UH1060' were obtained from shoots derived from nodal cuttings or excised laminae cultured in the dark on H1 (Kuehnle and Sugii, 1991 b) medium. Etiolated

internodes were plated on media Cmod and D (Kuehnle and Sugii, 1991b) and C (identical to D with 0.5 mg·liter⁻¹ kinetin and auxin reduced to 1.5 mg·liter⁻¹ 2,4-dichlorophenoxy acetic acid) for callus proliferation, and H1 and H2 (Kuehnle and Sugii, 1991 b) and CK7 for plant regeneration. Medium CK7 is half-strength MS (Murashige and Skoog, 1962) medium containing 0.2 mg·liter⁻¹ benzyladenine and 3 mg·liter⁻¹ 2-isopentenyl adenine. Culture conditions are specified below. 'Su' tobacco callus, obtained courtesy of E.D. Earle (Cornell Univ., Ithaca N.Y.), was grown in darkness on solidified Su (Gamborg et al., 1979) medium and subculture within 4 days before use in co-cultivation.

Kanamycin selection for callus formation. To determine suitable kanamycin concentrations for the selection of transformed cells, 40 explants each of 'Rudolph' and 'UH1060' laminae and etiolated internodes were cultured per treatment on C medium containing 0, 25, 50, 75, or 100 mg·liter⁻¹ kanamycin. The number of explants that formed callus in the dark at 25C was scored after 100 days.

Co-cultivation of 'Rudolph' explants and plant regeneration. To test transformation protocols, the plasmid pCa2Att (Destéfano-Beltrán, 1991) was obtained from J. Jaynes (Demeter Biotechnologies, Research Triangle Park, N.C.). The plasmid contains a chimeric gene, designated Ca2Att, comprised of *att* (encodes attacin, derived from the cecropia moth but lacking signal peptide sequences) under control of a double CaMV 35S promoter and NOS-3' terminal sequence and inserted at the *HindIII* site of pBI121 (Destéfano-Beltrán, 1991; Jefferson et al., 1987; Fig. 1). After introduction into *A. tumefaciens* LBA4404 either by triparental mating (Ditta et al., 1980) or the freeze and thaw method (An et al., 1988), overnight cultures were grown in 5 ml liquid YEP (An et al., 1988) medium, pH 7.2, or LB (Miller, 1972) medium, pH 7.0, with 25 mg·liter⁻¹ streptomycin and 50 mg·liter⁻¹ kanamycin at 220 rpm, 28C. The bacterial suspension then received 200 µM acetosyringone

Fig. 1. T-DNA region of plasmid pCa2Att showing the order of genes for neomycin phosphotransferase II (*neo*), attacin (*att*), and β -glucuronidase (*uidA*), and the *HindIII* restriction sites.

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and was diluted with YEP, pH 5.5, for a final concentration of 20 μ M acetosyringone.

Etiolated internodes of 'Rudolph' were cut into 0.5- to 1 -cm long segments and immersed for several minutes in bacterial suspension. Control internodes were immersed in LB medium only. Internodes and *Agrobacterium* were co-cultured on filter paper supported by 'Su' tobacco nurse cells on Su medium for 5 days in darkness at 25C. Twenty internodes per treatment were then incubated in darkness on Cmod medium containing 500 mg-liter⁻¹ carbenicillin or 250 mg-liter⁻¹ cefotaxime and 100 mg-liter⁻¹ kanamycin with subcultures at monthly or bimonthly intervals for a total of 8 months; antibiotic was reduced to 50 mg-liter⁻¹ kanamycin after the first 4 months. To accelerate regeneration, caulogenic calli were cultured for four months in liquid D and CK7 medium (two months each) with 50 mg-liter⁻¹ kanamycin at 100 rpm, 25C, under light (22 μ E·m⁻²·s⁻¹, 16 h light/8 h dark). All regenerated shoots were transferred to agar-solidified H 1 medium containing 50 mg-liter⁻¹ kanamycin with monthly or bimonthly subcultures under light (32 μ E·m⁻²·s⁻¹, 16 h light/8 h dark). Plants originating from distinct calli were assigned line designations, indicated by the construct name followed by a line number (e.g., Ca2Att 1-1). Plantlets were considered kanamycin-sensitive if a newly developed leaf was mottled or bleached, or if there was no further growth, and were subsequently discarded. After rooted, plants were transferred to H2 medium with 75 mg-liter⁻¹ kanamycin.

Effect of tobacco nurse cells on 'Rudolph' and 'UH1060' transformation. Two replications of 19 to 26 etiolated internode segments of 'Rudolph' and 'UH 1060' per replicated plate were incubated for 3 days on Cmod medium with or without tobacco nurse cells (Horsch et al., 1985) during co-culture with bacteria carrying pCa2Att. Other explants (controls) were immersed in LB medium without bacteria and plated with or without nurse callus. After co-cultivation, explants from all four treatments were transferred to Cmod medium containing 500 mg-liter⁻¹ carbenicillin and 50 mg-liter⁻¹ kanamycin and were subculture monthly for 3 months. Calli were transferred onto H 1 regeneration medium containing 50 mg-liter⁻¹ kanamycin and 250 mg-liter⁻¹ carbenicillin and incubated in low light (4 μ E·m⁻²·s⁻¹, 16 h light/8 h dark) at 25C with monthly subcultures until leafy shoots regenerated. Regenerated shoots, some with roots, were removed from the callus and transferred to H2 medium with 75 mg-liter⁻¹ kanamycin and maintained under higher light (32 μ E·m⁻²·s⁻¹, 16 h light/8 h dark) for further plant growth. Counts of explants forming shoots and total number of kanamycin-resistant and -sensitive plantlets were made about one year after co-cultivation. Regenerants which did not exhibit leaf bleaching or mottling compared to plants on nonselective medium were scored as resistant. Means were compared using the Students *t* test (unpaired, one-tailed) to determine any improvement in percentage shoot formation with nurse cells compared to without nurse cells.

GUS histochemical staining. Leaf and root segments from kanamycin-resistant plantlets were excised, treated at least overnight for GUS activity according to McCabe et al. (1988), and bleached in 95% ethanol to visualize any blue tissue. A total of 19 leaf and 17 root segments from 11 'Rudolph' and 8 'UH 1060' plantlets, previously transformed with construct pCa2Att, were examined.

Nucleic acid analysis using polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR), and genomic digests with Southern hybridization. Plant DNA extraction was according to Dellaporta et al. (1983), as described in Herrera-Estrella and Simpson (1987), using 0.5–1.2 g of in vitro 'Rudolph' leaf tissues

or caulogenic 'UH 1060' callus ground in dry ice. The final DNA pellet was RNase A-treated and stored in Tris-EDTA buffer at –20C until use. DNA-PCR amplification was carried out in a TempCycler model 60 (Coy Corp., Grass Lake, Mich.) using primers for *neo* (Chee et al., 1989; Beck et al., 1982), *uidA* (Nan and Kuehnle, 1995) and *att*. Primers for *att*, based on the gene sequence from Kockum et al. (1984), were a 5' primer of 5'-ATG,GAC,GCG,CAC, GGA,GCC,CTT-3' and a 3' primer of 5'-TCC,GAA,GTT,AGG, CTC,CCA,AGA-3', yielding a 546 bp product. Promega *Taq* DNA polymerase (1.25 units/50 μ l reaction) and each primer (0.25 μ g/50 μ l reaction) were added with 1.5 mM MgCl₂ and dNTPs at 200 μ M for reaction under the following conditions: initial denaturation at 94C for 3 min and 29 cycles at 94C for 1 min, 60C for 1 min, and 72C for 2 min. In the last cycle, the reaction at 72C was extended to 9 min.

PCR analysis was performed for *att* and *neo* on nucleic acids isolated from other plants recovered from the same experiment as those analyzed in vitro but grown for over 2 years in the greenhouse in a 3:1 composted bark: perlite potting mix. Frozen tissues stored from plants determined transgenic by PCR were subsequently used for RT-PCR and genomic DNA restriction enzyme digestion analysis.

For extraction of leaf RNA, total nucleic acids were isolated following the procedure of Dellaporta et al. (1983). RNA was precipitated with 2 M lithium chloride and DNase treated once using the conditions of Zaccarini et al. (1993). First strand cDNA was generated through reverse transcriptase using 1.5 μ g RNA with a 3' *att* primer of 5'-GAG,AAT,CCG, AAG,TTA,GGC,TC-3' specific for detection of *att* sequences. Reaction conditions were according to the Gene Amp Kit (Perkin-Elmer Cetus, Norwalk, Conn.). Second strand cDNA synthesis and amplification proceeded with addition of a 5' *att* primer of 5'-CTT,ACG,CTC,AAC, TCC,GAT,GG-3' (0.25 μ g/100 μ l reaction) under PCR conditions as described for DNA. Cycling was extended and modified for the TempCycler II (Coy Corp.): initial denaturation at 94C for 1 min and 45 cycles at 94C for 30 sec, 60C for 30 sec, and 72C for 1 min, extended to 5 min in the final cycle. This second set of *att* primers yields a 533 bp product.

DNA-PCR and RT-PCR products were separated by agarose gel electrophoresis and visualized using standard procedures. Southern hybridization (Maniatis et al., 1982) was performed to verify the amplified fragments. Bands in the blotted membrane were labeled and visualized using the Genius Nonradioactive Kit (Boehringer Mannheim Biochemical).

For genomic DNA restriction digestion analysis, about 10 μ g plant DNA were digested with HindIII according to manufacturer's directions (Promega, Madison, Wis.), separated, blotted, and bands visualized as described above.

Western analysis. Protein extraction from in vitro leaves of regenerated plants yielded insufficient quantities. Total protein was isolated instead from kanamycin-resistant calli newly formed on lamina excised from regenerated plants. Calli were obtained from six kanamycin-resistant regenerated plants representing 'Rudolph' lines Ca2Att 1-2, Ca2Att 1-3, and Ca2Att 1-4 using C medium containing 50 mg-liter⁻¹ kanamycin (this selection level inhibits lamina callusing by nontransformants). Protein from nonselected callus of one control plant was also used. Samples of 60 μ g total protein, as determined by the Lowry method (Lowry et al., 1951), were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Partially purified cecropia attacin, 4.5 μ g, (J. Jaynes, Demeter Biotechnologies) containing the 25 kDa native peptide served as a reference. Protein was electroblotted and immunodetected as

Table 1. Callus and shoot regeneration from 'Rudolph' and 'UH1060' etiolated internodes co-cultivated with *Agrobacterium tumefaciens* LBA4404 with and without tobacco nurse culture.

Nurse	Treatment	No. explants co-cultured	No. forming callus ^z (%)	No. forming shoots (mean % $\pm$ SD) ^y	No. Km-r ^x plants per total regenerated (%)	Average no. Km-r ^x plants per explant with shoots
Rudolph						
Yes	pCa2Att	46	38 (83)	38 (81.8 $\pm$ 9.6)	206/737 (28)	4.8
No	pCa2Att	44	33 (73)	24 (56.2 $\pm$ 17.3)	134/441 (30)	5.6
Yes	LB medium only	45	39 (87)	0	0	0
No	LB medium only	47	33 (70)	0	0	0
UH1060						
Yes	pCa2Att	50	16 (32)	13 (26.0 $\pm$ 14.1)	319/853 (37)	24.5
No	pCa2Att	51	9 (18)	10 (19.5 $\pm$ 5.0)	366/792 (46)	22.9
Yes	LB medium only	51	1 (2)	0	0	0
No	LB medium only	50	1 (2)	0	0	0

^xValues obtained 6 months after co-cultivation; percentage of total explants cultured.

^yvalues obtained 12 months after co-cultivation; mean of two replications.

^zKm-r, kanamycin-resistant based on green, unmottled leaves; final count on H2 medium with 75 mg/liter⁻¹ kanamycin.

described by Bio-Rad Labs using 400-fold diluted rabbit anti-attacin antibody (J. Jaynes, Demeter Biotechnologies) and 3000-fold diluted goat anti-rabbit IgG conjugated with alkaline phosphatase.

Results and Discussion

Kanamycin concentration for selection. 'Rudolph' laminae formed yellow, compact callus on medium with zero or 25 mg-liter⁻¹ kanamycin. The percentage of laminae with callus per total number cultured (40) was 60% and 22.5%, respectively. Concentrations of 50 mg-liter⁻¹ kanamycin or higher completely inhibited laminar callus formation. 'Rudolph' internodes formed callus on medium lacking kanamycin or containing 25 or 50 mg-liter⁻¹ kanamycin. The percentage of internodes with caulogenic callus per total number cultured was 70%, 5%, and 15%, respectively. No callus was observed on 75 and 100 mg-liter⁻¹ kanamycin. For UH 1060 laminae and internodes, explants formed callus in the absence of kanamycin (100% and 12.5%, respectively) or in the presence of 25 mg-liter⁻¹ kanamycin (12.5% and 2.5% respectively). Callusing of 'UH 1060' internode and lamina explants was completely inhibited by 50 to 100 mg-liter⁻¹ kanamycin. For both cultivars, calli on medium with kanamycin were substantially smaller than calli produced in the absence of kanamycin.

'Rudolph' callus formation and plant regeneration after co-cultivation 'Rudolph' internodes formed callus on selection medium within one month on 19 of 20 (95%) co-cultivated explants and on 8 of 20 (40%) control explants treated with LB broth lacking *Agrobacterium*. Calli from controls were, at 1 to 2 mm, less than half the size of those from all other treatments and lacked the yellow, compact appearance of calli on *Agrobacterium*-treated internodes. After 4.5 months of culture in the dark, calli in the *Agrobacterium* treatments produced etiolated shoots of about 4 mm. The percentage of total explants with shoots was 20%. Controls did not form shoots. Eighteen months after co-cultivation, rooted green plants were recovered from treatments with *Agrobacterium*.

Effect of nurse culture on transformation of 'Rudolph' and 'UH1060'. 70% to 87% of all 'Rudolph' etiolated internodes initially produced callus within 6 months (Table 1). Calli on LB broth-treated explants were less than 2 mm in diameter and less frequent per explant than those on *Agrobacterium*-treated explants

that grew to 5 mm or larger, with or without nurse culture. In the co-cultivation of 'UH1060' internodes with and without nurse cells, 32% and 18% produced callus, respectively. A few controls showed slight callusing. Calli from 'Rudolph' and 'UH1060' explants treated only with LB medium did not produce shoots (Table 1). Many calli from explants co-cultivated with *Agrobacterium* regenerated shoots, some with roots; some internodes also formed shoots directly with minimal callusing. Among those regenerants whose callus origin was tracked from this and the previous experiment, 14 lines of 'Rudolph' Ca2Att and 3 lines of 'UH1060' Ca2Att were identified.

Based on callus growth data and for convenience of medium preparation, 50 mg-liter⁻¹ kanamycin was chosen for culture of both 'Rudolph' and 'UH1060' internodes. As the number of explants forming callus was not necessarily an indication of transformation at this selection level (controls formed some callus), the effect of nurse culture on transformation was evaluated based on number of explants with shoot production (control callus

Fig. 2. (A), Southern hybridization of a PCR-amplified 1054 bp *neo* gene fragment and (B), gel of a PCR-amplified 546 bp attacin gene fragment, from DNA of kanamycin-sensitive (untreated) or kanamycin-resistant (from *Agrobacterium* treatment) 'Rudolph' plantlets and 'UH 1060' caulogenic callus. Lanes: 1, untreated 'Rudolph'; 2, 'Rudolph' line Ca2Att 1-1; 3, 'Rudolph' line Ca2Att 1-2; 4, 'Rudolph' line Ca2Att 1-3; 5, 'Rudolph' line Ca2Att 1-4; 6, 'UH1060' line Ca2Att2-0; 7, 'UH1060' line Ca2Att 4-0 (A) or pCa2Att plasmid (B); 8, pBII21 plasmid (A) or blank (B).

Fig. 3. RT-PCR analysis of RNA from wild-type and kanamycin-resistant (from *Agrobacterium* treatment) 'Rudolph' plants. Lanes: 1, 'Rudolph' line Ca2Att 2-2; 2, same as 1 but no reverse transcriptase; 3, 'Rudolph' line Ca2Att 10-0; 4, same as 3 but no reverse transcriptase; 5, 'Rudolph' wild-type; 6, 'Rudolph' line Ca2Att 2-2 DNA only; 7, 'Rudolph' line Ca2Att 10-0 DNA only; 8, pCa2Att plasmid.

was not caulogenic). Shoot regeneration among *Agrobacterium*-treated 'Rudolph' explants previously cultured with or without nurse cells averaged 81.8% and 56.2%, with an average of 4.8 and 5.6 kanamycin-resistant plants per explant with shoots, respectively (Table 1). Although the nurse treatments produced more explants with shoots, there was a low significant difference for 'Rudolph' between treatments ($P = 0.1$). While 'UH1060' also produced more shoots with nurse culture than without nurse culture (26% vs. 19.5%), there was no significant difference between treatments ($P = 0.3$). The cultivar 'UH1060' was more prolific than 'Rudolph' during shoot multiplication with an average of 22.9 to 24.5 kanamycin-resistant shoots produced per internode.

Selection on 75 mg·liter⁻¹ kanamycin during subsequent plant development was not sufficiently stringent, since only about 25% to 50% of the shoots recovered were kanamycin-resistant (Table 1). In some cases antibiotic-sensitive plants were not easily identifiable, as they bleached only after multiple subcultures of this slow-growing species. Therefore, the level of antibiotic that completely eliminates growth of nontransformed shoots needs to be determined and implemented during shoot regeneration. The antibiotic geneticin (G148) may be considered; 10 mg·liter⁻¹ geneticin completely inhibits shoot regeneration from 'UH1060' callus (A. Kuehnle, unpublished data).

In the first co-cultivation experiment with cultivar 'Rudolph' only, tissues were incubated in the dark for eight months and liquid culture was used for four months. In the subsequent experiment with 'Rudolph' and 'UH1060', explants were moved into low light three months after co-cultivation, soon after callusing was observed. This allowed faster shoot regeneration with decreased callus proliferation; a liquid culture phase was unnecessary. Using this scheme, kanamycin-resistant plantlets of cultivars 'Rudolph' and 'UH1060' were obtained within one year.

Early and high antibiotic selection pressure appeared effective in reducing escapes during production of caulogenic callus. Of the total number of 'Rudolph' internodes treated with pCa2Att, the portion producing at least one kanamycin-resistant shoot ranged from 55% to 83% in the nurse culture experiment using 50 mg·liter⁻¹ kanamycin, whereas it was 20% in the first co-cultivation experiment using 100 mg·liter⁻¹ kanamycin. The relatively high number of shoots on 50 mg·liter⁻¹ kanamycin can be explained by escapes which were manifested only later during plant development. To support the use of higher selection pressure to reduce possible escapes, a subsequent experiment using 100 mg·liter⁻¹ kanamycin has produced caulogenic calli on only 2.5% (1/40) of *Agrobacterium*-treated internodes. Regular subculture of etiolated stock plants before co-cultivation, combined with stringent selection conditions and transfer of explants into lighted culture conditions soon after callus formation should help improve the recovery of

Fig. 4. Genomic Southern blot analysis of HindIII-digested DNA from wild-type and transgenic plants probed with *att*. Lanes: 1, molecular weight marker *BstEII*-lambda digest; 2, 'Rudolph' line Ca2Att 9-1; 3, 'Rudolph' wild-type; 4, 'Turkish' tobacco line 2-3 obtained after transformation with pCa2Att; 5, pCa2Att plasmid.

Fig. 5. Western analysis of protein extracts from callus initiated on laminae of regenerated kanamycin-sensitive (untreated) or kanamycin-resistant (from *Agrobacterium* treatment) 'Rudolph' plants, reacted with polyclonal antibody against attacin. Lanes: 1, untreated 'Rudolph'; 2-4, three distinct plants of kanamycin-resistant 'Rudolph' line Ca2Att 1-2; 5, partially purified cecropia insect attacin.

transgenic anthurium.

Molecular analysis. PCR-amplified DNA fragments from co-culture-derived kanamycin-resistant 'Rudolph' and 'UH1060' were shown by Southern hybridization to correspond to *neo* and *att* fragments with expected sizes of 1054 bp and 546 bp, respectively. The amplified neogene fragment of three 'Rudolph' lines (Fig. 2A, lanes 2-5) and two 'UH1060' lines (Fig. 2A, lanes 6 and 7) showed the expected size of 1054 bp as seen in pBI121 (Fig. 2A, lane 8). An amplified *att* gene fragment of 546 bp from the same lines (Fig. 2B, lanes 2-6) had similar mobility as the *att* fragment from pCa2Att (Fig. 2B, lane 7). No amplification of *neo* and *att* was observed in DNA from untreated 'Rudolph' (Fig. 2, lane 1). The presence of *att* transcripts was verified for two selected greenhouse-grown plants by RT-PCR. The amplification products showed the expected size of 533 bp (Fig. 3) and were confirmed by Southern hybridization (not shown). No amplification occurred in RNA samples lacking reverse transcriptase (Fig. 3, lanes 2 and 4) or from wild-type 'Rudolph' RNA (Fig. 3, lane 5). The expected product of 533 bp was present in the DNA from both plants (Fig. 3, lanes 6 and 7) and from plasmid pCa2Att (Fig. 3, lane 8).

DNA from greenhouse-grown 'Rudolph' line Ca2Att 9-1 was used in genomic digest analysis. HindIII digestion generated the expected internal fragment of about 1.5 kb when probed for *att* (Fig. 4, lane 2). Bands of similar size were seen in transgenic tobacco and pCa2Att plasmid (Fig. 4, lanes 4 and 5). No hybridization was detected with wild-type 'Rudolph' DNA (Fig. 4, lane 3).

GUS activity was never detected in the anthurium leaf and root tissues examined. In some transgenic wheat and orchid plants

containing *uidA*, no GUS-positive staining was observed (Nan and Kuehnle, 1995; Vasil et al., 1992). Lack of GUS activity could be due to modifications at the gene or protein level (Diaz et al., 1992; Iturriaga et al., 1989). Effectiveness in anthurium of the *uidA* promoter, a single-copy CaMV 35S, is not known. It is also possible that *uidA* was not completely transferred during transformations. DNA from three plants recovered later from the nurse culture experiment, representing 'Rudolph' lines Ca2Att1-15 and Ca2Att 1-17 and 'UH1060' line Ca2Att 12-0, were tested by PCR for the presence of *uidA*. A gene fragment was amplified to the expected size in only one of three plants ('Rudolph' line Ca2Att1-17); all three of these plants were found positive for *neo* and *att* (Chen, 1993).

Since the naturally slow growth of anthurium prevented having enough leaf tissue from one plantlet for both nucleic acid and protein analysis, other regenerants derived from lines with plants found *att*-positive by PCR were used for western analysis. A polyclonal antibody raised against partially purified attacin from the cecropia insect cross-reacted with a 25 kDa band in kanamycin-resistant leaf calli from all six regenerated plants sampled. Results from 3 plants of 'Rudolph' line Ca2Att 1-2 are shown (Fig. 5, lanes 2-4). This band had the same mobility in the denaturing gel as the partially purified attacin (Fig. 5, lane 5). No cross-reaction for this specific band was detected in leaf callus from the untreated regenerant (Fig. 5, lane 1). Proteins of sizes other than 25 kDa (15 kDa and several proteins larger than 31 kDa) were also recognized by the antibody and were considered not to be due to expression of *att*.

In summary, we have shown that protocols tested for co-cultivation of etiolated internode explants with disarmed *A. tumefaciens* LBA4404 carrying the plasmid pCa2Att resulted in transformed anthurium plants of two distinct genotypes. Suggestions for improvement of these protocols with elimination of nurse culture during co-cultivation, more stringent selection during shoot growth, and faster plant regeneration by earlier exposure to light were made for future application. This study also showed that the attacin protein expressed in anthurium had the same apparent molecular weight as insect attacin, which indicates little or no processing of the protein in anthurium. As attacin may have only moderate antibacterial activity against the *Xanthomonas* responsible for anthurium blight (Chen, 1993), this gene transfer method has been successfully applied with more recently available antibacterial constructs to obtain transgenic plants for future evaluation of bacterial disease resistance and flowering characteristics.

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