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Characterization of an Unusual New *Agrobacterium tumefaciens* Strain from *Chrysanthemum morifolium* Ram†

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We characterized five isolates of *Agrobacterium tumefaciens* from naturally occurring galls on *Chrysanthemum morifolium*. The isolates are similar, possibly identical, members of a single strain of *A. tumefaciens* that we designate Chry5. The strain is a biotype I, as indicated by its response to both newly described and traditional biotype tests. Chry5 produces tumors on at least 10 plant species. It is unusual in its ability to form efficiently large tumors on soybean (*Glycine max*), a species normally refractory to transformation. Chry5 is unable to utilize octopine or mannopine as a carbon source. Although Chry5 can catabolize a single isomer each of nopaline and succinamopine, it differs from other known nopaline and succinamopine strains in its insensitivity to agrocin 84. This pattern of opine catabolism is unique among *Agrobacterium* strains examined to date. All five isolates of Chry5 contain at least two plasmids, one of which shares homology with pTiB6.

Crown gall, a disease that affects a wide variety of plant species (11), is caused by the bacterial pathogen *Agrobacterium tumefaciens*. The disease attracts considerable attention because of the ability of the causal organism to act as a natural genetic engineer. A piece of bacterial DNA, the T-DNA, is transferred into a plant cell, where it is integrated into the genome and expressed, giving rise to the gall (see references 36 and 43 for reviews).

Strains of *A. tumefaciens* are diverse and have been classified in several ways. Three biotypes have been recognized (18, 29) on the basis of pattern of responses to a variety of biochemical and physiological tests. *A. tumefaciens* strains also can be subdivided according to their abilities to catabolize opines or to produce them in tumors (13, 32); these characteristics are specified by the Ti plasmid (5, 24). Although early work dealt only with octopine and nopaline, increasing numbers of tumor-specific compounds are being described (33).

Alternatively, strains have been categorized by various aspects of host range. For example, an *A. tumefaciens* strain can be described as having wide or limited host range on the basis of the number of susceptible plant species. Host range appears to be an isolate-specific characteristic, since different isolates from a single tumor can show distinctive patterns of specificity (1). The ability of a strain to cause tumors on different subspecies, or cultivars, of a single host also may vary. This has been observed in a variety of plants (for some examples, see reference 14 and references therein). In all instances, some degree of specificity is apparent, indicating that even closely related plants can differ in susceptibility. This cultivar specificity can be exploited for the purpose of efficient genetic engineering, breeding for resistance to the pathogen, or as a model to examine the pathogen-host interaction.

Unfortunately, no single criterion comprehensively describes a strain of *A. tumefaciens*. While there is some overlap, there is little relationship among biotype, opine type, and host range (30, 40). For example, although isolates from grapevine are typically biotype III (18, 28) and have a limited host range (28, 41), both biotype I and II strains and wide-host-range strains have been isolated from this species (20, 31).

We have been interested in the cultivar specificity of *A. tumefaciens* isolates from chrysanthemum. These isolates were discovered in a Florida nursery, and although they were described as cultivar specific, they never have received much attention. In the process of initial comparison to common laboratory strains, we found that these isolates are highly virulent on soybean. Prior to detailed genetic investigations, we thought it desirable to provide a thorough description of the isolates as well as to document their pathogenicity on soybean.

MATERIALS AND METHODS

Bacterial strains. Lyophilized bacterial isolates from naturally occurring *Chrysanthemum morifolium* galls were obtained from R. E. Stall, University of Florida, Gainesville, in 1980. The bacteria originally had been isolated by Miller (22) and are described as Chry 1, -3, -5, -8, and -9. As the isolates appeared to be identical in most tests, they are treated here as a single strain designated Chry5, unless otherwise indicated. All other strains and their sources are indicated in Table 1. Working cultures were maintained at 5°C on gluconate-mannitol medium (4) containing the appropriate antibitoics (Table 1); long-term storage was in 15% glycerol at −70°C. Prior to use, cultures typically were grown overnight at room temperature in 10 ml of liquid gluconate-mannitol medium or in AB minimal medium (8); aliquots were centrifuged for 1 min in a table top microcentrifuge and then resuspended to 10^8 cells per ml in sterile phosphate-buffered saline (PBS; 0.43 g of KH_2PO_4, 1.48 g of Na_2HPO_4, 7.2 g of NaCl, final pH 7.2, per liter), unless otherwise specified.

Host range determination. The plant species tested and the number of plants inoculated in a representative experiment are listed in Table 2. All except kalanchoe, which was propagated from vegetatively produced plantlets, were raised from commercially available seed. Plants were grown in 15-cm pots in autoclaved soil, two or three plants per pot, under greenhouse conditions (usually 21 to 27°C, occasion-

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TABLE 1. *Agrobacterium* strains used in this study

Strain	Biotype	Characteristic(s)	Source
Reference biotype			
B6	I	Apple isolate	A. Matthysse
C58	I	Cherry isolate	J. Lippincott
Ag5	II	Peach isolate	R. Goodman
B234	II	Unknown	L. Moore
K27	II	Poplar isolate	L. Moore
Ag57	III	Grape isolate	R. Goodman
Ag63	III	Grape isolate	R. Goodman
CG64	III	Grape isolate	L. Moore
Others			
A136	ND[a]	C58, pTiC58$^-$	E. Nester
NT1	ND	C58, pTiC58$^-$	S. Farrand
NT1-A1	ND	NT1 (pAgK84::Tn5)	S. Farrand

[a] ND, not determined.

ally up to 38°C in the summer) without supplemental illumination. Plants were watered daily and fertilized biweekly with a commercial fertilizer (Peters 15-16-17). When the seedling stems were thick enough to be pierced without collapsing (approximately 2 to 3 weeks after germination), each plant was inoculated by piercing the stem once with a sterile hypodermic needle and depositing a droplet of inoculum (10^8 cells per ml of PBS) on the wounds created on opposing sides of the stem. Plants were inoculated with positive control strain B6 or with the five isolates of Chry5, either below the cotyledons or between the cotyledons and the first leaves. Kalanchoe was inoculated by scratching the leaf surface with the hypodermic needle and extruding a drop of inoculum into the wound. Controls consisted of plants wounded in an identical manner, but inoculated with avirulent strain A136. Plants were scored for gall production on a weekly basis, starting at 2 weeks and continuing to 4 (most species) or 8 (beet, collard, and soybean) weeks after inoculation. The tumorigenic response of each species was examined in two or three separate experiments.

Biotype determination, opine utilization, and sensitivity to agrocin 84. Tests for 3-ketolactose production, utilization of citrate, growth on NaCl, oxidase reaction, and alkali from L-tartaric acid, malonic acid, and propionic acid were performed for each of the five isolates of Chry5 as described before (37). A complementary test, which biotypes strains by their pH-dependent motility, was performed as described recently (6).

The ability of agrobacteria to utilize various opines was tested by monitoring increase in turbidity of cultures in medium containing the substrate to be tested as the sole carbon source. The following compounds were tested: DL-octopine and DL-nopaline (both from Sigma Chemical Co., St. Louis, Mo.), mannopine, mixed LL and DL isomers of succinamopine (isosuccinamopine and succinamopine, respectively), and mixed LL and DL isomers of nopaline (isonopaline and nopaline, respectively). Mannopine and the isomeric mixtures of succinamopine and nopaline were supplied by W. S. Chilton, North Carolina State University, Raleigh. Controls were grown in AB minimal medium lacking a carbon source or containing mannitol as a carbon source. Control *Agrobacterium* strains (and the corresponding opines known to support their growth) were B6 (DL-octopine and mannopine), C58 (DL- and LL-nopaline), and A281 (LL-succinamopine and mannopine).

All five isolates of Chry5 were included. Aliquots of 5×10^8 cells from overnight cultures were harvested, washed twice in AB medium lacking a carbon source, and then resuspended in 0.5 ml of AB medium lacking a carbon source. The wells of a flat-bottomed Immulon 2 microtiter plate (Dynatech Laboratories, Chantilly, Va.) were loaded with 200 μl of AB medium; carbon sources were supplied at a concentration of 2 mg/ml. Wells were inoculated with 10 μl of bacterial suspension. Plates then were covered with a Titertek adhesive-backed acetate plate sealer to minimize both evaporation and possible cross-contamination between wells and incubated at 30°C with gentle shaking. Culture density was measured over a 4-day interval with a Titertek Multiskan PLUS vertical photometer fitted with a 492-nm interference filter; the resultant absorbances were converted to cells per milliliter with a standard curve based on cell counts. Experiments with octopine and nopaline were performed once; those with mannopine and the mixed isomers of succinamopine and nopaline were repeated twice. Each experiment used at least two wells per strain or isolate per carbon source.

To test for sensitivity to agrocin 84, aliquots of 10 μl of the agrocin-producing strain NT1-A1 (12) were spotted in the center of agar plates of AB medium with L-glutamic acid (2 g liter^{-1}) substituted for glucose as a carbon source. Plates were incubated for 2 days at 30°C, and bacteria then were killed with chloroform. One-milliliter aliquots of overnight cultures of the bacterial strains to be tested were mixed with

TABLE 2. Host range of *A. tumefaciens* Chry5 and B6

Plant species	No. of plants inoculated with Chry5/B6[a]	% of plants with tumors 4 wk[b] after inoculation with:	
		Chry5	B6
Beet (*Beta vulgaris* cv. Detroit Dark Red)	76/10	58	60
Collard (*Brassica oleraceae* cv. Georgia Southern)	91/15	44	20
Kalanchoe (*Kalanchoe diagremontiana*)	5/1	100	100
Marigold (*Tagetes patula* cv. Dwarf Sparky Mix)	28/5	100	100
Pea (*Pisum sativum* cv. Laxton's Progress)	32/3	90	100
Soybean (*Glycine max* cv. Peking)	80/11	86	27
Sunflower (*Helianthus annuus* cv. Mammoth Russian)	12/2	100	100
Tobacco (*Nicotiana tabacum* cv. Havana)	50/4	100	100
Tomato (*Lycopersicon esculentum* cv. Early Pick Hybrid)	74/11	100	100

[a] Number of plants inoculated with Chry5 or B6, respectively.
[b] Beet and collard were scored 8 weeks after inoculation.

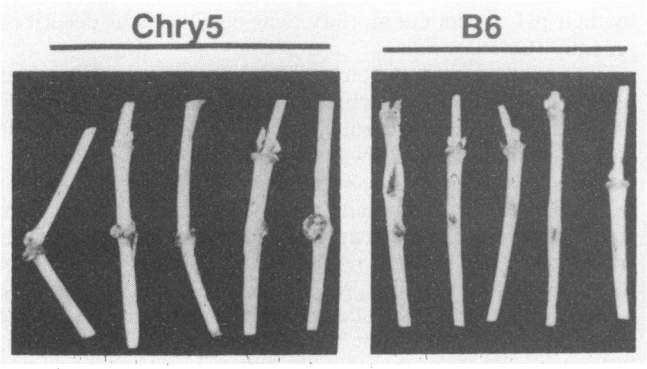

FIG. 1. Tumor formation on soybean.

9 ml of PBS containing molten 0.8% water agar; 3 ml was used to overlay each agrocin tester plate. Growth inhibition was scored after 5 days. Strains C58 and B6 were used as sensitive and resistant controls, respectively. Each experiment consisted of two replicate plates for each of the controls and three replicate plates for each Chry isolate. The experiment was repeated twice with identical results.

Plasmid profiles. Plasmids were visualized by two methods. Originally, the Eckhardt procedure, as modified by Heron and Pueppke (15), was used. Subsequently, a modification of the Schwinghamer protocol (38) was developed and found to give more reproducible results. This procedure was scaled down so that it could be performed in a microcentrifuge tube with approximately 10^9 cells. The volumes of lysozyme and EDTA were increased threefold, and the final concentration of Sarkosyl in the last step was increased to 0.96%. Agarose gels (0.7%) that had been prepared as described previously (15) were loaded with 100 to 200 μl of cell lysate per well. After electrophoresis, gels were prepared for Southern analysis (21) and probed with the DNA insert of plasmid p202 (25), which had been isotope labeled by nick translation (21). This approximately 7-kb fragment, which is designated *Eco*RI fragment B, contains the right third of the conserved region of T-DNA (25).

RESULTS AND DISCUSSION

Host range. The five Chry isolates were indistinguishable in abilities to cause tumors on all of the species tested, so their results were pooled and presented in Table 2 as a single strain, which we designated Chry5. All species formed at least some tumors after inoculation with Chry5, and with two important exceptions, the plants responded similarly to reference strain B6 (Table 2). The percentage of plants that formed tumors in response to inoculations, and the speed with which tumors developed, varied with the host species. Compared with other species, beet and collard were less responsive to Chry5 and B6, both in the percentage of plants that formed tumors and in the speed of response. At 4 weeks after inoculation, all of the other species in Table 2 were approaching or had attained 100% tumor formation in response to Chry5 inoculation, whereas the percentage of beet and collard plants that formed tumors increased slowly through 8 weeks.

TABLE 3. Characteristics of a chrysanthemum strain of *A. tumefaciens*

Diagnostic test	Biotype I[a]	Biotype II[b]	Biotype III	Chry5
3-Ketolactose	+	±	−	+
Utilization of citrate	−	±	+	−
Growth on 2% NaCl	+	±	+	+
Oxidase reaction	+	+(d)	+	+
Alkali from:				
L-Tartaric acid	−	+	+	−
Malonic acid	−	+	+	−
Propionic acid	−	−	−	−
Mucic acid	−	+	−	−

[a] Reference strains for biotype determination are given in Table 1.
[b] ±, + or − reaction, depending on strain; +(d), delayed positive reaction in one strain.

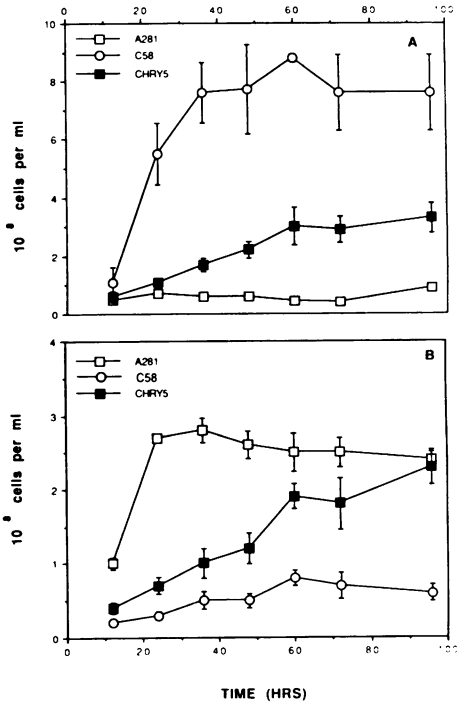

FIG. 2. Growth of *A. tumefaciens* strains on isomeric mixtures of opines: growth on mixed LL and DL isomers of (A) nopaline or (B) succinamopine.

Soybean and collard revealed significant differences between Chry5 and B6. In both cases, Chry5 produced tumors on a greater percentage of plants than did B6, and these tumors were visually larger than those induced by strain B6. This was especially dramatic on soybean, which formed large tumors rapidly in response to Chry5 inoculation (Fig. 1). Tumor formation on B6-inoculated soybean was delayed, and the tumors that formed were much smaller than those on the plants inoculated by Chry5. Thus, the percentage of plants that formed tumors increased only from 27 to 36 to 45%, respectively, from 4 to 6 to 8 weeks after B6 inoculation.

These results demonstrate that Chry5 is a wide-host-range strain, a conclusion supported by scattered observations that Chry5 also forms tumors on dahlia and carrot slices (22), potato (34), and cacao (35). The tumorigenicity of Chry5 thus is as great as, or greater than, the common wide-host-range strain B6. The soybean response to Chry5, however, is of particular interest. Although soybean is susceptible to *Agrobacterium* spp., transformation is inefficient and the tumors tend to be small and slow growing on many genotypes (27). A few laboratory constructs, including transconjugant

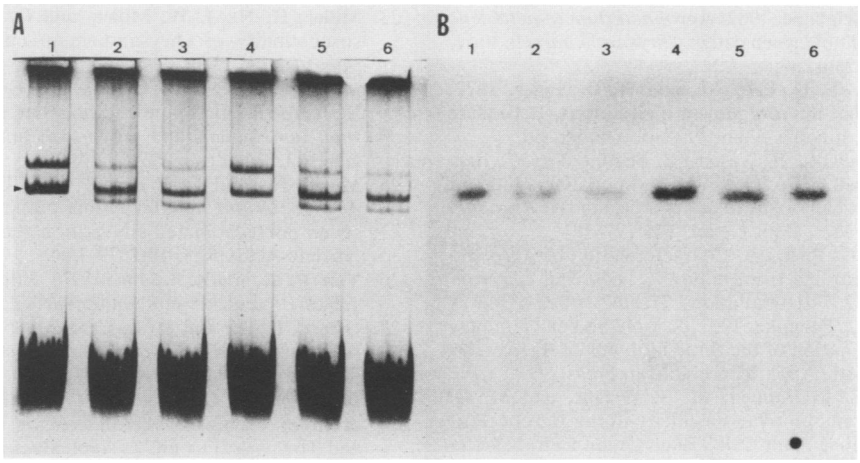

FIG. 3. Agarose gel electrophoresis of plasmid DNA in *A. tumefaciens* strains and isolates. (A) Ethidium bromide-stained gel. (B) Southern blot of gel as in panel A, probed with *Eco*RI fragment B, which contains highly conserved T-DNA. (Both panels) Lane 1, B6; lane 2, Chry 1; lane 3, Chry 3; lane 4, Chry 5; lane 5, Chry 8; lane 6, Chry 9. Arrowhead indicates pTiB6 (approximately 120 MDa).

strains A208 and "supervirulent" A281 (7, 16), form large tumors on this species. Chry5, a field isolate, appears to be as tumorigenic on soybean as these constructs (20a), and we suggest that it may be the strain of choice for pursuit of an efficient soybean transformation system.

Biotype, opine utilization, and sensitivity to agrocin 84. All five isolates of Chry5 were identical in these tests, so the results are presented for strain Chry5. Results from biochemical tests for biotype determination are summarized in Table 3. The response pattern of Chry5 indicates quite clearly that it is biotype I. Measurement of colony diameters in the pH-dependent motility test (23, 23, 30, and 31 mm for pH 5, 6, 7, and 8, respectively) confirms the biotype I designation.

Chry5 was unable to grow with DL-octopine, DL-nopaline, or mannopine as sole carbon source (data not shown). This is in accordance with our previous negative data that octopine and nopaline are absent in the tumors and that the DL-octopine or DL-nopaline content of liquid medium does not decrease in the presence of Chry5 (5a). Chry5 thus is not a standard octopine or nopaline strain (33). When carbon was supplied as a mixture of the LL and DL isomers of nopaline, however, Chry5 achieved a culture density approximately one-half that of the control strain, C58 (Fig. 2A). Since Chry5 is unable to use DL-nopaline as a carbon source, we conclude that Chry5 is using the LL isomer, isonopaline. Chry5 grew to the same extent as A281 on the mixed LL and DL isomers of succinamopine, indicating that both strains were able to metabolize the same amount of carbon (Fig. 2B). As A281 is able to catabolize only LL-succinamopine (9), this indicates that Chry5 also is able to utilize only one isomer. We presume this to be LL-succinamopine, but our tests are unable to make this distinction. This pattern of opine catabolism is unique among *A. tumefaciens* strains investigated to date. These investigations, as well as chemical analysis of opines produced in Chry5 tumors, are being pursued further (8a).

Chry5 is insensitive to agrocin 84. Since sensitivity to agrocin 84 is characteristic of nopaline-type strains (19) and at least one succinamopine-type strain (10), it is interesting that Chry5 is not sensitive. Despite its ability to catabolize isonopaline, and one isomer of succinamopine, Chry5 might lack the permease that allows uptake of agrocin 84 in sensitive nopaline strains.

Plasmid profiles. Chry5 contains two plasmids (Fig. 3A), one of approximately 120 MDa which hybridized to a Ti plasmid-specific probe (Fig. 3B) and one somewhat larger. Also, as shown in Fig. 3A, the Chry 1, -3, -8, and -9 isolates inconsistently appeared to contain a third smaller band, the nature of which has not yet been determined.

Conclusion. Although the bacterial etiology of crown gall first was confirmed with *C. frutescens* (39), little is known about strains that naturally infect this plant. In addition to Chry5 (22), biotype I strains have been reported on chrysanthemum in New Zealand (26), the Italian Riviera (2), and the Republic of South Africa (42). In none of the cases were the strains well characterized. Biotype III strains also have been isolated from *C. morifolium* (3), but their relationship to the well-known biotype III strains from grapevine is unclear. Chry5 is clearly a biotype I strain, but it has some interesting characteristics. Foremost among these are its tumorigenicity on soybean and its unique pattern of opine catabolism and agrocin insensitivity.

The cultivar specificity of Chry5 on *C. morifolium* (5a, 23) is a characteristic too rarely investigated to determine whether it is unusual or not, but a differential cultivar response has been reported for another strain from chrysanthemum (17). As much of what is known about *A. tumefaciens* as a species is based on relatively few individual strains, it is important to build a larger data base to increase understanding of the areas of similarity and diversity in the species as a whole. This strain illustrates both of these extremes. Further work will focus on an examination of the cultivar specificity of Chry5 on chrysanthemum.

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