The Chromosomal Background of *Agrobacterium tumefaciens* Chry5 Conditions High Virulence on Soybean

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***Agrobacterium tumefaciens* Chry5, a wild-type strain from chrysanthemum, is highly virulent on soybean stems (Bush and Pueppke, Appl. Environ. Microbiol. 57:2468-2472, 1991). Using soybean cotyledons, we have developed a quantitative tumorigenesis assay and show here that only strain A281, the so-called "supervirulent" strain, matches Chry5 in tumor-inducing ability. Six of the remaining 19 test strains failed to induce tumors, and the other 13 produced no more than 60% of the tumor mass elicited by Chry5 and A281. The Chry5 Ti plasmid has been genetically dissected from the remainder of the bacterial genome to independently study the relative contributions of the Ti plasmid and the chromosomal background to virulence. This was achieved by curing Chry5 of its Ti plasmid and then independently mobilizing three heterologous Ti plasmids, pTiAch5, pTiT37, and pTiBo542, into the Ti plasmidless Chry5 derivative. The tumor-inducing abilities of the resulting constructions and of the wild-type Ti plasmid donors were quantified and compared. We document that the chromosome and the cryptic plasmid of Chry5 provide a genetic background that potentiates the tumorigenic abilities of all three Ti plasmids in comparison to their normal genetic backgrounds.**

Additional keywords: soybean–*Agrobacterium* interaction, Ti plasmid-curing, quantitative tumorigenesis assay.

Agrobacterium tumefaciens (Smith and Townsend) Conn incites tumorous growths, called crown galls, on a wide range of plant species. The tumorigenic process involves the transfer and integration of a fragment of bacterial DNA (T-DNA) into the plant nuclear genome. In the bacterium, the T-DNA resides on a large plasmid, the so-called tumor-inducing (Ti) plasmid. Most of the genetic information that controls the T-DNA transfer mechanism also resides on the Ti plasmid, but outside of the T-DNA region. Several additional loci that also affect the tumorigenic process do not map to the Ti plasmid (Cooley *et al.* 1991; Douglas *et al.* 1985; Huang *et al.* 1990; Thomashow *et al.* 1987; Wirawan *et al.* 1993). Thus, the Ti plasmid does not contain all of the genetic information required for the T-DNA transfer process, and it is not surprising that the chromosomal background, i.e., the chromosome and cryptic plasmid(s), influences the extent to which a Ti plasmid can realize its tumorigenic potential.

This effect of the chromosomal background is best illustrated by virulence studies with strains Bo542 and A281. The wild-type strain Bo542 consists of pTiBo542 and its native Bo542 chromosomal background, whereas the transconjugant strain A281 consists of pTiBo542 in the C58 chromosomal background (Montoya *et al.* 1977). Although tumor induction by Bo542 is meager on tobacco and tomato and undetectable on alfalfa and soybean, A281 induces substantial numbers of tumors on all four plant species (Hood *et al.* 1986, 1987). These results suggest that the C58 chromosomal background is a key factor in the high virulence of A281, a strain that has been termed supervirulent. Although the unusually high tumor-inducing potential of pTiBo542 has attracted considerable scientific attention (An *et al.* 1985; Chen *et al.* 1991; Hood *et al.* 1984, 1986, 1987; Jin *et al.* 1987; Owens and Smigocki 1988; Pythoud *et al.* 1987), there is no experimental explanation for the ability of this Ti plasmid to realize a much higher tumor-inducing potential in the C58 chromosomal background than in its native chromosomal background.

Our laboratory recently reported that the tumor-inducing ability of *A. tumefaciens* Chry5, a naturally occuring strain that originally was isolated from chrysanthemum (Miller 1975), equals that of A281 on several plant species, including soybean (Bush and Pueppke 1991). As a first step in our efforts to understand the high virulence of Chry5, we developed a quantitative assay to measure tumorigenesis on soybean. We then genetically dissected the Chry5 genome to independently study the relative contributions of its Ti plasmid and of its chromosomal background to tumorigenesis. Chry5 was cured of its Ti plasmid, and three heterologous Ti plasmids were successfully conjugated into the resulting tumor-inducing abilities of these strains suggest that the

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high virulence of Chry5 is determined to a great extent by the chromosomal background.

RESULTS

Quantitative virulence assay.

Our first objective was to confirm and to quantify the observation that Chry5 is exceptionally virulent on soybean, as reported by Bush and Pueppke (1991). We began by studying the tumor-inducing ability of Chry5 relative to that of other *A. tumefaciens* strains. In our initial whole-plant inoculation experiments, the variation in the data was so high that it was impossible to establish significant differences between the tumorigenic abilities of *A. tumefaciens* strains (data not shown). We thus set out to develop a rapid alternative assay that would provide data adequate for statistical analysis. Virulence in this new assay is measured as tumor mass induced on a defined area of wounded cotyledonary tissue from the soybean cultivar Peking. The results of two independent experiments are presented in Table 1. The data were subjected to an *F*-test, and least significant differences were calculated at the 95% level ($P = 0.05$) for each experiment.

On the basis of this analysis, the 21 strains included in the study can be divided into seven groups, some of which overlap, and one of which contains six nontumorigenic strains. The group of highest virulence, which includes only strains Chry5 and A281, did not overlap with any other group, even at the stringent probability level of 99.9% ($P = 0.001$, LSD = 15.4 and 12.7 mg for experiments 1 and 2, respectively). Therefore, Chry5 and A281 are statistically indistinguishable from one another in virulence, and the virulence of these two strains is considerably higher than that of any other strain assayed in these experiments. Tumors induced by Chry5 and A281 were similar in morphology as well. The most conspicuous characteristic of these tumors is their light green color, which distinguishes them from the darker tumors induced by other strains. The Chry5- and A281-induced tumors also grew faster and had a smoother appearance than tumors induced by other strains.

This study also demonstrated that the chromosomal background significantly influences tumorigenesis on soybean. We examined five pairs of strains, each of which consisted of a strain with a Ti plasmid in its native background and a strain with the Ti plasmid in a C58-derived background. These native/exconjugant pairs include T37/A208, Bo542/A281, 15955/NT1(15955), B6/NT1-(B6), and A6/A348. Except for NT1(B6), each exconjugant exhibits significantly higher virulence than its wild-type donor strain. The difference was most extreme with pTiBo542, which induced nearly 100× more tumor tissue in the A136 background than in the Bo542 background (Table 1).

Curing Chry5 of its Ti plasmid.

We originally intended to cure Chry5 of its Ti plasmid by culturing the bacterium at elevated temperature or in the presence of a mutagenic agent. Neither of these techniques enabled us to obtain a Ti plasmid-cured Chry5 strain, and so we adopted an alternative curing technique devised by Hynes *et al.* (1989). The technique allows the direct selection for plasmid-loss in gram-negative bacteria by exploiting the *sac* operon (*sacB* and *sacR* genes) of *Bacillus subtilis* (Ehrenberg) Cohn. The *sacB* gene specifies the synthesis of the enzyme levansucrase, whose catalytic product is lethal to gram-negative bacteria grown in the presence of sucrose. When a large number of cells carrying the *sac* operon on a plasmid are plated on sucrose-containing medium, colonies are formed only by those cells that have lost the plasmid or have an otherwise inactivated *sacB* gene.

The *sac* operon carried on the *nptI-sacB-sacR* cartridge (Ried and Collmer 1987) was integrated into the Chry5 Ti plasmid via marker exchange. An isolate carrying the *nptI-sacB-sacR* cartridge on its Ti plasmid was amplified in liquid medium and plated on YEM agar supplemented with 3% sucrose. Sucrose-resistant colonies emerged at frequencies ranging from 1.2 to 2.8×10^{-6} colony-forming units per input cell. Approximately 50% of such colonies remained resistant to kanamycin and were assumed to retain their Ti plasmids.

Nineteen isolates that were both sensitive to kanamycin and resistant to sucrose were selected to confirm the loss of the Ti plasmid by Southern hybridization. DNA from these isolates did not hybridize to radioactively labeled pTiChry5 DNA, indicating that they lacked a Ti plasmid (data not shown). Based on this assumption, the frequency of plasmid loss in this experiment was approximately 10^{-6} per input cell. One of the 19 isolates was purified and used to inoculate soybean cotyledons and leaves of *K. daigremontiana*. This isolate, designated Chry5C, did not induce tumors on either plant.

Table 1. Tumor induction by *Agrobacterium tumefaciens* strains on soybean cotyledons

Strain	Mean tumor weight (mg)	
	Experiment 1	Experiment 2
Chry5	54.8 ± 7.7[a] A[b]	58.3 ± 9.7 A
A281	53.1 ± 8.7 A	60.9 ± 12.2 A
A208	28.3 ± 2.9B	30.9 ± 10.9 B
C58	23.8 ± 5.6 B	23.7 ± 6.5 BC
S1005	22.8 ± 6.4 BC	25.7 ± 8.9 BC
AT-181	22.2 ± 4.4 BC	23.5 ± 9.4 C
Ach5	14.1 ± 2.2 CD	14.4 ± 5.7 D
NT-1(15955)	11.2 ± 5.0 DE	12.2 ± 6.2 DE
CG1C	5.9 ± 4.4 EF	5.8 ± 3.8 EF
B6	2.2 ± 2.1 F	3.4 ± 1.5 F
NT-1(B6)	2.2 ± 0.8 F	2.1 ± 1.3 F
338	1.6 ± 2.3 F	3.2 ± 2.2 F
T37	1.5 ± 1.7 F	2.3 ± 2.1 F
Bo542	0.6 ± 1.7 F	0.8 ± 1.8 F
A348	0.4 ± 0.7 F	1.5 ± 2.0 F
A6	0.0	0.0
15955	0.0	0.0
B2A	0.0	0.0
Ag63	0.0	0.0
AT-1	0.0	0.0
Tm-4	0.0	0.0

[a] Mean ± SD, mean is based on 12 replicates.
[b] Values followed by the same letter do not differ significantly according to *F*-test ($p = 95\%$).

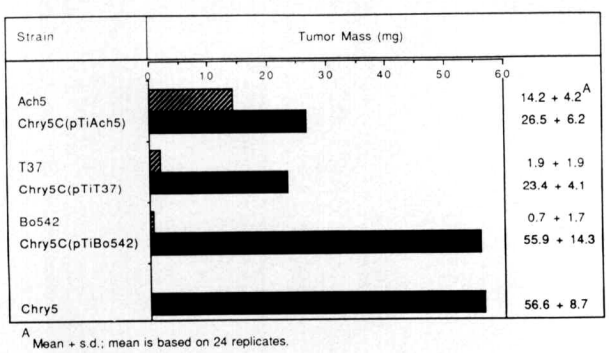
Ti plasmid conjugation.

Strain Chry5C was marked by rifampicin- and streptomycin-resistance to facilitate the recovery of Chry5C transconjugants that contained heterologous Ti plasmids. A281 and Chry5C then were mated *in planta* on an axenically cultured soybean tumor line as host. The bacteria were allowed to mate for 7 days, and subsequently plated on selective medium, which supplied mannopine as a sole nitrogen source. We obtained several mannopine-utilizing colonies on this medium. One proved to be tumorigenic on soybean tissue, and was named Chry5C(pTiBo542). We also attempted to conjugate several other Ti plasmids into the Chry5 chromosomal background. We mated Chry5C with octopine strains Ach5, B6, A6, 15955, Ag63, and Tm-4 on octopine-containing medium. Transconjugants never appeared in experiments with biotype 3 strains Ag63 and Tm-4. Octopine-utilizing colonies from experiments with four other octopine strains as donors did arise on selective medium, but when these colonies were purified and inoculated onto soybean tissue, only the pTiAch5 exconjugant proved to be tumorigenic. This strain was named Chry5C(pTiAch5). Nopaline-type strain T37 was mated with Chry5C to take advantage of the constitutive conjugation properties of the T37 Ti plasmid (Zhang and Kerr 1991). A tumorigenic transconjugant that resulted from this mating was named Chry5C(pTiT37).

The transfer of the Ti plasmids into Chry5C was confirmed by Southern blot analysis. Genomic DNAs from donors and transconjugants were probed first with T-DNA-specific sequences and then with cloned DNA from the Chry5 chromosome (data not shown). Each of the transconjugants contained T-DNA-specific sequences, and each also displayed a hybridization pattern indistinguishable from the Chry5 chromosome itself. This provides proof that the Ti plasmids were present in the background of the Chry5 chromosome.

The Chry5 chromosomal background potentiates virulence on soybean.

The novel transconjugants allowed us to assess the role of the Chry5 chromosomal background in tumorigenesis.



A Mean + s.d.; mean is based on 24 replicates.

Fig. 1. The effect of chromosomal background on virulence of *A. tumefaciens* on soybean 'Peking' cotyledons. Tumor mass induced by Ti plasmids pTiAch5, pTiT37, and pTiBo542 in their native chromosomal backgrounds is indicated by hatched bars, and in the background of the Chry5 chromosome by solid bars. Tumor induction by wild-type Chry5 is shown for reference.

The tumorigenic abilities of the three donor/transconjugant pairs, as well as that of Chry5, were tested with the quantitative soybean assay as described above (Fig. 1). A *t*-test ($P = 0.05$) was used to compare the masses of tumor tissue incited by the transconjugant strains to those incited by the wild-type Ti plasmid donors. All three Ti plasmids mediated the induction of significantly more tumor tissue in combination with Chry5C than in combination with their native chromosomal backgrounds (Figs. 1 and 2). Compared to the native background, the Chry5 background doubled the tumorigenicity of pTiAch5, increased that of pTiT37 by more than 10-fold, and enhanced that of pTiBo542 by more than 80-fold (Figs. 1 and 2). Figure 2 confirms that the tumor morphology on soybean tissue is specified by the Ti plasmid. pTiAch5 specifies the induction of rooty tumors (Fig. 2E, F). Tumors specified by pTiT37 are made up of dark green, dense tissues (Fig. 2A, B), whereas those elicited by pTiBo542 are lighter in color and more diffuse in contexture (Fig. 2C, D). Tumors elicited by pTiT37 and most other Ti plasmids are often covered with white, friable tissues (Fig. 2A, B), which are characteristically absent from tumors elicited by pTiBo542 (Fig. 2C, D) and by pTiChry5.

The tumorigenic potential of pTiChry5 exceeds that of pTiT37 or pTiAch5.

The novel iso-chromosomal transconjugant strains allowed us to compare the tumor-inducing potential of pTiChry5 to that of pTiT37, pTiAch5, and pTiBo542 without the confounding effect of heterologous genetic backgrounds. An *F*-test of the tumor weight data ($P = 0.05$; LSD = 12.87 mg) revealed that in the Chry5C background, pTiChry5 mediated the induction of approximately twice as much tumor tissue as did pTiT37 or pTiAch5 (Figs. 1 and Fig. 2B, D, F). No significant difference was found between the mass of tumors induced by pTiChry5 and pTiBo542, indicating that the tumorigenic potentials of these two plasmids on soybean are equal (Fig. 1).

DISCUSSION

Soybean is a key candidate for *Agrobacterium*-mediated plant improvment, and the soybean–*A. tumefaciens* interaction has been the subject of intensive investigation (Byrne *et al.* 1987; Hood *et al.* 1987; Owens and Cress 1985; Owens and Smigocki 1988; McKenzie and Cress 1992). Originally, we utilized whole plants to assay *A. tumefaciens* virulence on soybean, but variables, such as the size of the wound, the number of the interacting *A. tumefaciens* cells, and the growth conditions could not be precisely controlled in these experiments. This fact prompted us to devise a quantitative laboratory method to measure *A. tumefaciens* tumorigenesis on soybean. The new assay is based on the observation of Owens and Cress (1985) that cotyledons of soybean cultivar Peking respond to *A. tumefaciens* infection with the rapid formation of easily recognizable tumor tissue. Statistical analysis of two independent experiments confirms that the bioassay

provides reproducible results (Table 1). The variability of the data generated in these experiments is reasonably low in the case of the highly and moderately virulent *A. tumefaciens* strains, but tends to be greater in the case of weakly virulent strains (Table 1). An unexpected benefit of the new bioassay is that, in addition to tumor size, tumor morphology also correlates well with the inducing *A. tumefaciens* strains. The relationship between the

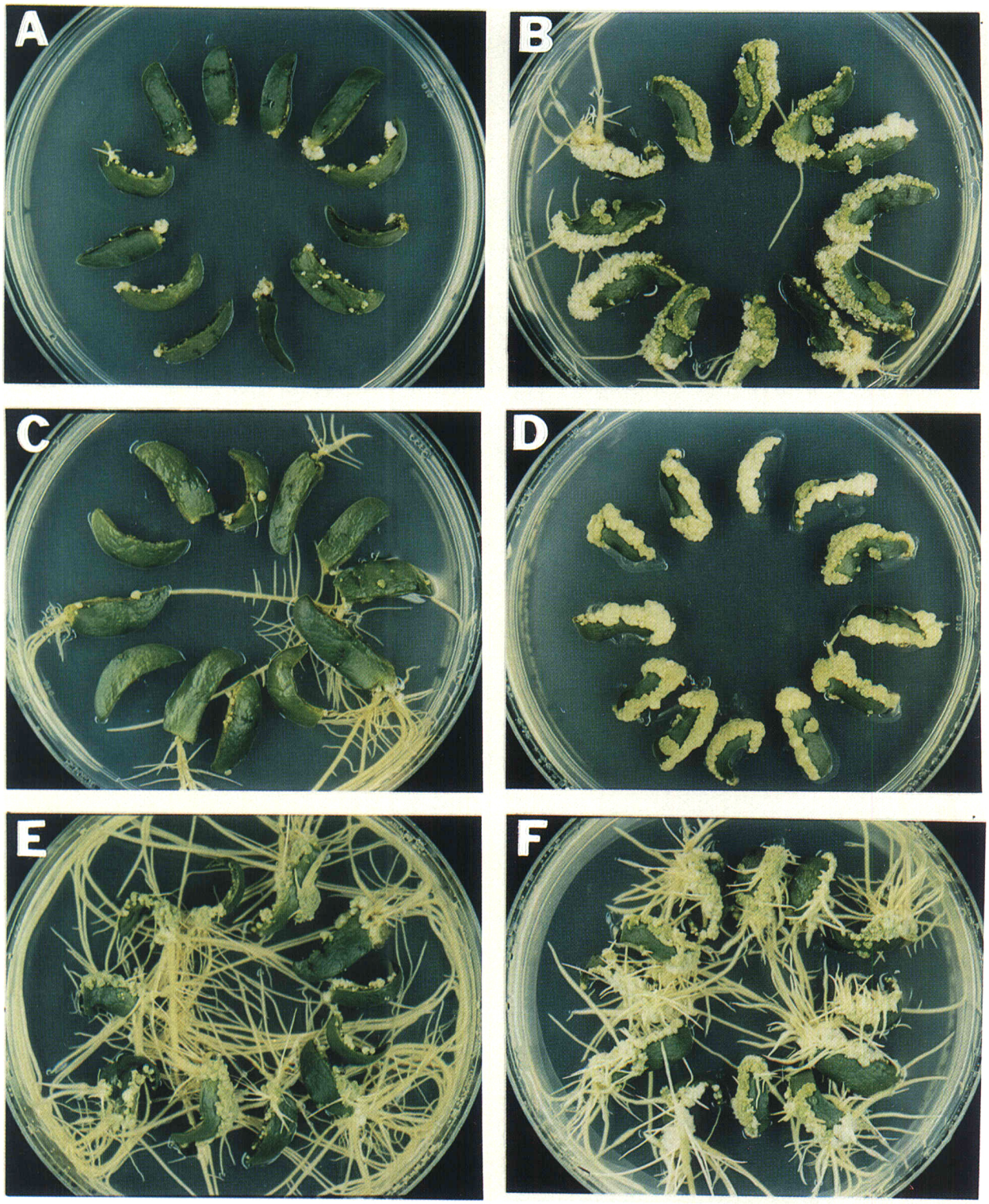


Fig. 2. Tumor induction on soybean 'Peking' cotyledonary tissue by *A. tumefaciens* strains **A,** T37; **B,** Chry5C(pTiT37); **C,** Bo542; **D,** Chry5C(pTiBo542); **E,** Ach5; and **F,** Chry5C(pTiAch5). The hyperplasic growths shown in each horizontal pair of photographs were induced by the same Ti plasmid; on the left, the Ti plasmid is harbored in its wild-type chromosomal background, whereas on the right, it is in the background of Chry5. Tumors were photographed 12 days after inoculation.

morphology of the hyperplasic tissue and the challenger *A. tumefaciens* strain is particularly conspicuous for strains harboring pTiAch5, which induce the proliferation of roots in addition to tumors (Fig. 2).

Our data on the strain-specificity of the *A. tumefaciens*–soybean interaction are in good agreement with the more limited findings of previous investigators, who commented on the high virulence of strains A281, C58, and A208 (Hood *et al.* 1987; Byrne *et al.* 1987) on soybean stems. We have extended this list with such strongly virulent strains as Chry5, AT-181, and S1005 (Table 1). We also have shown that biotype 3 strains (also called *Agrobacterium vitis* Ophel and Kerr) Ag63, Tm-4, and AT-1 are nontumorigenic on soybean (Table 1). These strains induced a characteristic necrosis on the wounded surface of the cotyledons within 72 hr following inoculation. This symptom was not produced in response to any biotype 1 or 2 strains, and it probably is the result of the extracellular polygalacturonase activity specifically associated with biotype 3 isolates (McGuire *et al.* 1991).

We were able to confirm and quantify the original observation of Bush and Pueppke (1991) that the tumor-inducing ability of *A. tumefaciens* Chry5 is unusually strong on soybean, being equaled only by that of strain A281 (= A136[pTiBo542]). Tumors induced by Chry5 and A281 are indistinguishable from each other, but differ significantly from those induced by other strains tested here. The similarity between the A281- and the Chry5-induced tumors is manifested not only in their unusually high abundance, but in their characteristic morphology as well. These observations were confirmed by the comparative study of pTiChry5 and pTiBo542 in isogenic chromosomal backgrounds. The conspicuous similarity between the tumorigenic features of these two Ti plasmids clearly sets them apart from other Ti plasmids studied here, and raises the possibility of their relatedness. This comparison becomes more pronounced if one considers that the plasmids share a common opine marker, L,L-succinamopine (Bush and Pueppke 1991), and that they originate from agrobacteria that were isolated from *Chrysanthemum* and *Dahlia*, two closely related genera of the Compositae (Table 2). This pattern of related features between the two plasmids has prompted us to undertake a detailed molecular analysis of pTiChry5, which will be published elsewhere (Kovács and Pueppke 1993).

Hood and associates (1986, 1987) have shown that the C58 chromosomal background is a key component of the high virulence of strain A281. Our studies with soybean cotyledonary tissue confirm the virulence-potentiating effect of the C58 chromosomal background in combination with pTiBo542, pTiT37, pTiA6, or pTi15955, but not with pTiB6 (Table 1). The lack of any virulence-potentiating effect in combination with pTiB6 is unexpected in the light of the near identity of this Ti plasmid to pTiA6 and pTi15955. At present we can not offer an explanation to this phenomenon. We demonstrate that the chromosomal background of strain Chry5 also conditions high virulence when combined with pTiBo542, pTiT37, or pTiAch5. In fact, the Chry5 and C58 backgrounds potentiate virulence to a similar extent in combination with pTiBo542 or pTiT37 (Table 1 and Fig. 1). This resemblence is intriguing, because the C58 and

Table 2. Bacterial strains and plasmids used in this study

Strain	Salient characteristics	Source or reference
A. tumefaciens		
Chry5	L,L-Succinamopine-type; *Chrysanthemum morifolium* Ram. strain	R. E. Stall, University of Florida
Bo542	L,L-Succinamopine-type; *Dahlia* strain	R. O. Morris, University of Missouri
A281	L,L-Succinamopine-type; pTiBo542 in A136	L. D. Owens, USDA, Beltsville
AT-181	D,L-Succinamopine-type; *Dahlia* strain	R. N. Goodman, University of Missouri
C58	Nopaline-type; *Prunus* (cherry) strain	J. Lippincott, Northwestern University
T37	Nopaline-type; *Juglans* strain	E. W. Nester, University of Washington
A208	Nopaline-type; *pTiT37 in A136*	R. O. Morris, University of Missouri
Ach5	Octopine-type; *Achillea millefolium* L. strain	J. Lippincott, Northwestern University
CG1C	Octopine-type; *Pyrus communis* L. strain	L. Moore, Oregon State University
B6	Octopine-type; *Malus domestica* Borkh. strain	A. G. Matthysse, University of North Carolina
NT-1(B6)	Octopine-type; pTiB6 in NT-1	W. B. Gurley, University of Florida
A6	Octopine-type; *Rubus occidentalis* L. strain	E. W. Nester, University of Washington
A348	Octopine-type; *pTiA6 in A136*	E. W. Nester, University of Washington
B2A	*Octopine-type; Lycopersicon esculentum* Mill. strain	R. O. Morris, University of Missouri
15955	Octopine-type strain of unknown origin	W. B. Gurley, University of Florida
NT-1(15955)	Octopine-type; pTi15955 in NT-1	W. B. Gurley, University of Florida
S1005	Octopine-type; unknown origin	W. B. Gurley, University of Florida
Ag63	Octopine-type; *Vitis vinifera* L. strain	R. N. Goodman, University of Missouri
Tm-4	Octopine-type; *V. vinifera* strain	E. Szegedi, Institute for Viticulture, Hungary
AT-1	Nopaline-type; *V. vinifera* strain	R. N. Goodman, University of Missouri
338	Strain of unknown origin	L. D. Owens, USDA, Beltsville
E. coli		
DH5α	*recA, hsdR, hsdM, pro, leu*	Miller 1972
HB101	*recA, endA, hsdR, gyrA, thi*	Maniatis *et al.* 1982
Plasmids		
pUM24	*nptI-sacB-sacR* cartridge	Ried and Collmer 1987
pCH85	pTiChry5 insert in pLAFR1	Kovacs and Pueppke 1993
pRK2013	Transfer functions for pRK2-derived replicons	Ruvkun and Ausubel 1981
pPH1J1	Incompatibility with pRK2-derived replicons	Beringer *et al.* 1978
p202	*Eco*RI fragment B of *A. tumefaciens* 15955 T-region	Gurley *et al.* 1979

the Chry5 chromosomes are associated with distantly related Ti plasmids in nature (Table 2) and appear to be only remotely related at the level of DNA homology (L. Kovács and S. G. Pueppke, unpublished observations).

Our observations raise an interesting question: Are the virulence-conditioning effects of different chromosomal backgrounds due to positive or negative factors? More specifically, is there a positively acting factor(s) in the C58 and the Chry5 backgrounds that potentiates virulence with certain Ti plasmids, or, alternatively, is there a negatively acting factor(s) in the Bo542 and the T37 backgrounds that prevents certain Ti plasmids from realizing their tumorigenic potentials? Hood and associates (1986) favor the later hypothesis, reasoning that one of the three other plasmids of Bo542 may specify a function that impairs tumorigenesis. Such a possibility has recently found support in results by Close and Kado (1991), who showed that the *osa* gene of plasmid pSa from *Shigella flexneri* Castellani and Chalmers suppresses the virulence of *A. tumefaciens* strain LBA4301(pTiC58) on *Datura stramonium* L. As of now, however, no direct experimental evidence is available to demonstrate the virulence-suppressing effect of any cryptic plasmid from *Agrobacterium*, and therefore the hypothesis that C58 and Chry5 backgrounds contain a positively acting factor can not be rejected.

More detailed analysis of the virulence-potentiating effect of the C58 and the Chry5 backgrounds may lead to a better understanding of the interactions between the replicons of the *Agrobacterium* genome. Moreover, a comprehensive study of Ti plasmid combinations with different chromosomal backgrounds holds potential to identify *A. tumefaciens* constructions that are especially virulent on certain plant species. Such organisms would have considerable practical value in the genetic engineering of hard-to-transform plants such as soybean.

MATERIALS AND METHODS

Organisms and culture conditions.

Seeds of soybean (*Glycine max* (L.) Merr.) 'Peking' were obtained from the University of Missouri Bradford Research Farm. Bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* (Migula) Castellani and Chalmers and *A. tumefaciens* were cultured routinely in LB and YEM media, respectively (Maniatis *et al.* 1982; Vincent 1970). All bacterial strains were stored in 15% glycerol at –70° C. *E. coli* strains were kept on LB agar plates, and *A. tumefaciens* strains were kept on gluconate-mannitol agar plates (Bhuvaneswari *et al.* 1977) at 4° C for short-term storage.

Quantitative virulence assay.

Seeds of soybean cultivar Peking were submerged for 2 min in 70% ethanol, then for 10 min in 50% commercial bleach, and subsequently rinsed thoroughly in sterile distilled water. Seeds were germinated on water agar plates at 30° C for 4 days. The emerging cotyledons were aseptically excised at the nodes and sliced longitudinally into approximately equal halves. Twelve cotyledon halves from three plants were submerged and gently shaken in 10 ml of an *A. tumefaciens* suspension. This suspension had been prepared as follows: Cells were cultured for 24 hr in 50 ml of YEM medium on a gyratory shaker at 175 rpm, washed in phosphate-buffered saline (PBS; 0.43 g of KH_2PO_4, 1.48 g of Na_2HPO_4, 7.2 g of NaCl per liter, pH 7.2), and suspended to a concentration of about 10^9 cells per milliliter of PBS. After a 10-min submersion in this suspension, the soybean cotyledon halves were blotted briefly between sterile paper towels and placed on water agar plates. The cotyledon halves were cocultivated with bacteria at 24° C under a 12-hr photoperiod. After 3 days, the tissue pieces were transferred to B5 medium (Gamborg *et al.* 1968), supplemented with 200 mg of carbenicillin and 200 mg of vancomycin per liter. Tumors were allowed to form under continuous light for 12 days. At the end of this period the stem-proximal end of each cotyledon piece was removed so that the remaining tissue slice was 15 mm long. Tumor tissue along the cut edge of this tissue was excised and weighed. For each treatment 12 tumor-weights were measured, and each experiment was repeated twice.

Curing Chry5 of its Ti plasmid.

The Ti plasmid of Chry5 was cured after introduction of the *nptI-sacB-sacR* cartridge (Ried and Collmer 1987), the generous gift of Alan Collmer, Cornell University. The *nptI-sacB-sacR* cartridge was removed from pUM24 as a 3.8-kb BamHI fragment and cloned into the single *Bam*HI site of pCH85, a clone carrying pTiChry5 DNA in cosmid pLAFR1 (L. Kovács and S. G. Pueppke, 1993). The resulting plasmid pCH85*sac* was introduced into Chry5 by triparental mating, utilizing the mobilization functions of helper plasmid pRK2013. The *nptI-sacB-sacR* cartridge was marker exchanged from pCH85sac to the Ti plasmid through a double homologous recombination event which was selected for the introduction and maintenance of plasmid pPH1JI. Colonies containing the integrated cartridge were identified on the basis of resistance to kanamycin, spectinomycin, and gentamycin, and sensitivity to tetracycline and sucrose. A colony of this phenotype was transferred to YEM medium and incubated for 8 hr at 28° C. The culture then was diluted to a concentration of 10^7 cells per milliliter, and 200 ml of this suspension was plated on YEM agar supplemented with 3% sucrose. Colonies that arose on sucrose-containing plates were transferred to kanamycin-containing medium. Strains that were sensitive to kanamycin and that presumably had lost the Ti plasmid with its *nptI-sacB-sacR* cartridge were examined by Southern blot analysis. Genomic DNAs derived from 19 kanamycin-sensitive strains were digested with restriction enzyme *Eco*RI, immobilized on nitrocellulose and probed with radioactively labeled DNA from pTiChry5. Radioactive labeling was done with the Random Primed DNA Labeling Kit (United States Biochemical Corporation, Cleveland, OH), according to the directions of the manufacturer. The probe consisted of 10 *Eco*RI fragments whose original locations were widely scattered around the Ti plasmid. These fragments were obtained from a pTiChry5 cosmid library (Kovács and

Pueppke 1993). The loss of the Ti plasmid was confirmed by loss of tumor-inducing ability on soybean cotyledons and leaves of *Kalanchoë daigremontiana* Hamet and Perr.

Ti plasmid conjugation.

One of the strains that had become avirulent by losing its Ti plasmid as a result of the curing experiment was made resistant to rifampicin (100 mg/L) and streptomycin (500 mg/L) by sequential selection for two spontaneous mutation events. This strain, designated Chry5C, was used as a recipient for different Ti plasmids. Prior to mating, the sensitivity of the donor strains to rifampicin (100 mg/L) and streptomycin (500 mg/L) was confirmed by plating on YEM medium supplemented with these antibiotics. Mating between Chry5C and A281 was carried out on soybean tumor tissue. The tumor tissue had been induced by Chry5, and then axenically cultured on carbenicillin- and vancomycin-containing B5 medium to free it from the bacterium. The sterility of the tissue was confirmed by the absence of bacterial growth in cultures on antibiotic-free medium. Five microliters of a mixed suspension of strains Chry5C and A281 was deposited on the tissue with a micropipette tip, and the cells were allowed to mate for 7 days. Cells subsequently were resuspended in sterile water and plated on SMNO agar (Lichtenstein and Draper 1985) supplemented with 0.1 g of mannopine per liter as the sole nitrogen source; rifampicin (100 mg/L) and streptomycin (500 mg/L) also were added. Cells that combine Chry5C, which is resistent to rifampicin and streptomycin, and pTiBo542, which confers the ability on the cell to utilize mannopine as a nitrogen source, form colonies on this medium. One colony was purified and tested for virulence to confirm the transfer of the Ti plasmid.

Chry5C also was mated separately with the nopaline-type strain T37 and octopine-type strains Ach5, B6, A6, 15955, Ag63, and Tm-4. Bacteria for these experiments were grown on YEM agar for 24 hr, resuspended in PBS buffer to a concentration of 5×10^7 cells per milliliter, and suspensions of both donor and recipient were mixed and deposited on a sterile filter, that had been placed on SMNO agar supplemented with 2 g of the appropriate opine per liter. After 4 days of mating at 30° C, the bacteria were resuspended in sterile water and plated on minimal salts agar that had been supplemented with the appropriate opine at 0.1 g/L as the sole nitrogen source; rifampicin and streptomycin also were added. Colonies that arose on this medium were purified and tested for virulence to confirm the transfer of the Ti plasmid.

Genomic DNAs of the tumorigenic transconjugants were tested for the presence of Ti plasmid sequences by Southern blot analysis. *Eco*RI fragment B of strain 15955 was used as a probe. This fragment contains the so-called "common DNA" that is highly conserved in most Ti plasmids (Gurley *et al.* 1979). To prove that the transconjugants were indeed based on Chry5C, their *Eco*RI-digested genomic DNA samples were subsequently probed with a 12-kb stretch of the Chry5 chromosome. This 12-kb DNA contains a sequence that is homologous to the *chvA* gene (Douglas *et al.* 1985), and is cloned in cosmid pCH6005 (L. Kovács and S. G. Pueppke, unpublished). The probe prepared from this insert produces a pattern of radiactive signals that is characteristic for the Chry5 chromosome. Southern blotting was performed according to the guidelines of Maniatis *et al.* (1982).

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