Agropine in “null-type” crown gall tumors: Evidence for generality of the opine concept

(Ptumors/Ti plasmid/octopine/nopaline)

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ABSTRACT Agrobacterium Ti (tumor-inducing) plasmids, the causative agents of crown gall disease, fall into four genetic groups based on the patterns of octopine and nopaline synthesis (by crown gall tumors) and catabolism (by Agrobacterium tumefaciens) for which they are responsible. Two classes of Ti plasmids induce tumors that synthesize neither octopine nor nopaline. The existence of these Ti plasmids challenged the view that opines such as octopine and nopaline play a central role in crown gall biology. We now report the occurrence of an opine in tumors induced by one of these classes of Ti plasmids, the "null-type" plasmids typified by pTi Bo542. The opine was purified by biological enrichment based on its utilization by bacteria containing pTi Bo542 but not by bacteria lacking a Ti plasmid. The mass spectrum and biological properties of this opine are identical to those of agropine, an opine recently discovered in octopine-type tumors. We propose that null-type Ti plasmids now be named for their signal opine, agropine-type Ti plasmids.

Transformation of plant tissues to malignancy by Agrobacterium tumefaciens is an example of natural genetic modification of a host by a pathogen. The oncogenicity of the bacterium is conferred by a class of large plasmids called Ti (tumor inducing) plasmids (1–4). A part of the Ti plasmid, called T-DNA, is transferred to the plant cell during the transformation process and is maintained in the tumor tissue in the absence of the inciting bacterium (5). RNA transcripts of T-DNA have been detected in axenic tumor tissue (6–8), consistent with the view that there is expression of foreign genetic information in the transformed cell. New metabolites are synthesized by tumor cells at the direction of Ti plasmid genetic information (9–11). These compounds, generally called opines (12), include the octopine group (pyruvic opines) and the nopaline group (α-ketoglutaric opines) (Fig. 1) (13–19) as well as agropine (19), whose structure has not been determined. The gene for nopaline synthesis maps in the T-DNA portion of pTi CS8 (20).

The Ti plasmid confers on A. tumefaciens the ability to use specifically those opines whose synthesis it elicits in the tumor (2, 3, 9–11). In addition to serving as nutrients, the opines act as specific inducers for conjugal transfer of Ti plasmids to other Agrobacteria (21–23). Thus the bacterium harboring a Ti plasmid creates, in the tumor it induces, a specific ecological niche for itself, rich in metabolites that are useful to itself but not to the plant or to other microorganisms.

The production of opines by crown gall tumors at the direction of Ti plasmid-borne genes thus appears to be a biological rationale for the existence of the crown gall tumor. If the gall indeed exists in order to fulfill nutritional needs of the oncogenic bacterium, then all crown gall tumor lines should produce opines. Thus far, such a model is supported by two classes of Ti plasmids: the octopine-type and the nopaline-type, which elicit the synthesis of octopine and nopaline, respectively, by the tumor lines they induce. The model is challenged by two other classes of Ti plasmids that induce tumors that fail to produce any known opines. The first is the “unusual nopaline plasmids,” pTi AT181 and pTi EU6 (9) and pTi T10/73 (24), which confer nopaline utilization on the host bacterium but induce nopaline-free tumors. These three Ti plasmids, by the criterion of Sma I fingerprinting (24), resemble one another closely and are very similar to pTi T37, a standard nopaline-type Ti plasmid (24). One can thus view them as “defective” nopaline-type Ti plasmids. A second group of exceptional plasmids is the “null-type” Ti plasmids, pTi 542 and pTi AT1, which confer no known opine utilization on the host bacterium and elicit no known opine production by their tumors (9, 24).

Examples of both groups of plasmids have been observed to conjugate in planta (24). Such conjugation is inducible by octopine and nopaline in the case of octopine- and nopaline-type Ti plasmids (21–23); it is therefore reasonable to suppose the existence of opines in the tumors incited by unusual nopaline and null-type Ti plasmids. The present study was undertaken to seek unknown opine compounds in the tumors induced by null-type Ti plasmids. The biological properties of opines, inferred from the characteristics of the opine and nopaline groups, are not dependent on their chemical structure. An opine is a substance produced by the plant in response to a stimulus from the pathogen; its presence creates favorable environmental con-

Abbreviation: Ti, tumor inducing.

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‡ See Note Added in Proof.
ditions for the pathogen and contributes to its dissemination. The specific nutritional characteristics of crown gall opines were exploited for detection and isolation of a compound possessing opine characteristics without prior knowledge of its chemical properties.

**MATERIALS AND METHODS**

**Bacterial Strains.** The *A. tumefaciens* strains used in this study are listed in Table 1, together with their characteristics and plasmid content. The *SmaI* cleavage patterns (fingerprints) of many of the plasmids have been reported (24).

**Culture Media.** AT medium contained, per liter: KH$_2$PO$_4$, 10.9 g; MgSO$_4$·7H$_2$O, 0.160 g; FeSO$_4$·7H$_2$O, 0.005 g; CaCl$_2$·2H$_2$O, 0.011 g; and MnCl$_2$·4H$_2$O, 0.002 g. The pH was adjusted to 7.0 with 6 M KOH and the solution was boiled for 15 min. A precipitate was removed by filtration prior to sterilization. Carbon and nitrogen sources were sterilized separately. As carbon source, mannitol or glucose was added at a final concentration of 0.2%.

Stock cultures were maintained on yeast agar slants consisting of AT medium supplemented with (NH$_4$)$_2$SO$_4$ (0.2% final), mannitol (0.5% final), and Difco yeast extract (5 g/liter) and solidified with agar (16 g/liter).

Solid media containing purified opine extract were prepared with Difco noble agar in order to minimize background growth due to extraneous nitrogen sources.

YE medium used in bacterial transformation was that of Holsters et al. (28).

**Inoculation of Plants.** Tumors were induced on *Kalanchee tubiflora* plants about 15 cm in height. Plants were decapitated and inoculated with bacterial suspension at the apex and in two lateral stab wounds in the uppermost internodes. The bacterial inoculum was prepared by suspending bacteria from a freshly grown yeast agar slant in 5 ml of distilled water by vigorous agitation.

**Preparation and Purification of Opine Extracts.** *Crude extracts.* Tumors were harvested 6–10 weeks after inoculation with strain Bo542 or strain A281 (containing pTi Bo542) (Table 1). Tumor tissue (2–3 g per plant) was minced and extracted for 4–5 min with 10 vol of boiling 50 mM HCl. The mixture was diluted with 2 vol of ice-cold water, homogenized, and stored overnight at 4°C. After centrifugation, the extract was evaporated to dryness under reduced pressure and yielded about 3 g of dried extract per 100 g of fresh tissue. The crude extract was subjected either to biological enrichment or to ion-exchange chromatography followed by biological enrichment, as detailed below.

**Assay of Extracts as Growth Substrates.** The presence of growth substrates specific for strains carrying a Ti plasmid was assessed by comparing growth of C58C1 with that of C58C1(pTi X) on a medium containing the extract. The medium to be tested was either used directly after the biological enrichment described above or prepared by addition of extract and glucose or mannitol to AT salts. The medium or the extract was sterilized by filtration through 0.45-μm Millipore filters. Cultures were grown in glass tubes (12 × 120 mm) containing 2 ml of medium. Turbidity of cultures was assayed by OD$_{580}$ measurements with a Bausch and Lomb Spectronic spectrophotometer.

**Conjugation.** Donor and recipient bacteria were grown from single colonies in minimal medium to exponential phase. The recipient was a mutant of C58C1 selected for resistance to streptomycin (500 μg/ml) and rifampicin (100 μg/ml) in two successive steps. Donor and recipient were mixed in a 10:1 ratio, and drops of the mating mixture were deposited on minimal agar containing the extract as a sole source of nitrogen and carbon. The concentration of the extract in the medium was equivalent to that giving a turbidity of OD$_{580}$ = 1.0 in liquid culture. After 72 hr on the conjugation medium at 27°C, the mating mixture was resuspended in distilled water and about 10$^6$ cells were plated on agar consisting of AT medium containing mannitol (2 g/liter), the extract as nitrogen source (one-fifth of the concentration used above), rifampicin (100 μg/ml), and streptomycin (500 μg/ml).

**Transformation.** Plasmid DNA was isolated as described (23). Recipient bacteria (strain A136) were prepared and treated

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**Table 1.** *A. tumefaciens* strains

<table>
<thead>
<tr>
<th>Designation of strain</th>
<th>Ti plasmid content*</th>
<th>Octopine utilization</th>
<th>Nopaline utilization</th>
<th>Source†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bo542</td>
<td>pTi Bo542</td>
<td>–</td>
<td>–</td>
<td>(23)</td>
</tr>
<tr>
<td>A281</td>
<td>pTi Bo542</td>
<td>–</td>
<td>–</td>
<td>(23)</td>
</tr>
<tr>
<td>AT4</td>
<td>pTi Bo542</td>
<td>–</td>
<td>–</td>
<td>(23)</td>
</tr>
<tr>
<td>AT1</td>
<td>pTi AT1</td>
<td>–</td>
<td>–</td>
<td>(25)</td>
</tr>
<tr>
<td>K15/73</td>
<td>NC</td>
<td>–</td>
<td>–</td>
<td>LWM</td>
</tr>
<tr>
<td>H100</td>
<td>pTi H100</td>
<td>+</td>
<td>+</td>
<td>(23)</td>
</tr>
<tr>
<td>T37</td>
<td>pTi T37</td>
<td>+</td>
<td>+</td>
<td>(23)</td>
</tr>
<tr>
<td>R10</td>
<td>pTi R10</td>
<td>+</td>
<td>+</td>
<td>(26)</td>
</tr>
<tr>
<td>B683</td>
<td>pTi B683</td>
<td>+</td>
<td>+</td>
<td>(27)</td>
</tr>
<tr>
<td>C58C1</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>(2)</td>
</tr>
<tr>
<td>A136</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>(3)</td>
</tr>
</tbody>
</table>

* The designation NC indicates that the plasmid content of the strain has not been characterized.
† The designation LWM indicates a strain kindly provided by Larry W. Moore (Oregon State University, Corvallis, Oregon).
by the method of Holsters et al. (28). A mixture of 0.15 ml of bacterial suspension in 10 mM Tris (pH 7.4) (≈2 × 10/ml) was added to 0.1 ml of pTi Bo542 DNA (4 µg) in 1 mM Tris (pH 7.5). The mixture was immediately frozen in dry ice/ethanol; after 5 min, it was thawed at 37°C. After 30 min, 0.5 ml of YEP broth was added. After 1 hr of incubation at 37°C without shaking, the bacteria were centrifuged and washed three times in AT medium without carbon and nitrogen sources before plating on AT agar with the purified extract described above as a source of carbon and nitrogen. Transformation frequency was approximately 4 X 10⁶, similar to the frequency observed with octopine-type Ti plasmid transformations performed in parallel with the same recipient cells.

Isolation and Characterization of Plasmid DNA. Plasmid DNA was isolated as described by Sciky et al. (24). Before addition of CsCl and ethidium bromide, an aliquot of the plasmid extract was reserved for agarose gel electrophoresis. Purified plasmid DNAs were subjected to restriction endonuclease cleavage as described (24), and fragments were separated by horizontal agarose gel electrophoresis (24).

RESULTS

We set out to isolate from null-type tumors a substance that could be used as a source of carbon or nitrogen by bacteria with null-type Ti plasmids but not by strain C58C1, which lacks a Ti plasmid. The strategy of the isolation procedure is summarized in the flow diagram of Fig. 2. A tumor tissue homogenate was freed from cellular debris and tested for ability to support the growth of strain Bo542 and C58C1 when used in AT medium as a sole source of nitrogen. No significant difference was detected, presumably because of the preponderance of nonopinic growth substrates in the crude extract. Accordingly, a biological enrichment procedure was used, as indicated in Fig. 2. The crude extract was added to AT growth medium as a sole source of nitrogen and C58C1 was allowed to use from it the nonopinic substances. When the culture supernatant was tested for opine activity, it was found to serve as nitrogen source for strain Bo542 approximately 2-fold more effectively than for strain C58C1 (Table 2). Similar results were obtained when the extract served as carbon source or as carbon and nitrogen source (Table 2).

In order further to enrich the opinic substance detected in the extract, a series of steps outlined in the right half of the flow diagram of Fig. 2 was used. Starting from the crude tumor extract (supernate of the homogenate), cationic substances were first isolated by adsorption and elution from Dowex 50W resin. This fraction was subjected to the biological enrichment regimen described above, and the culture supernatant was again tested for opine activity. As shown in Table 3, the extract supported the growth of strain A281 (containing pTi Bo542) but not that of C58C1 (containing no Ti plasmid). The opine extract also supported the growth of strain R10, an octopine-using strain, but not of strain H100, a nopaline-using strain.

Cationic substances were once again recovered from the culture supernatant by adsorption and elution from Dowex 50W resin as described in Materials and Methods. The resulting extract was subjected to chromatography on Dowex 50W, and individual fractions were assayed for opine activity. Fig. 3 presents the results of such a chromatographic analysis. Opine activity eluted in a peak that was in some cases not symmetrical. The peak fractions were pooled and evaporated to dryness. This "opine fraction" was used in the growth studies described below.

The known opines, octopine and nopaline, are inducers of conjugal transfer of their respective Ti plasmids (21–23). Accordingly, we tested the ability of the opine extract isolated in this study to induce conjugal transfer of the null-type Ti plasmid, pTi Bo542. Donor and recipient bacteria (Bo542 and C58C1) were mixed and plated on AT agar with the opine extract as sole source of carbon and nitrogen. After 72 hr, the bacteria were replicated on medium selective for the transconjugant characteristics (drugs to which the recipient is resistant, and opine extract as sole source of nitrogen). Four transconjugant colonies were observed. These were purified and found to possess the phage sensitivity pattern of the recipient strain, as expected of true transconjugants (Table 4).

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**Table 2. Biological assay 1: Opine activity in extract after biological enrichment**

<table>
<thead>
<tr>
<th>Strain</th>
<th>C and N</th>
<th>N</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>C58C1</td>
<td>0.085</td>
<td>0.070</td>
<td>0.060</td>
</tr>
<tr>
<td>Bo542</td>
<td>0.230</td>
<td>0.145</td>
<td>0.110</td>
</tr>
</tbody>
</table>

AT medium was supplemented with glucose (1 g/liter), (NH₄)₂SO₄ (2 g/liter), or neither when opine extract served as nitrogen, carbon, or carbon and nitrogen source. Per 100 ml of medium, 45 mg of extract from 15 g of tumor was added as carbon and nitrogen source; half that amount was added as carbon or nitrogen source.

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**Table 3. Biological assay 2: Opine activity in cationic fraction after biological enrichment**

<table>
<thead>
<tr>
<th>Strain</th>
<th>OD₆₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>C58C1</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>A281</td>
<td>0.215</td>
</tr>
<tr>
<td>H100</td>
<td>0.01 (0.01)</td>
</tr>
<tr>
<td>R10</td>
<td>0.145 (0.39)</td>
</tr>
</tbody>
</table>

The cationic fraction was used as nitrogen source in AT medium supplemented with mannitol (1 g/liter). Per 100 ml of medium the opinic material from 7 g of tumor was added. The tabulated values are OD₆₅₀ readings of maximally grown cultures. The values in parentheses indicate the results of a second experiment performed on a separate lot of opine extract.
Plasmid isolated from the four transconjugants (542TC1–542TC4) was compared with that of the donor strain (Bo542) (Fig. 4 Upper). All five strains exhibited plasmids of two size classes, one of which is $2.5 \times 10^6$ daltons (two cryptic plasmids of Bo542) (23) and the other of which must be the Ti plasmid size class, $150 \times 10^6$ daltons (23). Contrary to evidence from contour length measurements cited previously (23), the large cryptic plasmid of Bo542 must have a size very close to that of pTi542, $150 \times 10^6$ daltons, for it is not visible as a separate band in Fig. 4 Upper.

The Sma I cleavage pattern of the plasmids from Bo542 was compared with that of the four transconjugants. Of the four plasmids in Bo542 (23), two were visible in the (identical) fingerprints of 542TC1 (Fig. 4 Lower), TC2, TC3, and TC4 (data not shown). In the transconjugant fingerprints the pattern of pTi Bo542 was present together with a bright seven-band pattern ascribable to one of the two 25-megadalton plasmids of the parent strain (24). [Small plasmids are recovered in better yield and thus yield a brighter pattern of fragments (24).]

The opine extract was also used to prepare selective agar medium that allowed us to isolate transformants that received the Ti plasmid of strain Bo542. On AT medium with the opine extract as sole carbon and nitrogen source we obtained four transformant colonies when strain A136 was transformed with purified plasmid from strain Bo542. Sma I digest patterns of the plasmid isolated from each of the transformants showed that they received only pTi Bo542 (data not shown) (24).

The recently discovered opine named agropine that was found in octopine tumor extracts shares with the opine extract isolated here the ability to support the growth of octopine strains of Agrobacterium (Table 3) (19). A sample of agropine, kindly provided by Firmin and Fenwick (19), was compared with our...
characteristics identical to those of authentic agropine. We have confirmed the identity of mass spectra of our opine and an agropine sample provided by Firmin and Fenwick.

**DISCUSSION**

The results presented here demonstrate that an opine is present in null-type crown gall tumors, formerly not known to contain any. This finding supports the view that opines may be an essential and central feature of crown gall tumors and Ti plasmid evolution. Opine synthesis by the tumor and catabolism by the bacterium harboring the corresponding Ti plasmid have apparently played an important role in the dissemination of Ti plasmids in nature. However the "unusual nopaline Ti plasmids" induce tumors that synthesize no known opine: thus they remain a challenge to the generality of the opine concept.

The approach used here can be used to detect and isolate other opinic substances. Indeed, we have not actually exploited the approach in its most general form, but have limited our analysis to the cationic fraction of the tumor extract. The general definition of an opine does not limit the concept to crown gall tumors. It is plausible that opines may play a role in other host/pathogen interactions. The approach described here could be used to test this concept.

The opine isolated from null-type crown gall tumors is presumably identical to agropine, an opine of unknown chemical structure recently isolated from octopine tumors (19). The data presented here show that this compound is a nutritional substrate for both null-type and octopine-type Ti-plasmid-bearing strains. This new opine does not, however, appear to be an efficient inducer of conjugation for the null-type Ti plasmid, for very few transconjugants were obtained compared to the numbers observed for octopine- and nopaline-induced conjugation of the corresponding Ti plasmids (21–23). The presence of a second smaller plasmid in the transconjugants together with pTi Bo542 remains unexplained; it is possible that the small plasmid mobilizes the large one, whose transfer was therefore not induced by agropine. Because of the method used, the four transconjugants obtained may be siblings that resulted from a single rare conjugal event.

We propose that the null-type tumors and plasmids now be designated "agropine type." It is of great interest that an opine detected in these tumors is the same as one of the octopine tumor opines. There is a high degree of DNA homology between the octopine Ti plasmid and pTi Bo542 (29, 30). It is thus tempting to speculate that the agropine Ti plasmid might be an evolutionary ancestor of the wide host-range octopine Ti plasmid, whose surprising DNA conservatism (24) might reflect its recent and successful emergence after acquisition of the octopine trait. Genetic mapping of the opine catabolism and production traits on these Ti plasmids should give further insight into this interesting evolutionary question.

**Note Added in Proof.** The structure of agropine has recently been reported (31).

We thank Dr. Firmin (John Innes Institute, Norwich, England) for a sample of agropine and for communication of unpublished data. We thank Dr. Das (Institut des Substances Naturelles, Centre National de la Recherche Scientifique, Gif/Yvette, France) for mass spectral data. This research was supported in part by Centre National de la Recherche Scientifique Grant 3583 and AI032750 to J.T. and by American Cancer Society Grant NP194 to M.-D.C.