Plant Physiol. (1982) 69, 37–40
0032-0889/82/69/0037/04/$00.50/0

Characteristics of Teratomas Regenerated *in Vitro* from Octopine-Type Crown Gall

Received for publication April 3, 1981 and in revised form August 13, 1981

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ABSTRACT

Crown galls induced by infection of tobacco plants with *Agrobacterium tumefaciens* strain C58-Cl(pTiB6S3) were excised and cultured *in vitro*. After about one year of culture on medium-lacking phytohormones, two noncloned lines spontaneously formed shoots. Leaf explants from shoots of tumor-line T5 were capable of growing on hormone-free medium, and the resulting mixture of organized and unorganized tissue synthesized octopine. Detached leaves from T5 shoots also synthesized octopine. These results establish that shoots from this octopine-type tumor contain transformed cells and are true crown-gall teratomas.

Crown gall is a plant tumor caused by the soil bacterium, *Agrobacterium tumefaciens* (4). The oncogenic trait in the bacterium is borne on the Ti[1] plasmid. During oncogenic transformation of plant cells, a piece of Ti DNA is transferred from the bacterium to the plant cell, wherein it becomes integrated into plant nuclear DNA (3, 18). The integrated piece of plasmid DNA is called T-DNA.

Because of the unique ability of this DNA sequence to integrate into plant nuclear DNA, the T-DNA may potentially be used as a gene vehicle. Bacterial transposons inserted into the T-DNA region of the Ti plasmid are maintained in tumors incited by agrobacteria carrying this modified Ti plasmid (8). However, the question of whether information borne on the transposon is functionally expressed in the tumor remains unanswered.

The ultimate goal of most genetic modification schemes is to regenerate whole plants from modified cells. Of the three classes of crown galls—octopine, nopaline and 'null type' or, possibly, agropine (7)—only certain of the nopaline class commonly have been observed to differentiate shoot-like structures called teratomas. Recently, however, cultured octopine tumors of tobacco (16) and *Arabidopsis thaliana* (1) were reported to have spontaneously regenerated teratomas. The classification of these shoot-like structures as crown-gall teratomas was based mainly on the criterion of ability of their leaves to produce octopine, a tumor trait specified by the T-DNA. Although opine synthesis is not an obligatory phenotype of crown-gall teratomas, its expression does indicate the presence of tumor cells in the tissue. Further, preliminary hybridization evidence for the presence of T-DNA in the leaf tissue of *Araliidopsis thaliana* was also cited (1). The third criterion that can be used to distinguish between crown-gall teratomas and normal shoots which have regenerated from normal cells is the ability of the teratoma tissue to grow on medium devoid of phytohormones. This criterion was not investigated in either of the instances cited above.

We report here the spontaneous formation of crown-gall teratomas from cultured octopine tobacco tumor based upon two criteria, octopine production by the teratomas and hormone-independent growth of teratoma leaf explants.

MATERIALS AND METHODS

Crown-Gall Induction. Tumors were induced on *Nicotiana tabacum* L. cv. Bright Yellow plants by pricking the stems several times with a hypodermic needle containing a suspension of *Agrobacterium tumefaciens* strain C58-Cl(pTiB6S3). The plants used were greenhouse-grown, in which case they were about 15 cm tall at time of inoculation, or the seedlings were cultured axenically, resulting in 5-cm-tall plants.

Axenic Culture of Tobacco. Seeds were placed on a screen and immersed successively in 70% ethanol (8 s), 1% NaOCl (8 min), and three beakers of sterile distilled H_2O (20 s each). The screen was suspended, and an additional 50 ml of sterile H_2O was poured over the seeds.

The surface-sterilized seeds were germinated and cultured *in vitro* on 0.25 strength MS salts (GIBCO) in 1% agar (27°C; 16-h photoperiod, cool-white fluorescent lights, 30 $\mu Em^{-1}s^{-1}$).

***In Vitro* Tumor Culture.** Large pieces (3 × 4 cm) of crown gall were excised from plants grown in the greenhouse and surface-sterilized by immersing in 70% ethanol for 1 min and in 5% NaOCl for 10 min. After three rinses in sterile H_2O, the outer tissue was removed and discarded. Slices (about 2 mm thick) of the remaining tissue or of crown gall excised from axenically cultured plants were placed on the callus medium of Uchimiya and Murashige (17) without hormones but supplemented with vancomycin (1 mg/ml). Tumor calli that subsequently showed bacterial growth were placed for several days in liquid medium containing either penicillin G (100 unit/ml) plus streptomycin (250 µg/ml) or gentamycin (500 µg/ml) alone, following which they were returned to the vancomycin agar medium for several weeks before transfer to medium without antibiotics. These noncloned tumor lines have been maintained, since their inception in 1978, on medium A: Linsmaier and Skoog agar medium (9) minus hormones and supplemented with 0.1 g/L NZ amine, type AS (Humko Sheffield, Memphis, TN), an enzymic hydrolysate of casein.

***In Vitro* Shoot Culture.** Shoots were induced from a normal callus line of tobacco cv. Bright Yellow in the manner described previously (13). Small shoots (1 cm) were removed and cultured on hormone-free, agar medium A. Stock cultures of both normal and teratoma shoots were maintained on medium A and subcultured at roughly 2-month intervals by transferring small shoots.

Bacteria Culture. *Agrobacterium tumefaciens* strain C58-Cl(pTiB6S3) was kindly provided by J. Schell. It consists of the nopaline strain C58, which has been converted to an octopine strain by elimination of its Ti plasmid and replacement with the

[1] Abbreviations: Ti, tumor-inducing; T-DNA, the segment of Ti DNA maintained in tumor cells; MS, Murashige and Skoog (11).

Ti plasmid from the octopine strain B6 (15) by conjugation. The bacterium was cultured in yeast-extract broth (5 g beef extract, 1 g yeast extract, 5 g peptone, and 5 g sucrose/L, 2 mM $MgSO_4$, pH 7.2). Stocks were maintained on the same medium solidified with agar (1.0%).

Hormone-Independent Growth Test. With the exception of the first experiment (in which 60 leaf explants were tested), 100 leaf sections (about 2 × 3mm) taken from uniform sized leaves of each test line were aseptically excised with a scalpel and placed on hormone-free medium (16 sections/20 ml of medium in a 100- × 15-mm Petri dish). The dishes were sealed with polyvinyl film and cultured at 27°C under a daily 16-h photoperiod (cool-white fluorescent, 12 $\mu Em^{-1}s^{-1}$). At 40 to 50 days, the resulting callus/shoot tissue was assayed for its ability to biosynthesize octopine, as described in the following section.

In one experiment, leaf explants were passaged on hormone-containing medium B: MS medium (11) containing 1.2 μM thiamine HCl, 1.37 mM L-glutamine, 0.75 μM 2,4-D, 0.5 μM kinetin, and 1% agar.

Octopine Assay. The method of Aerts *et al.* (1) was used. Briefly, about 50 mg (fresh weight) of tissue was cultured on hormone-free medium A containing 5 mM arginine (an octopine precursor) for 1 to 18 days. The tissue was then crushed in a 1.5-ml polypropylene test tube and centrifuged in an Eppendorf microcentrifuge (15,000 rpm) for 2 min. Five μl aliquots of the supernate were then analyzed by electrophoresis on paper (Whatman 3MM, 21 × 53 cm), using formic acid:acetic acid:water (5:15:80), v/v) as the buffer, at a constant current (25 mamp and about 1,500 v) for 1.25 h. The dried electropherogram was dipped in a freshly prepared mixture (1:1, v/v) of 0.02% phenanthrene-quinone in 95% ethanol and 10% NaOH (in 60% aqueous ethanol). After drying, the spots were visualized by UV illumination (366 nm) and photographed through a UV filter.

RESULTS

Shoot Formation. Two of the four octopine-type tumor lines established in culture in 1978 spontaneously regenerated shoots after 12 to 15 months of culture on medium lacking phytohormones. None of the lines were cloned from single cells. One of the shoot-forming lines (T5) originated from a tumor on a greenhouse-grown plant and the other (T10) from a tumor induced on a small tobacco seedling cultured axenically. The shoots that developed from these tumor calli were abnormal in morphology (Fig. 1), having narrow leaves and profuse axillary branching. Only the shoot line from T5 was maintained in culture. Roots have never formed from this shoot line.

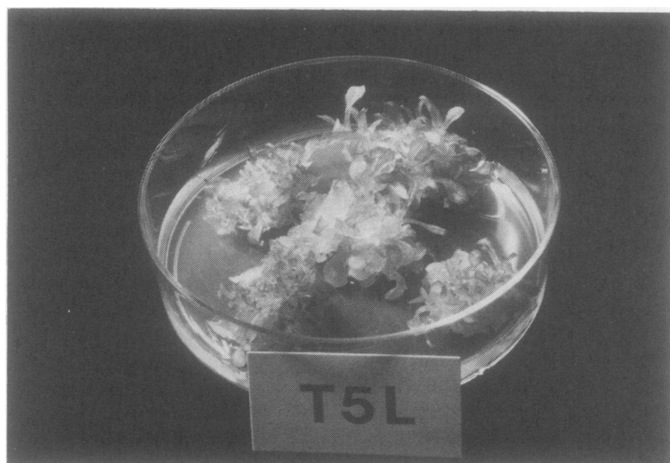

FIG. 1. Teratoma shoots derived from cultured octopine-type tumor callus.

Hormone-Independent Growth. Leaf sections from shoots of tumor line T5 were tested for their ability to grow on hormone-free medium. In the first experiment, performed when the shoot line had been subcultured for about 11 months, 60 leaf pieces were tested. Of these, 16 (26%) formed calli which, in addition, usually regenerated new shoots (Fig. 2). All of these calli produced elevated levels of octopine when cultured for several days on a medium containing the octopine precursor, arginine. The results of an octopine analysis are depicted in Figure 3. Lanes 1 to 5 and 6 to 10 represent analyses of tumor tissue cultured on medium without and with arginine, respectively, for various lengths of time. The octopine spots in lanes 7 through 10 are clearly evident in the print but were even more so in the original electropherogram, where they emitted a greenish-yellow fluorescence. This clearly distinguished them from nearby purple-fluorescing compounds. Only one of 60 control leaf pieces cultured on hormone-free medium produced a small callus/shoot growth. The callus died after one subculture, before it was large enough to assay for octopine production. Leaf pieces from its shoot failed to grow when placed on hormone-free medium.

In a second round of testing (100 explants of each line tested), 57% of the leaf sections from newly derived cycle 1 teratomas (Fig. 2) grew on hormone-free medium and produced octopine while only 22% of the explants from the original teratoma line did so (not indicated in Fig. 2). The cycle 1 teratomas were about 2 months old when tested and had not been subcultured but merely transferred to fresh medium as whole shoots with small amounts of adhering unorganized tissue. None of the leaf pieces from control shoots grew.

In a repetition of this experiment with cycle 2 teratoma leaf explants (Fig. 2), 79% (100 explants of each line tested) displayed hormone-independent growth and octopine production compared to 28% for the original teratoma line (not indicated in Fig. 2). The cycle 2 teratomas had been regenerated from leaf explants about 4 months before the experiment. Of the control leaf explants, 1% grew but did not produce octopine.

Leaf sections (100) of T5 shoots were also tested in a different way. They were first cultured for 4 weeks on hormone-containing medium B, following which a part of each primary callus was transferred to hormone-free medium A (Fig. 2). Of these, 95% (86 explants tested) continued to grow in the absence of hormone for the duration of the experiment (50 days) and produced octopine (58 randomly chosen calli assayed). None of the primary calli from 100 control leaf sections treated in the same manner continued to grow when placed on hormone-free medium A. In a second kind of control, all 100 calli from leaf sections of a habituated callus/shoot line of tobacco continued to grow upon transfer to hormone-free medium A; however, they did not produce octopine.

Octopine Synthesis by Excised Teratoma Leaves. Several teratomas were cultured in pint jars to permit the leaves to grow larger. Subsequently, whole leaves (about 1.5 cm long) were excised and the cut ends inserted into arginine-containing agar medium. After 3 days (before visible callus growth occurred), the leaves were removed and assayed for octopine. All contained octopine (Fig. 4, lane 4). Detached leaves from habituated or normal shoots treated in the same manner did not produce octopine (Fig. 4, lanes 7 and 10, respectively).

DISCUSSION

Octopine-type crown galls have rarely been observed to form shoots in nature. In two reports of their occurrence, on decapitated stems of *Datura metaloides* (10) and on certain strains of *Bryophyllum* (2), infected in both instances with *Agrobacterium tumefaciens* strain B6, the shoots were not proved to be teratomas, *i.e.* to contain cells which express tumor traits or maintain T-DNA. The possibility that the shoots had regenerated from normal plant cells residing in the gall was not ruled out. In fact, in studies where

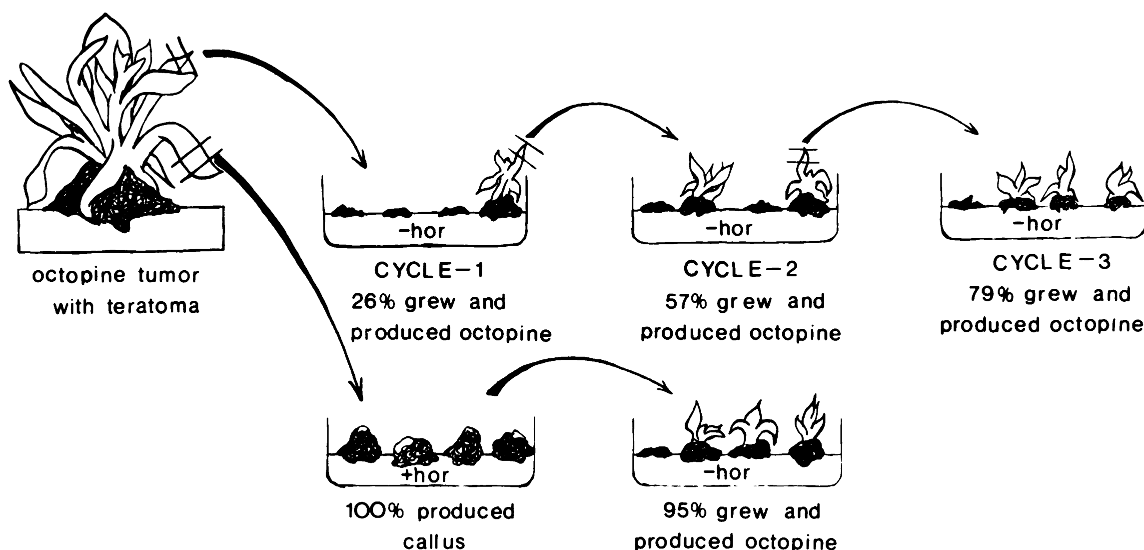

FIG. 2. Diagrammatic summary of successive tests of teratoma leaf explants for their ability to grow on hormone-free medium and, subsequently, to produce octopine when transferred to an arginine-containing medium.

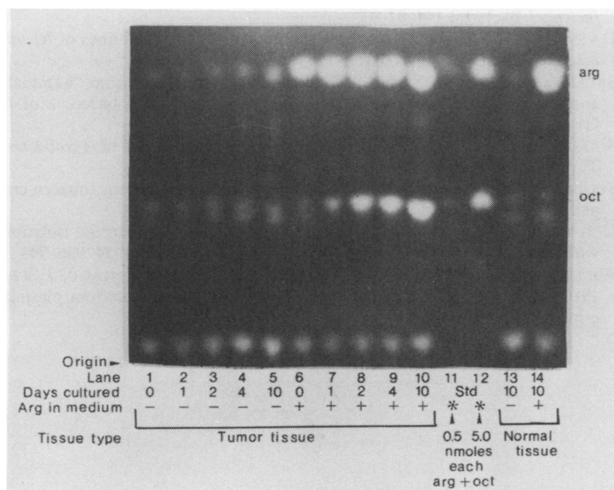

FIG. 3. Fluorograph of a paper electrophoretogram showing the production of octopine by crown-gall tumor tissue cultured on medium without (lanes 1–5) or with (lanes 6–10) 5 mM arginine as a function of time. That the faintly fluorescing spots from extracts of normal tissue (lanes 13 and 14) coincident in position with octopine are not octopine was indicated by their blue rather than greenish-yellow fluorescence characteristic of guanidino reaction products and by the failure of the spot to increase in intensity in extracts from tissue cultured on arginine (lane 14 compared with lane 13).

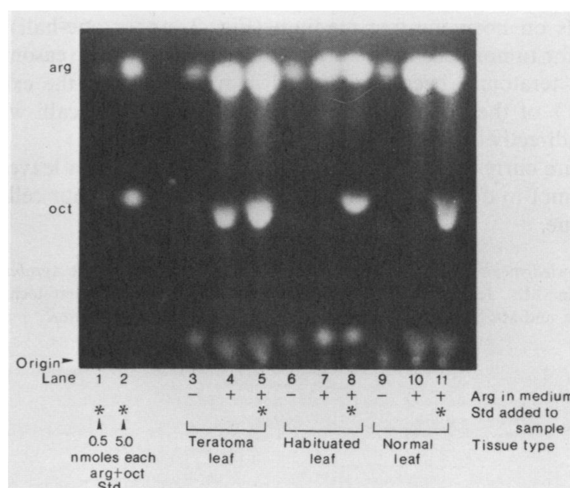

FIG. 4. Fluorograph of an electrophoretic analysis of excised teratoma (lanes 3–5), habituated (lanes 6–8) and normal (lanes 9–11) leaves incubated for 3 days with their basal ends inserted into agar medium with or without arginine.

hormones were used to induce shoots on cultured octopine tumors (5, 14), the resulting shoots apparently were not teratomas. They appeared normal, developed roots, and failed to produce octopine (5). In addition, explants of these shoots failed to grow on hormone-free medium (5, 14). We have obtained similar results (L. D. Owens, unpublished) with hormone-induced shoots from cultured, noncloned tumors. Unlike shoots induced by hormone treatment, the spontaneously derived shoots described here expressed traits of authentic crown-gall teratomas. Detached leaves synthesized octopine (Fig. 4), and small explants of leaves were capable of growing on medium lacking phytohormones. This teratoma line has been stably maintained by shoot subculture for 2 years. The line apparently is similar to the teratoma line of Scott (16), which also spontaneously regenerated from a noncloned, cultured tumor originally incited by a B6 Ti plasmid. However, their sources differ somewhat. The tumor line of Scott (16) originated from a crown gall induced on a tobacco plantlet (*N. tabacum* cv. White Burley) cultured *in vitro*, while our tumor line T5 originated from a presumably larger plant (about 15-cm tall at time of infection), cv. Bright Yellow, cultured in the greenhouse.

The formation of teratomas by tumor line T5 appears to be an infrequent event. On only two other occasions have shoots subsequently regenerated from this line. In both instances, they proved to be normal shoots. The original teratomas may have arisen from an unusual tumor cell, one in which the T-DNA had mutated or been partially lost or repressed. We are currently analyzing DNA from the teratomas to discern whether either of these explanations is plausible. There is growing evidence that T-DNA alterations can, in some way, affect tumor morphology. For example, insertions of transposons into T-DNA of octopine-type plasmids subsequently caused the development of crown galls with excessive abnormal roots (6, 12) or shoots (6). Whether these aberrant morphological structures actually arose from cells transformed with altered T-DNA seems unlikely, however, since investigations of the shoots failed to reveal any tumor traits. Appar-

ently, they regenerated from nontransformed cells in the tumor tissue under the influence of nearby tumor cells.

The propensity of teratoma leaf explants to exhibit increasing vitality in growth and new teratoma formation with each cycle of subculture on hormone-free medium (Fig. 2) may be evidence that these organized tissues are chimeras, *i.e.* mixtures of tumor and normal cells. Normal cells could have been propagated along with tumor cells from the initial crown-gall explant or, alternatively, could have originated at a later stage of culture through loss or repression of T-DNA. Possibly, the proportion of tumor cells in leaf tissue of the original teratoma line is very low. We do know, from the experiment depicted in the lower one-half of Figure 2, that at least 95% of the small explants (2 × 3 mm) indeed contained at least one tumor cell. After suitable multiplication on a medium containing hormones, tumor calli were obtained which were capable of growth on hormone-free medium.

In contrast to these results, the low viability of original teratoma explants placed directly on hormone-free medium (26%; Fig. 2) may simply mean that there were too few tumor cells in most explants to adequately condition the surrounding medium. The requirement for a minimum density of viable cells, per unit volume of medium, to permit cell division is a well-documented phenomenon in plant-cell culture. Possibly, the successive culturing of leaf explants on hormone-free medium (Fig. 2, upper one-half) selected for tumor cells in the tissue outgrowths. By this reasoning, cycle 2 teratomas became enriched in tumor cells to the extent that 79% of their leaf explants could produce tumor calli when placed directly on hormone-free medium.

We are currently culturing protoplasts from teratoma leaves in an attempt to determine directly the proportion of tumor cells in the tissue.

Acknowledgments—The author thanks Dr. J. Schell for supplying the *Agrobacterium* strain, Mrs. Jean Bellows and Ms. Bonnie Mattingly for expert technical assistance, and Ms. Sandra Sutherland for the creative artwork in Figure 2.

LITERATURE CITED

1. Aerts M, M Jacobs, J-P Hernalsteens, M van Montagu 1979 Induction and *in vitro* culture of *Arabidopsis thaliana* crown gall tumors. Plant Sci Lett 17: 43–50
2. Bopp M, F Resende 1966 Crown-Gall-Tumoren bei verschiedenen Arten und Bastarden der *Kalanchoideae*. Port Acta Biol Ser A 9: 327–366
3. Chilton M-D, RK Saike, N Yadav, MP Gordon, F Quetier 1980 T-DNA from agrobacterium Ti plasmid is in the nuclear DNA fraction of crown gall tumor cells. Proc Natl Acad Sci USA 77: 4060–4064
4. Drummond M 1979 Crown gall disease. Nature (Lond) 281: 343–347
5. Einset JW, A Cheng 1979 Regeneration of tobacco plants from crown gall tumors. In Vitro 15: 703–708
6. Garfinkel DJ, EW Nester 1980 *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J Bacteriol 144: 732–743
7. Guyon P, M-D Chilton, A Petit, J Tempe 1980 Agropine in "null-type" crown gall tumors: Evidence for generality of the opine concept. Proc Natl Acad Sci USA 77: 2693–2697
8. Hernalsteens J-P, F Van Vliet, M de Buckeleer, A Depicker, G Engler, M Lemmers, M Holsters, M van Montagu, J Schell 1980 The *Agrobacterium tumefaciens* Ti plasmid as a host vector system for introducing foreign DNA in plant cells. Nature (Lond) 287: 654–656
9. Linsmaier EM, F Skoog 1965 Organic growth factor requirements of tobacco tissue cultures. Physiol Plant 18: 100–127
10. Maier JT, PK Chen 1971 Growth hormones in relation to crown gall development in two *Datura* species. Phyton 28: 95–108
11. Murashige T, F Skoog 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473–497
12. Ooms G, PK Klapwijk, JA Poulis, RA Schilperoort 1980 Characterization of Tn 904 insertions in octopine Ti plasmid mutants of *Agrobacterium tumefaciens.* J Bacteriol 144: 82–91
13. Owens LD 1981 Characterization of kanamycin-resistant cell lines of *Nicotiana tabacum.* Plant Physiol 67: 1166–1168
14. Sacristan MD, G Melchers 1977 Regeneration of plants from "habituated" and "agrobacterium-transformed" single-cell clones of tobacco. Mol Gen Genet 152: 111–117
15. Sciakey D, AL Montoya, M-D Chilton 1978 Fingerprints of *Agrobacterium* Ti plasmids. Plasmid 1: 238–253
16. Scott IM 1979 Opine content of unorganized and teratomatous tobacco crown gall tissues. Plant Sci Lett 16: 239–248
17. Uchimiya H, T Murashige 1974 Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant Physiol 54: 936–944
18. Willmitzer L, M de Buckeleer, M Lemmers, M van Montagu, J Schell 1980 DNA from Ti plasmid present in nucleus and absent from plastids of crown gall plant cells. Nature (Lond) 287: 359–361