Hormonal Control of Tobacco Crown Gall Tumor Morphology

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ABSTRACT

The endogenous levels of auxin and cytokinin in teratoma and unorganized tobacco (Nicotiana tabacum L. var Wisconsin #38) crown gall tumor tissues were determined. Teratoma tissues contain levels of auxin and cytokinin favorable to shoot formation, whereas unorganized tumors contain levels of auxin that suppress shoot formation. This conclusion is based upon the observation that when levels of auxin and cytokinin similar to those found in a teratoma were added to the growth medium of nontumorous tobacco tissue, shoot formation resulted; when levels similar to those found in unorganized tumors were added, the normal tissue grew as unorganized callus.

Crown gall tumors can be induced on many species of plants by inoculation of a wound with a virulent strain of Agrobacterium tumefaciens (20). The ability of the bacteria to induce tumors depends on a class of large plasmids, the Ti plasmids (25, 27, 30). Tumor induction results from a transfer of a portion of the Ti plasmid, the T-DNA, from the bacterium to the nucleus of the plant cell where it is stably maintained and transcribed (4–6, 28). In addition to its oncogenic properties, the T-DNA directs crown gall tumor cells to synthesize unusual metabolites called opines that can serve as nutrients for the bacteria (11, 24, 26).

One manifestation of the transformed state is the capacity of crown gall tumor cells to grow in culture on basal medium without added auxin and cytokinin (3); normal plant tissues generally require exogenously added auxin and cytokinin for growth in vitro. The phytohormone autonomy of crown gall tumor cells is apparently a result of the production of auxin and cytokinin by these cells (3, 7, 13, 15, 17, 18, 23).

Crown gall tumors of tobacco and certain other species of plants can be organized (teratoma) or unorganized (2, 21). Tumor morphology, and also which opines are produced by the tumor tissue, is controlled by the strain of bacteria (i.e., the type of Ti plasmid) used to induce the tumor (1, 2, 9, 10, 16).

The different morphologies observed in tobacco crown gall tumors correspond to patterns of growth that can be observed in normal tissues of tobacco by manipulation of the levels of auxin and cytokinin in the growth medium (19). The results presented here show that in a similar manner the levels of endogenously produced auxin and cytokinin appear to be controlling the morphology of tobacco crown gall tumors.

MATERIALS AND METHODS

Establishment of Tumor Lines. In all studies reported herein, crown gall tumors were induced on plants of Nicotiana tabacum L. var. Wisconsin #38 of clonal origin. A leaf fragment from one plant was grown in vitro on basal medium (14) supplemented with 2 mg/l IAA and 1 mg/l BA to promote shoot formation. Shoots from this tissue were rooted in sterile culture on basal medium and leaves inoculated with various strains of A. tumefaciens. After 3 to 4 weeks, the resulting tumors were excised from the plants and grown on basal medium supplemented with 100 mg/l each of carbenicillin, streptomycin, and vancomycin. The tissues were free of bacteria after 2 to 3 weeks on this medium and were subsequently maintained on basal medium without antibiotics. Tumor lines are designated by W38 (for the variety of tobacco) followed by the symbol for the inducing strain of bacterium. Nontumorous tissue of the same origin was maintained on basal medium supplemented with 2 mg/l IAA and 1 mg/l BA and was used for the comparative growth studies.

Examination of Cytokinins. A minimum of 5 g of 3- to 4-week-old tissue was ground in 3 volumes of 95% ethanol (w/v) in a mortar at −15°C and allowed to stand at least 4 h at −15°C with occasional mixing. The homogenate was centrifuged for 15 min at 18,000g and the supernatant reduced in an air stream to an aqueous phase. The aqueous extract was lyophilized and redissolved in a small amount of 70% ethanol and streaked on Whatman 3MM paper for ascending paper chromatography. For the determination of cytokinin levels in tumor tissues grown on 5 mM kinetin, the crude aqueous extract was boiled 2 min in the presence of Dowex 50 (H+) to convert cytokinin metabolites to the free base. The cytokinins were eluted from the resin with 5 N NH4OH and the ammonia removed from the eluate by evaporation under reduced pressure. The extract was brought to a volume of 1 ml/g tissue extracted with 3 equal volumes of ethyl acetate and the aqueous layer (containing the natural cytokinins) assayed directly for the remaining cytokinin activity. Cytokinin contents were determined by bioassay with soybean callus tissue (12).

Measurement of IAA. Ten g 3-week-old tissue was ground in a mortar with 5.8 to 6.2 ml 0.25 N formic acid (to which 4 mg/ml sodium dithionite and 2 mg/ml ascobic acid had just been added) and 10 ml ethyl acetate (that had been washed with 10 mM sodium carbonate). The pH after grinding was 2.8 to 3.0. All operations were performed at 0 to 4°C except where indicated. The homogenate was transferred to a screw-top centrifuge tube and centrifuged for 5 min at 200g. Five ml of the ethyl acetate phase was added to another centrifuge tube, mixed with 2.0 ml 0.5 M sodium carbonate (pH 9.0) to which 4 mg/ml sodium dithionite had just been added and centrifuged for 2 min at 120g. The ethyl acetate layer was discarded and the aqueous phase adjusted to pH 3.0 with 0.40 to 0.46 ml 2.8 M phosphoric acid. One ml ethyl acetate was added and the tube vortexed at low speed for 3 s and centrifuged for 2 min at 120g. The ethyl acetate phase was removed (0.75 ml) and dried under reduced pressure at 35°C. IAA in the residue was converted to indole-3-pyruvine according to the method of Stoeussl and Venis (22). The tube was transferred to an

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4 Abbreviation: Ti, tumor-inducing.
ice bath and 0.2 ml of a mixture of equal parts of acetic anhydride and trifluoroacetic acid was added. After 15 min, the reaction was stopped by the addition of 3.0 ml water:ethanol:acetic acid (5:4:1, v/v/v) (prevents turbidity) and the fluorescence of the sample immediately measured on an Aminco Bowman spectrofluorimeter. Excitation was at 440 nm and emission at 490 nm. A standard curve was constructed for each trial by linear regression analysis and the amount of IAA in each unknown determined from the curve. This amount was adjusted for the aliquots used and for percent recovery. To determine the recovery, a known amount of IAA was added to some of the samples in each trial before grinding and the increase in yield of IAA compared to the original amount of IAA added. After 2 h in strong light or UV light there was no fluorescence remaining in any of the samples indicating that the fluorescence was due to indolo-α-pyrene, which is unstable under these conditions (8).

Detection of Octopine and Nopaline. Tumor tissue grown on basal medium was used for the detection of nopaline. Tissues used for the detection of octopine were grown for 4 days in liquid medium containing the mineral salts and vitamins of Miller (14) and 5 g/l sucrose, 15 g/l glucose, and 50 mm arginine. Tissues were ground in 2 volumes of 95% ethanol and centrifuged for 5 min at 18,000g. The supernatant was dried to an aqueous phase under reduced pressure and the aqueous phase extracted with 1 volume of ethyl acetate. The ethyl acetate phase was discarded and a 5 to 10 µl aliquot of the aqueous phase was spotted on a sheet of Whatman No. 3MM paper and electrophoresed for 1 h in 12 mm sodium tetraborate buffer (pH 9.5) at 16 v/cm. The paper was dried and stained for guanidines by the phenantthrenequinone method (29). Octopine and nopaline were run as standards both alone and mixed with extracts of nontumorous tissue.

Chemicals. IAA and zeatin were purchased from Calbiochem. Octopine was purchased from Sigma. Nopaline was the generous gift of M.-D. Chilton.

RESULTS AND DISCUSSION

Tumor Lines. During the first few passages of the tumor tissues in vitro, a few normal-appearing shoots were formed from each line. Unlike tumorous shoots, these shoots readily rooted and grew into normal-appearing, fertile plants. Since tissues from these plants did not produce octopine or nopaline and required auxin and cytokinin for growth in vitro, it is possible that the tumor tissues were originally a mixture of normal and tumorous cells and that subculture on basal medium selects for transformed cells.

With repeated subculture, the capacity of the tumors to form normal shoots gradually disappeared and tumor tissues used in this study were from stable lines that had been in culture for at least 1 year.

A total of nine tumor lines induced by six different strains of A. tumefaciens were examined (Fig. 1). Lines W38T37, W38C58, and W38EU6 are shoot-forming tissues (teratomas). When tumors induced by A. tumefaciens strains A6 and B6 were initially established in culture, the tissues developed sectors with different morphologies. Upon further subculture, these sectors retained their particular characteristics. Lines W38A6-1 and W38B6-1 are composed of densely packed cells that grow as a greenish-white half sphere of tissue (compact unorganized). Lines W38A6-2, W38B6-2, and W38542 are composed of large cells that grow as a yellowish, friable, completely unorganized callus (friable unorganized). Line W38A6-3 occasionally forms shoots, although most of this tissue is in a compact unorganized form resembling line W38A6-1. Isolated shoots of line W38A6-3 revert to the predominantly compact unorganized morphology. All of the A6-induced tumor lines produce octopine; however, the B6 induced tumor lines do not produce detectable levels of octopine. Lines W38T37 and W38C58 produce nopaline. Lines W38EU6 and W38542, as expected (11), produce neither octopine nor nopaline.

Cytokinins in the Tumor Lines. Chromatography and subsequent bioassay of concentrated ethanolic extracts of all the tumor lines showed the presence of at least two distinct forms of cytokinin, as illustrated for W38A6-1 in Figure 2. More than 60% of the cytokinin activity in every line was associated with a slow migrating, polar form that chromatographed to the position expected for zeatin ribonucleotide when water saturated 2-butanol (Fig. 2), water saturated 1-butanol, or 1-butanol:acetic acid:water (12:3:5, v/v/v) was used as the developing solvent. When incubated with calf intestinal alkaline phosphatase, the polar cytokinin was converted to a form that co-chromatographed with ribosylezatin (Fig. 3). The polar cytokinin therefore most likely is zeatin ribonucleotide. Virtually all of the remaining cytokinin co-chromatographed with ribosylezatin in the solvent systems mentioned above and with 0.03 M sodium borate (pH 8.4) which provides good resolution of zeatin and ribosylezatin (Fig. 4). The levels of cytokinins in these tumor tissues (Table I) are similar to those found in other tobacco crown gall tumor tissues (7, 15, 18), but in contrast to other reports the predominant cytokinin in these lines appears to be zeatin ribonucleotide. However, the significance of the various forms of cytokinin (i.e. which may be the active form(s)) is unknown. The bioassay method of measurement employed in this study detects cytokinins active in promoting cell division, but the conversion of one form of cytokinin to a more active form by the tissue during the course of bioassay cannot be ruled out. Cytokinin levels are, therefore, expressed collectively as zeatin equivalents in Table I.

IAA in the Tumor Lines. The endogenous level of IAA in the tumor lines was also determined (Table I). The level of IAA in
these tissues appeared to be age-dependent. The highest levels were found in tissues that were still rapidly growing, but about to fill the culture flask. This stage was reached 3 weeks after the start of subculture, and IAA levels reported here are from tissues at this stage of growth. The levels of IAA are lower than those previously reported for two other tobacco crown gall tumor lines (15, 17). This may reflect differences in the tumor lines, inasmuch as the tumors were isolated from different varieties of tobacco, or perhaps it is due to the use of different methods of IAA measurement. The lack of a labeled internal standard in the method employed in this study makes an absolute quantitation of IAA levels dubious. However, it is the relative levels of auxin and cytokinin among the various tumor lines that are emphasized here.

The Relationship of Auxin and Cytokinin Levels to Tumor Morphology. Each of the tumor types had a level of auxin or auxin and cytokinin that was characteristic of a particular tumor morphology (Table I). All of the friable unorganized lines (W38A6-2, W38B6-2, W38542) had relatively high levels of IAA. The cytokinin contents of these lines varied. All of the nopaline-type teratomas (W38T37, W38C58, W38EU6) had relatively low levels of IAA and intermediate levels of cytokinin. The compact unorganized tumor lines (W38A6-1, W38A6-3, W38B6-1) had high levels of cytokinin and levels of IAA that were much lower than in the friable unorganized lines but 2- to 3-fold higher than in the nopaline-type teratomas. Additionally, we have examined tissues derived from single cell clones of W38T37 and W38B6-2; these had phytohormone levels similar to those of the parent lines.

When cultured on basal medium supplemented with a high level (5 μM) of the auxin 2,4-D, all of the tissues grew as friable unorganized callus resembling the tumor lines that contain a high endogenous level of IAA (Fig. 5). Also, when teratoma tissues were cultured on intermediate levels of auxin (1 μM 2,4-D), they grew as a compact unorganized callus. Thus, increasing the auxin concentration in the medium of tumor tissue having a low (teratoma) or intermediate (compact, unorganized) endogenous level of auxin will cause that tissue to grow with the same morphology as a tumor type having a higher endogenous level of auxin.

Each of the tumor lines was cultured on medium containing 5 μM 2,4-D to eliminate morphological differences among the tissues (Fig. 5), and auxin and cytokinin levels measured. Within the limits of variation (see legend to Table I), tumor tissues grown on 5 μM 2,4-D contained the same endogenous concentrations of IAA and cytokinin as tissues grown on basal medium. Therefore, the production and levels of phytohormones in these tumor lines are not a consequence of tumor morphology. It is interesting that the presence of shoots in teratoma tissue is not necessary for phytohormone production. This type of study also shows that the production of auxin by these tumor cells is not detectably repressed by the presence of a high concentration of auxin in the growth medium. Additionally, the presence of 5 μM kinetin in the growth medium did not influence the levels of naturally produced cytokinin in the tumor tissues.

Comparative Studies with Normal Tissue. When levels of auxin and cytokinin similar to those found in a particular tumor type are added to the growth medium of normal, phytohormone-requiring tobacco tissue, the normal tissue grows with the same
CONCLUSIONS

These results confirm and extend previous work showing that the phytohormone autonomy of crown gall tumor cells results from the production of auxin and cytokinin by these cells. Furthermore, we have demonstrated that the endogenous levels of auxin and cytokinin in several tobacco crown gall tumor lines can vary considerably, and this variation is likely to be responsible for the differences in tumor morphology. Teratomas contain endogenous levels of auxin and cytokinin favorable for shoot formation, whereas unorganized tumors contain levels of auxin that suppress shoot formation. The mechanism by which the T-DNA is causing the production and controlling the levels of phytohormones in tumor cells is unknown.

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