Plant Physiol. (1982) 69, 389–392
0032-0889/82/69/0389/04/$00.50/0

Hormonal Control of Tobacco Crown Gall Tumor Morphology[1]

Received for publication August 4, 1981 and in revised form October 6, 1981

RICHARD M. AMASINO[2] AND CARLOS O. MILLER[3]
Department of Biology, Indiana University, Bloomington, Indiana 47405

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 for 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[4] 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.

[1] Supported by Grants PCM80-03749 and PCM76-12306 from the National Science Foundation and represents part of the doctoral thesis of R. M. A.

[2] Present address: Department of Microbiology and Immunology, University of Washington, Seattle, WA 98195.

[3] To whom reprint requests should be sent.

[4] Abbreviation: Ti, tumor-inducing.

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 18,000*g* 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 Whatmann 3MM paper for ascending paper chromatography. For the determination of cytokinin levels in tumor tissues grown on 5 μM 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 NH_4OH 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 ascorbic 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 200*g*. 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 120*g*. 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 120*g*. The ethyl acetate phase was removed (0.75 ml) and dried under reduced pressure at 35°C. IAA in the residue was converted to indolo-α-pyrone according to the method of Stoessl and Venis (22). The tube was transferred to an

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 per cent 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-pyrone, 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 Whatmann 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 phenanthrenequinone 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.

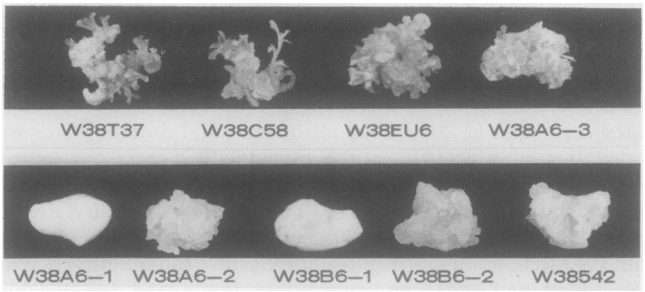

FIG. 1. Tumor lines. Tumors were induced on plants of *N. tabacum* L., var. Wisconsin #38, and established in sterile culture as described in the text. The lines are labeled according to which strain of *A. tumefaciens* was used for tumor induction.

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 ribosylzeatin (Fig. 3). The polar cytokinin therefore most likely is zeatin ribonucleotide. Virtually all of the remaining cytokinin co-chromatographed with ribosylzeatin in the solvent systems mentioned above and with 0.03 M sodium borate (pH 8.4) which provides good resolution of zeatin and ribosylzeatin (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

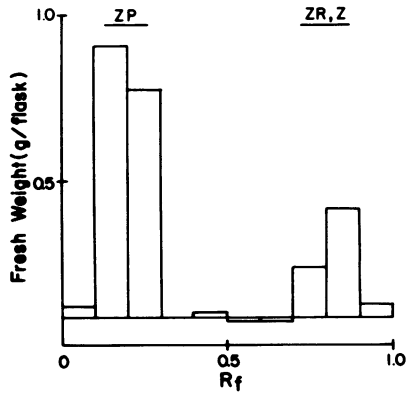

FIG. 2. Chromatography and bioassay of a concentrated ethanolic extract of W38A6-1. Developing solvent was water saturated 2-butanol. An equivalent of 1.5 g tissue/flask containing 50 ml assay medium was used. Z, zeatin; ZR, ribosylzeatin; and ZP, zeatin ribonucleotide.

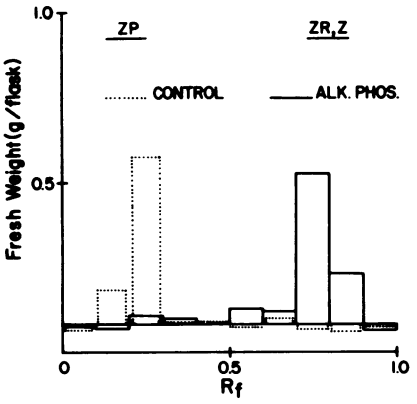

FIG. 3. Enzyme treatment, chromatography, and bioassay of the cytokinin eluted from R_F 0.1 to 0.3 of the chromatogram in Figure 2. The incubation mixture consisted of 1 mg/ml calf intestinal alkaline phosphatase, 50 mM Tris (pH 9.0), and 5 mM $MgCl_2$. Incubation was for 1 h at 32°C. The control sample received the same treatment minus enzyme. Developing solvent was water saturated 2-butanol. An equivalent of 2.5 g tissue/flask containing 50 ml assay medium was used.

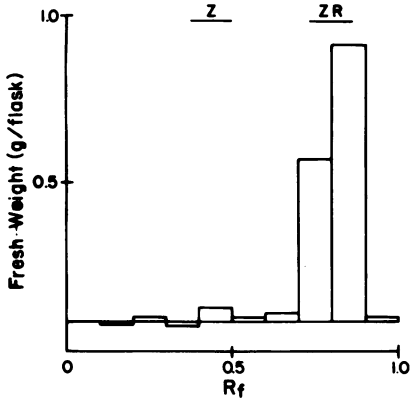

FIG. 4. Chromatography and bioassay of an 1-butanol extract of the aqueous phase remaining after reducing a crude ethanolic extract of W38A6-1. The aqueous phase was extracted three times with an equal volume of 1-butanol. Developing solvent was 0.3 M borate (pH 8.4). An equivalent of 3 g tissue/flask containing 50 ml assay medium was used.

Table I. *Auxin and Cytokinin Levels in the Tumor Lines*

Phytohormone levels are based on averages of the values from at least three separate extractions with different batches of tissue. Levels varied by as much as 30% among replicates. Cytokinin levels are expressed as a summation of the various forms in the tissue.

Tumor Line	Cytokinin Content	IAA Content
	nmol zeatin eq/kg fresh wt	*nmol IAA/kg fresh wt*
Friable unorganized		
W38A6-2	230	290
W38B6-2	23	350
W38542	14	180
Teratoma		
W38T37	24	8–10
W38C58	14	8–10
W38EU6	27	8–10
Compact unorganized		
W38A6-1	200	34
W38B6-1	150	29
W38A6-3	170	28

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

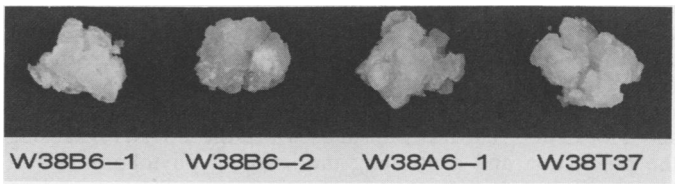

FIG. 5. Representative tumor lines grown on basal medium containing 5 μM 2,4-D.

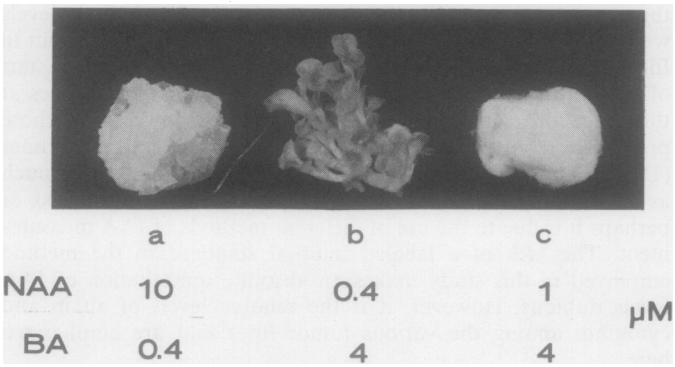

FIG. 6. Normal, phytohormone-requiring tobacco tissue grown on basal medium supplemented with various amounts of α-naphthaleneacetic acid (NAA) and BA.

morphology as the corresponding type of tumor (Fig. 6). With a high level of auxin in the growth medium, tobacco tissue grows as a friable unorganized callus very similar to tumor lines W38A6-2, W38B6-2, and W38542 (Fig. 6a). This type of morphology is observed over a wide range of cytokinin concentrations; in fact, if the tissue is grown on medium containing 5 μM 2,4-D, a friable unorganized callus results regardless of the concentration of cytokinin. Lowering the auxin and increasing the cytokinin concentration results in shoot formation and the tissue resembles a teratoma (Fig. 6b). If the level of auxin is increased to 3-fold that in the shoot promoting medium, shoot formation is suppressed and a compact unorganized callus similar to lines W38A6-1 and W38B6-1 is formed (Fig. 6c). With this level of auxin, the compact unorganized phenotype is observed over a wide range of cytokinin concentrations. Line W38A6-3, which occasionally forms small shoots, has phytohormone levels similar to those of the compact unorganized lines. The cause of sporadic shoot formation in this line is unknown.

The levels of auxin and cytokinin added to the growth medium of the normal tissue do not correspond exactly to the levels of endogenous phytohormones in the tumor lines. However, the levels of auxin and cytokinin in the growth medium needed to achieve a particular intracellular concentration are not known. In addition, synthetic auxin and cytokinin, which differ in effectiveness from the naturally occurring compounds, were used because of their greater stability and possibly greater resistance to destructive metabolism in the plant cell. This hopefully provided a more constant level of these phytohormones over the long period of time required for this type of study. Therefore, only a correlation between the relative levels of phytohormones in the growth medium of normal tissue required to achieve a particular morphology and the endogenous levels of phytohormones in tumor tissues of similar morphology can be demonstrated.

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.

Acknowledgments—We thank J. A. Lippincott, Northwestern University, Evanston, IL, for providing strains A6, B6, C58, and T37, and E. W. Nester, University of Washington, Seattle, WA, for providing strains EU6 and 542 of *A. tumefaciens*.

LITERATURE CITED

1. BOMHOFF, G, PM KLAPWIJK, HCM KESTER, RA SCHILPEROORT, JP HERNALSTEENS, J SCHEEL 1976 Octopine and nopaline synthesis and breakdown genetically controlled by a plasmid of *Agrobacterium tumefaciens*. Mol Gen Genet 145: 177–181
2. BRAUN AC 1953 Bacterial and host factors concerned in determining tumor morphology in crown gall. Bot Gaz 114: 363–371
3. BRAUN AC 1958 A physiological basis for the autonomous growth of the crown-gall tumor cell. Proc Natl Acad Sci USA 44: 344–349
4. CHILTON M-D, MJ DRUMMOND, DJ MERLO, D SCIAKY, AL MONTOYA, MP GORDON, EW NESTER 1977 Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11: 263–271
5. CHILTON M-D, R SAIKI, 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
6. DRUMMOND MH, MP GORDON, EW NESTER, M-D CHILTON 1977 Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumors. Nature 269: 535–536
7. EINSET JW 1980 Cytokinins in tobacco crown gall tumors. Biochem Biophys Res Commun 93:510–515
8. ELIASSON L, L-H STROMQUIST, E TILLBERG 1976 Reliability of the indolo-α-pyrone fluorescence method for indole-3-acetic acid determination in crude plant extracts. Physiol Plant 36: 16–19
9. GARFINKEL DJ, EW NESTER 1980 *Agrobacterium tumefaciens* mutants affected in crown gall tumorigenesis and octopine catabolism. J Bacteriol 144: 732–743
10. GRESSHOFF PM, ML SKOTNICKI, BG ROLFE 1979 Crown gall teratoma formation is plasmid and plant controlled. J Bacteriol 137: 1020–1021
11. GYON 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
12. MILLER CO 1963 Kinetin and kinetin-like compounds. *In* HF Linskens, MV Tracey, eds, Modern Methods of Plant Analysis, Vol VI. Springer-Verlag, Berlin, pp 194–202
13. MILLER CO 1974 *Ribosyl-trans-zeatin*, a major cytokinin produced by crown gall tumor tissue. Proc Natl Acad Sci USA 71: 334–338
14. MILLER CO 1978 Cytokinin modification of metabolism of *p*-coumaric acid by a cell suspension of soybean. Planta 140: 193–199
15. NAKAJIMA H, T YOKOTA, T MATSUMOTO, M NOGUCHI, N TAKAHASHI 1979 Relationship between hormone content and autonomy in various autonomous tobacco cells cultured in suspension. Plant Cell Physiol 29: 1489–1499
16. OOMS G, PM KLAPWIJK, JA POULIS, RA SCHILPEROORT 1980 Characterization of Tn904 insertions in octopine Ti-plasmid mutants of *Agrobacterium tumefaciens*. J Bacteriol 144: 82–91
17. PENGELLY W, F MEINS 1977 A specific radioimmunoassay for nanogram quantities of the auxin, indole-3-acetic acid. Planta 136: 173–180
18. SCOTT IM, G BROWNING, J EAGLES 1980 Ribosylzeatin and zeatin in tobacco crown gall tumor tissue. Planta 147: 269–273
19. SKOOG F, CO MILLER 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp Soc Exp Biol XI, pp 118–131
20. SMITH EF, CO TOWNSEND 1907 A plant tumor of bacterial origin. Science 25: 671–673
21. SMITH EF 1917 Embryomas in plants produced by bacterial inoculations. Bull Johns Hopkins Hosp 28: 277–294
22. STOESSEL A, MA VENIS 1970 Determination of submicrogram levels of indole-3-acetic acid: a new highly specific method. Anal Biochem 34: 344–351
23. TEGLEY JR, FH WHITHAM, M KRASNUCK 1971 Chromatographic analysis of a cytokinin from tissue cultures of crown gall. Plant Physiol 47: 581–585
24. TEMPE J, P GUYON, D TEPFER, A PETIT 1979 The role of opines in the ecology of the Ti-plasmids of *Agrobacterium*. *In* K Timmis, A Puhler, eds, Plasmids of Medical, Environmental, and Commercial Importance. Elsevier, Amsterdam, pp 353–363
25. VAN LAREBEKE N, G ENGLER, M HOLSTERS, S VAN DEN ELSACKER, I ZAENEN, RA SCHILPEROORT, J SCHELL 1974 Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing activity. Nature (Lond) 252: 169–170
26. VAN MONTAGU M, M HOLSTERS, P ZAMBRYSKI, JP HERNALSTEENS, A DEPICKER, M DE BEUCKELEER, G ENGLER, M LEMMERS, L WILLMITZER, J SCHELL 1980 The interaction of *Agrobacterium* Ti-plasmid DNA and plant cells. Proc R Soc London Ser B 210: 351–365
27. WATSON B, TC CURRIER, MP GORDON, M-D CHILTON, EW NESTER 1975 Plasmid required for virulence of *Agrobacterium tumefaciens*. J Bacteriol 123: 255–264
28. WILLMITZER L, M DE BEUCKELEER, M LEMMERS, M VAN MONTAGU, J SCHELL 1980 DNA from Ti-plasmid present in nucleus and absent from plastids of crown gall cells. Nature (Lond) 287: 359–361
29. YAMADA S, HA ITANO 1966 Phenanthrenequinone as an analytical reagent for arginine and other monosubstituted guanidines. Biochim Biophys Acta 130: 538–540
30. ZAENEN I, N VAN LAREBEKE, H TEUCHY, M VAN MONTAGU, J SCHELL 1974 Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. J Molec Biol 86: 109–127