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Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA

(*Agrobacterium tumefaciens*/immunoaffinity chromatography/Tn5 mutagenesis/tumor morphology)

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ABSTRACT **Insertion of the transposon Tn5 into the T-region of the octopine Ti plasmid of *Agrobacterium tumefaciens* gives rise to crown gall tumors having altered morphology. Three loci within the T-DNA that control tumor morphology have been detected [Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P. & Nester, E. W. (1981) *Cell* 27, 143–153]. They influence tumor size (*tml*), production of roots (*tmr*), or production of shoots (*tms*). Cytokinin and auxin levels in such mutant tumors were examined by HPLC/radioimmunoassay and HPLC/fluorescence assay, respectively. Free indoleacetic acid levels (in pmol/g) were: uninfected tobacco stem tissues, 128; wild-type A348 tumors, 295; *tml* mutant tumors, 307; *tmr* mutant tumors, 129; and *tms* mutant tumors, 70. Average *trans*-ribosylzeatin levels were correspondingly: 0.97, 48, 40, 0.54, and 1,400 pmol/g. *trans*-Ribosylzeatin/indoleacetic acid ratios were as high as 24 in shoot-producing tumors and as low as 0.003 in root-producing tumors. The evidence strongly suggests that tumor phytohormone levels are determined by genes in the T-DNA.**

It is known from the work of several laboratories that a segment of DNA, the transferred (T) region of a large plasmid (the Ti plasmid) harbored by the inciting bacterium, *Agrobacterium tumefaciens*, is integrated into the host genome during crown gall tumorigenesis (1). Although seven T-DNA transcripts have been reported (2, 3) in octopine crown gall tumors, no T-DNA-coded gene product has been identified with certainty other than octopine synthase. Other transcripts are presumably determinative in tumor growth and maintenance, but their functions are unknown (4–6). However, crown gall tumors do grow in culture in the absence of added auxin or cytokinin (7), and recent studies (8–10) indicate that cytokinin levels are increased in many, but not in all, tumor lines.

The morphology of crown gall tumors is determined by at least three factors: (*i*) the strain of *A. tumefaciens* used to incite the tumor, (*ii*) the plant host, and (*iii*) the site of inoculation on the host (11). The work of Skoog and Miller (12) established that the morphology of normal tobacco tissue in culture is determined by the cytokinin/auxin ratio in the medium. High and low exogenous cytokinin/auxin ratios lead respectively to shoot and root production. It seems very plausible, therefore, that hormone overproduction and imbalance may be general characteristics also of crown gall tumors.

Transpositional inactivation of regions of the T-DNA (4–6) has defined several loci that control tumor morphology. We describe here the application of some new (13–14) analytical techniques to the measurement of hormone levels in tumors that have altered morphologies as a result of Tn5 insertion in the T-DNA. The data indicate clearly that endogenous tumor hormone levels are determined by specific loci in the T-DNA that also control tumor morphology.

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MATERIALS AND METHODS

The strains used in these experiments have been described in detail (4). All mutants were derived from strain A348, which was constructed by introducing the pTiA6NC plasmid into the cured *A. tumefaciens* derivative A136 by transformation (15).

Tobacco plants (*Nicotiana tabacum* var. Xanthi nc) were grown in a glasshouse in 20-cm pots of UC mix (16) at a temperature of 23°C and under ambient light conditions. Ten-week-old plants, 25 cm tall, were inoculated in the seventh stem internode with parental strain A348 or Tn5-inserted mutant A348 strains by the procedure of Garfinkel and Nester (15). Tumors and uninfected stem tissues were harvested 5 wk after inoculation and frozen at −70°C prior to analysis. All harvests were at the same time of day to minimize the effect of diurnal changes in auxin concentration.

Cytokinin Analysis. *Extraction.* Frozen tumor tissue (fresh weight, 1–3 g) was thawed rapidly and homogenized in 2 vol of dimethyl sulfoxide or methanol containing sodium diethyldithiocarbamate (10 mM) and 2-mercaptoethanol (300 mM) to inhibit oxidation. After centrifugation (20,000 × *g* for 10 min), the supernatant was diluted with 9 vol of ammonium acetate (40 mM at pH 6.5), incubated with almond β-glucosidase (0.04 units/ml) and wheat germ acid phosphatase (2.5 units/ml) (Sigma) at 25°C for 30 min, and passed rapidly through a small DEAE-cellulose column to remove the bulk of the impurities. This procedure converts any cytokinin *O*-glucosides or 5′-phosphates to free nucleosides or bases.

The cytokinins were enriched from the extract by adsorption onto either a cytokinin-specific immunoaffinity matrix or octadecylsilica (17, 18) and elution with methanol, and the eluate was subjected to HPLC. [^{3}H]Kinetin riboside was added as an internal standard prior to homogenization and was measured after HPLC.

HPLC. A previous method (14) was used with minor modification. The HPLC column of 5-μm octadecylsilica (25 cm × 4.6 mm; Ultrasphere ODS, Altex, Berkeley, CA) was equipped with a short (2.5 cm × 2.6 mm) guard column of 5-μm Spherisorb ODS (Alltech Assoc., Deerfield, IL). The aqueous buffer was 50 mM triethylammonium acetate (pH 3.45), and the organic phase was acetonitrile or acetonitrile/methanol, 60:40 (vol/vol). Samples were applied at 10% organic phase (flow rate, 1 ml/min) and eluted with a linearly increasing gradient of organic phase (10–15% over 30 min; 15–45% over 20 min). Under these conditions the cytokinins were eluted in order of

Abbreviations: IndAcOH, indoleacetic acid; RIA, radioimmunoassay.

increasing hydrophobicity. *trans*-Zeatin, *cis*-zeatin, dihydrozeatin, and their ribosides were all well resolved. Cytokinins were detected (if present at 5 ng or more) by their absorbance at 254 nm or by radioimmunoassay (RIA) (14) or by both.

Auxin Analysis. *Preparation of tritiated indoleacetic acid [(3-indolyl)acetic acid; IndAcOH].* L-[G-^{3}H]Tryptophan (New England Nuclear) was converted into [^{3}H]IndAcOH by the use of tryptophan 2-monooxygenase and indoleacetamide hydrolase. The enzyme preparation was obtained by $(NH_4)_2SO_4$ precipitation (25–55%) as described by Kosuge *et al.* (19). Reaction mixtures contained 5 μmol of Tris buffer (pH 7.2), L-[G-^{3}H]tryptophan (specific activity, 4.5 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), 0.2 ml of enzyme preparation, and water to a final 1.2-ml volume. After a 30-min incubation at 25°C, IndAcOH was isolated from the reaction mixture by adsorption onto DEAE-cellulose, eluted with acetic acid, adsorbed onto octadecylsilica, and eluted with methanol. Its radiochemical homogeneity was verified by HPLC to be greater than 99%.

Extraction and measurement. IndAcOH was isolated from tumor and normal tobacco stem tissue samples (0.7–0.85 g) by a rapid chromatographic procedure (unpublished data) onto DEAE-cellulose and octadecylsilica and analyzed by HPLC with an on-line fluorescence spectrophotometer (13). As an internal standard, 320–350 pmol of [^{3}H]IndAcOH (specific activity, 4 Ci/mmol) was added to each sample before homogenization. Recoveries were estimated by measuring the radioactivity in the IndAcOH peak after HPLC. The amount of [^{3}H]IndAcOH added was about 1% of the total IndAcOH present.

RESULTS

Tumor Morphology. Insertion of the bacterial transposon Tn*5* into the T-region of the Ti plasmid pTiA6NC defines three genetic loci that affect tumor morphology; these are *tml*, *tmr*, and *tms* (4). A tumor on tobacco induced by the parental strain A348 is characterized 5 wk after inoculation by an unorganized morphology and a weight of up to 10 g. Insertion of Tn*5* into the *tml* locus also results in unorganized tumors. However, on tobacco stems and more particularly on *Kalanchoë* leaves, *tml* tumors are significantly larger than the parental type. Insertions into the *tmr* and *tms* loci give tumors on tobacco that are characterized either by root production or shoot production, respectively. A map of the T-DNA illustrating the positions of the loci and the morphologies of the normal and mutant tumors is shown in Fig. 1. The locus, *ocs*, controls production of octopine by the tumor.

Cytokinin Levels in Tumors. Initial experiments indicated that there were large differences in the cytokinin content of different lines. In these experiments, cytokinins were extracted from whole primary tumors, purified by an immunoaffinity procedure that allowed their isolation at near homogeneity in a single-pass chromatographic protocol (17) and were then subjected to HPLC/RIA. Typical traces from *tms* (*tms*-328::Tn*5*) and *tmr* (*tmr*-149::Tn*5*) tumors are shown in Fig. 2.

The *tms* tumor contained two major and three minor cytokinins as determined by absorbance at 254 nm and by RIA with ribosylzeatin or isopentenyladenosine antisera. The peak eluting at 37.4 min had a retention time identical to that of authentic *trans*-ribosylzeatin and was strongly reactive towards *trans*-ribosylzeatin antiserum. It was present at sufficiently high levels

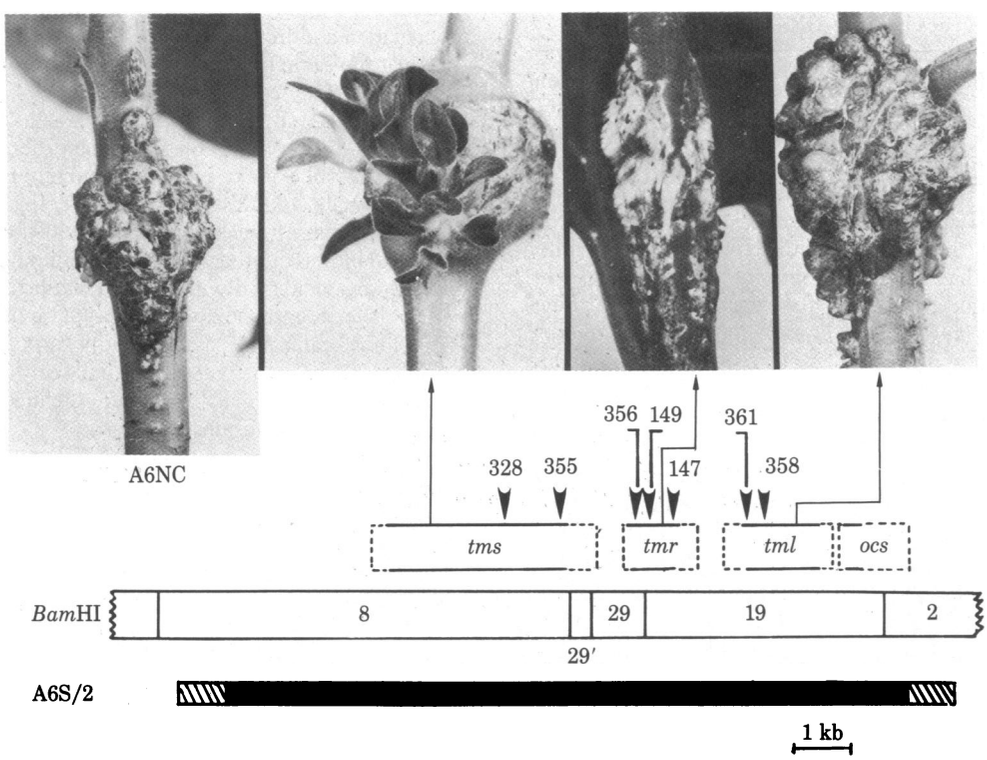

FIG. 1. Functional organization of pTiA6NC T-DNA and tumor morphology resulting from the Tn*5* insertion. The *Bam*HI restriction endonuclease map of the T-DNA is from Thomashow *et al.* (20). Arrows attached to vertical lines are sites of Tn*5* insertions used in these experiments, accurate to ± 0.2 kilobase. The *tms*, *tmr*, *tml*, and *ocs* (octopine synthase) loci are represented by rectangular boxes above the map; the dashed lines of each box represent the distance from the outermost transposon-induced mutation in a given locus to the nearest insertion that is phenotypically silent. The solid lines of each box show regions in which the transposon insertion confers a phenotypic change. ■, Physical extent of the pTiA6 T-DNA present in the A6S/2 tumor line (20). ▨, Junction regions where the T-DNA is joined to plant DNA (21). The scale is in kilobases. Tumor morphologies shown are (from left to right): *N. tabacum*, parental A6NC tumor (identical to that produced by strain A348); *N. tabacum*, shoot-producing tumor (*tms*-328::Tn*5*); *N. tabacum*, root-producing tumor (*tmr*-149::Tn*5*); *N. tabacum*, large phenotype tumor (*tml*-355::Tn*5*).

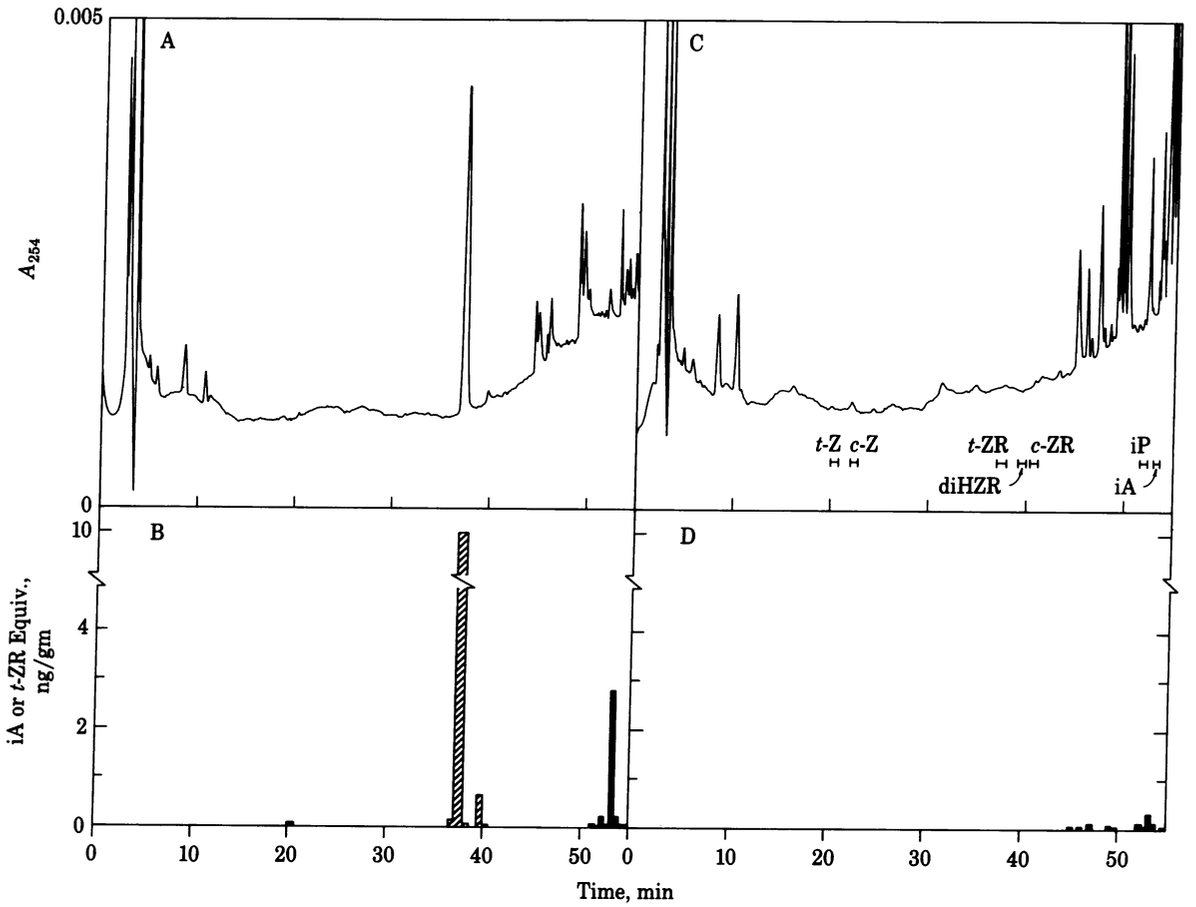

FIG. 2. Cytokinins present in typical shoot-producing and root-producing tumors. Cytokinins from *tms*-328::Tn*5* and *tmr*-149::Tn*5* tumors were isolated by immunoaffinity chromatography and fractionated by HPLC. (*A* and *B*) A_{254} and RIA data, respectively, from the *tms* line. (*C* and *D*) Corresponding data from the *tmr* line. RIA (*B* and *D*) was performed on all fractions using both *trans*-ribosylzeatin (▨) and isopentenyladenosine (■) antisera. Retention times of authentic cytokinin standards are indicated by the horizontal bars (⊢⊣) in *C*: *t*-Z, *trans*-zeatin; *c*-Z, *cis*-zeatin; *t*-ZR, *trans*-ribosylzeatin; diHZR, ribosyldihydrozeatin; *c*-ZR, *cis*-ribosylzeatin; iP, isopentenyladenine; iA, isopentenyladenosine. From UV absorbance measurements, the peak in *A* at 37.4 min represents about 113 pmol of *trans*-ribosylzeatin; by RIA, it contained 102 pmol. Values in this experiment were not corrected for extraction loss.

(100 pmol) to be observable as a UV-absorbing peak. In addition, isopentenyladenosine was present as a visible peak (retention time, 53.2 min; 9 pmol) crossreactive towards isopentenyladenosine antiserum. Smaller quantities of free *trans*-zeatin, ribosyldihydrozeatin, and isopentenyladenine were detected by RIA but were not present in sufficient amounts to be seen by UV absorbance (Fig. 2*B*). By contrast (Fig. 2 *C* and *D*), the *tmr* tumor contained much lower levels of all cytokinins. No significant UV absorbance was seen at any of the appropriate retention times, and RIA indicated that, at most, the extract contained 0.14 pmol of *trans*-ribosylzeatin together with about 2 pmol of isopentenyladenosine and 1 pmol of isopentenyladenine.

It should be noted that since β-glucosidase pretreatment was

Table 1. Cytokinins and IndAcOH in tobacco Tn*5* insertion tumors*

Inciting bacterial strain	IndAcOH	Cytokinins			*t*-ZR/IndAcOH ratio
		t-ZR	*t*-Z	iA	
Control†	128	0.97	0.71	7.8	0.008
A348	295	48	22	9.3	0.2
tmr-147::Tn*5*	117	0.47	0.62	6.0	0.004
tmr-149::Tn*5*	147	0.49	<0.6	7.2	0.003
tmr-356::Tn*5*	124	0.66	<0.6	6.6	0.005
tms-328::Tn*5*	73	1,200	>220	25	17
tms-355::Tn*5*	68	1,600	160	31	24
tml-358::Tn*5*	337	54	8.7	5.1	0.2
tml-361::Tn*5*	278	27	22	6.3	0.1

iA, Isopentenyladenosine; *t*-Z, *trans*-zeatin; *t*-ZR, *trans*-ribosylzeatin.

* Phytohormone levels are expressed as pmol/g of fresh weight and are corrected for recovery. Analyses were carried out on samples derived from pools of tissue from seven individual plants inoculated with the strains indicated. Values represent the average of triplicate samplings.

† Uninfected tobacco stem internode tissue.

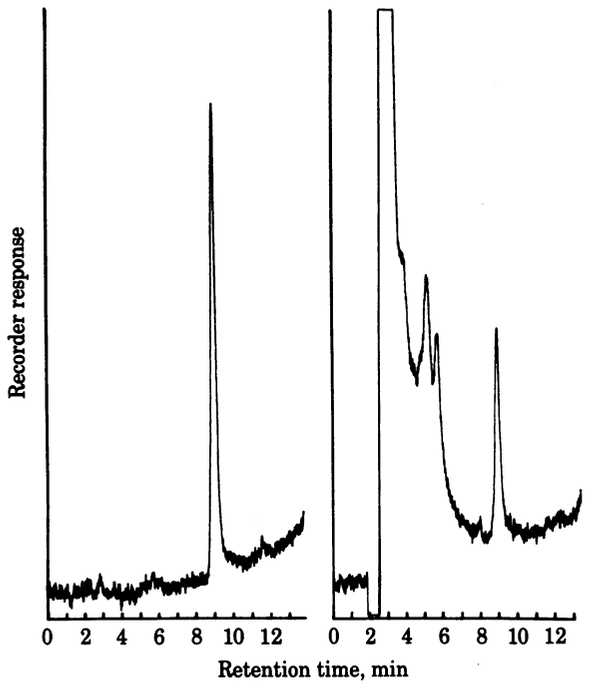

FIG. 3. Fluorometric monitoring of effluent from HPLC separations of standard IndAcOH and tumor samples. (*Left*) Standard IndAcOH sample (4.3 pmol). (*Right*) Preparation from tobacco tumor incited by *A. tumefaciens* parental strain A348.

included in the extraction protocol, some of the *trans*-zeatin and *trans*-ribosylzeatin observed here may have been present in the tissue as *O*-glucosides, which are thought to be storage forms of the hormones.

A survey was made of the hormone levels of a set of parental, *tms*, *tmr*, and *tml* tumors. In these experiments, the cytokinins were isolated onto octadecylsilica and accurately quantitated by HPLC/RIA with [^{3}H]kinetin riboside as an internal standard. The results are summarized in Table 1.

Control uninfected tobacco stem tissue at the same developmental stage as that on which the tumors were incited was found to contain very low levels of *trans*-zeatin, *trans*-ribosylzeatin, and isopentenyladenosine. Parental A348 tumors had levels of *trans*-ribosylzeatin that were elevated ≈50-fold over uninfected controls. However, the three *tmr* tumors had levels that were essentially the same as those of uninfected tissue. The most startling increases were seen in the two *tms* lines. Here the *trans*-ribosylzeatin levels were about 1,600 pmol/g of fresh weight, increased by factors of at least 30 over the parental tumors and at least 1,500 over that of the control stem tissue. In addition, significant increases were noted in the levels of *trans*-zeatin and isopentenyladenosine. Estimation of *trans*-ribosylzeatin levels in *tms* tumors harvested prior to the appearance of shoots gave values very similar to those above (data not shown), suggesting that the large increases in these lines precede the appearance of altered morphology.

Auxin Levels in Tumors. Free IndAcOH was extracted into methanol, purified on DEAE-cellulose and octadecylsilica and quantitated by measurement of fluorescence intensity of the appropriate peak on HPLC. Addition of an internal standard of [^{3}H]IndAcOH allowed accurate measurements to be made of the recovery during extraction and chromatography.

Fig. 3 *Left* shows the HPLC/fluorescence trace of the IndAcOH standard, while Fig. 3 *Right* shows a typical profile obtained from tumor tissue. The identity and purity of the peak eluting at 8.9 min was established by methylation and rechromatography. Its structure was confirmed by mass spectroscopy (data not shown). The peak was essentially homogeneous and at least 95% IndAcOH.

Free IndAcOH levels were lowest in tumors incited by *tms* mutants 328 and 355 and highest in tumors incited by *tml* mutants 358 and 361 and by the parental strain A348 (Table 1). They were intermediate in tumors incited by *tmr* mutants 147, 149, and 356.

Correlation of Phytohormone Ratios with Tumor Morphology. Although changes in free IndAcOH levels were not as large as those of *trans*-ribosylzeatin, changes in the *trans*-ribosylzeatin/IndAcOH ratio did correlate well with tumor morphology (Table 1). Uninfected stem tissue and *tmr* tumors had the lowest ratios (0.003–0.008); *tml* and parental tumors had intermediate ratios (0.1–0.2); and *tms* tumors had the highest ratios (17–24).

DISCUSSION

The early findings of Braun (7) showed that crown gall tumors could be maintained indefinitely in culture on a simple hormone-free medium. One possible explanation is that the tumors produce endogenous levels of auxins and cytokinins required for growth in culture. Recent studies (8–10, 22) showed that elevated phytohormone levels do indeed occur in many tumor lines, although in some plant species (10) tumor growth is not accompanied by increased hormone content. The data presented here permit two general conclusions to be drawn: specific T-DNA loci do indeed influence phytohormone levels in tumors and, second, there is a correlation between cytokinin/auxin ratio and primary tumor morphology. This correlation follows the pattern originally described by Skoog and Miller (12) for normal tobacco tissue in culture; namely, that shoot formation is favored by high cytokinin/auxin ratios, whereas root formation is favored by low ratios. Amasino and Miller (9) noted similar correlations between phytohormone content and the morphology of tumors incited by natural isolates of *A. tumefaciens*.

Previous studies suggested that the *tmr* and *tms* loci play roles in cytokinin and auxin metabolism. These assignments were based upon indirect evidence involving stimulation of tumor growth by phytohormones (5, 6) or upon demonstration of a phytohormone requirement by a cloned tumor line in culture (ref. 23; M-D. Chilton, personal communication). The data here provide direct support for such a role in the case of the *tmr* locus. Tumors incited by *tmr* mutants formed roots and had *trans*-ribosylzeatin/IndAcOH ratios of about 0.005. These ratios resulted primarily from the low content of the physiologically active cytokinin *trans*-ribosylzeatin. Thus, insertional inactivation of the *tmr* locus dramatically reduces the *trans*-ribosylzeatin content of tumor tissue, suggesting that this locus plays a central role in cytokinin metabolism in the tumor.

The situation with the *tms* locus is more complex. The data presented here and the results of a more extensive analysis (unpublished data) suggest that inactivation of the *tms* locus results in shoot-producing tumors that have *trans*-ribosylzeatin/IndAcOH ratios of 17 or greater. These ratios arise as a consequence of both a decrease in free IndAcOH and a dramatic increase in *trans*-ribosylzeatin accumulation. Thus, the *tms* locus appears to be involved not only with auxin but also with cytokinin metabolism. Elevated cytokinin levels persist after *tms* tumors are propagated in axenic culture although not with such extreme values (unpublished data). Therefore, it is likely that the high levels present in *tms* primary tumors are due neither to the continuing activity of the inciting bacteria nor to an accumulation of *trans*-ribosylzeatin from other regions of the plant. The cytokinin levels measured here are effectively averaged over the whole tumor mass. Their distribution within

the tumor in relation to tissue differentiation has yet to be determined.

Under the conditions of these experiments, we are unable to assign a function to the *tml* locus. Phytohormone ratios in tumors incited by two different *tml* mutants were close to the values seen for parental tumors. The results of a more extensive survey (unpublished data) showed that there was no statistically significant difference between the cytokinin levels of *tml* and parental tumors. The *tml* phenotype is not strongly pronounced on tobacco, although the tumors remain undifferentiated. Examination of phytohormone levels in *tml*-induced tumors on plants such as *Kalanchoë daigremontiana*, where the *tml* phenotype is more clearly evident, may reveal *tml*-specific hormone patterns.

The crown gall system with its well-defined genetics should provide a useful tool for probing the details of the relationship of the enzymology of cytokinin and auxin production and plant morphology.

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