Planta (1983) 158:272–279

Planta

Host and T-DNA determinants of cytokinin autonomy in tobacco cells transformed by *Agrobacterium tumefaciens*

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Abstract. The hormone autonomy of tobacco (*Nicotiana tabacum* L.) cells transformed by *Agrobacterium tumefaciens* containing mutations at *tmr* (the "rooty" locus) of the pTiT37 plasmid has been examined. These cells require cytokinin, but not auxin for continuous growth in culture, indicating that the function of the *tmr* locus is to specify or induce cytokinin autonomy. Examination of tissues from plants regenerated from cells transformed by the mutant bacteria showed that the auxin independent phenotype is suppressed, but can be reinitiated in culture by exposure to an exogenous supply of auxin. In addition the developmental state of the cells from such regenerated plants can exert a profound influence on their cytokinin autonomy phenotype.

Key words: *Agrobacterium*, mutants – Auxin autonomy – Cytokinin habituation – Mutant (*Agrobacterium*) – *Nicotiana* (cell transformation).

Introduction

Crown-gall tumors are induced on a wide variety of dicotyledonous plants by virulent strains of *Agrobacterium tumefaciens*. During the transformation process a portion of the Ti ("Tumor inducing") plasmid present in such bacteria is transferred into the host-plant cells and stably incorporated into their nuclear DNA (Chilton et al. 1977, 1980; Lemmers et al. 1980; Thomashow et al. 1980a, b; Hernalsteens et al. 1980; Willmitzer et al. 1980; Yadav et al. 1980; Zambryski et al. 1980). This transferred DNA (T-DNA) encodes several RNA transcripts (Gelvin et al. 1982; Willmitzer et al. 1982; Bevan and Chilton 1982) and has a variety of functions. One function of T-DNA is to direct the synthesis of novel metabolites termed opines, the best characterized of which are the arginine derivatives octopine and nopaline (reviewed by Tempé and Goldmann 1982). The type of opine synthesized depends on the type of Ti plasmid used for transformation and serves as an easily identifiable marker of the transformed state (Petit et al. 1970; Bomhoff et al. 1976; Montoya et al. 1977).

The T-DNA is also involved in the initiation and maintenance of the tumorous state. It has been proposed that the T-DNA functions by causing the transformed cell to produce or utilize the plant hormones auxin and cytokinin in an abnormally regulated fashion, thereby stimulating growth and cell division (Braun 1978). Several lines of evidence support this hypothesis. First, tumorous cells grow in culture in the absence of hormones whereas non-transformed cells require these substances in order to grow continuously (Braun 1958). Second, cultured crown-gall tumors contain both auxins and cytokinins at levels sufficient to promote cell growth and division (Pengelly and Meins 1977, 1982; Scott et al. 1980; Amasino and Miller 1982). Third, if transformed cells lose the T-DNA they revert to a hormone-requiring state (Braun and Wood 1976; Turgeon et al. 1976; Yang et al. 1980). Finally, strains carrying mutations at specific loci in the T-DNA can initiate tumors (in certain host plants) with morphologies distinctly different than those induced by the wild-type strain. For example, mutations in the T-DNA of a strain that usually induces unorganized tumors in tobacco result in bacterial strains that induce shoot-forming or root-forming tumors, depending on the site of the mutation (Ooms et al. 1980, 1981; Garfinkel and Nester 1980; Garfinkel et al. 1981; Holsters et al. 1980). Because auxins, at high concentrations, inhibit shoot formation and cytokinins in-

hibit root formation in tobacco cell cultures it has been proposed that genes at the "shooty" loci (*tms*, Garfinkel et al. 1981) are involved in auxin autonomy and those at the "rooty" locus (*tmr*) are involved in cytokinin autonomy.

Evidence for the role of *tms* genes in auxin autonomy has been presented elsewhere (Binns et al. 1982). In this case tobacco cells transformed by strain A66, the Ti plasmid of which carries a spontaneous insertion at the *tms* locus of pTiA6, are auxin dependent in culture unless shoots are formed. Akiyoshi et al. (1983) analyzed the hormone content of primary tumors induced by both *tms* and *tmr* mutants and found, as expected, that *tms*-induced tumors had decreased auxin levels (and, interestingly, increased cytokinin levels) whereas *tmr* induced tumors had decreased cytokinin levels. While these data support the hypothesis that the *tmr* gene is involved in cytokinin autonomy, the experiments were conducted on tumors excised directly from the plant and probably contained large numbers of both non-transformed cells and bacteria. I report here on experiments examining the hormone autonomy of cloned tobacco cell lines transformed by strains of *Agrobacterium* containing genetically engineered derivatives of the nopaline-type plasmid, pTiT37, carrying insertions at the *tmr* locus (Matzke and Chilton 1981; Barton et al. 1983). These lines are cytokinin dependent but auxin independent. In addition, the hormone requirements of various tissue types of plants regenerated from such transformed cells have been analyzed. These experiments demonstrate that (1) the auxin autonomy system is suppressed in the regenerated plants, and (2) the developmental (epigenetic) state of cells carrying the mutant T-DNA profoundly affects their hormone autonomy.

Materials and methods

All cell lines used in this study were derived from *Nicotiana tabacum* cv. Havana 425. HT37-8-10-25 is a cloned line from a tumor initiated by the wild-type T37 strain of *Agrobacterium tumefaciens*, and was isolated in May 1980 (Binns et al. 1981). HADH4–11 is a cloned line transformed by the mutant strain T37ADH4, while HADH2–3 and HADH2–5 are clones transformed by the mutant strain T37ADH2. Clone H14a/a-41 was transformed by mutant strain T3714a/a. These four lines were isolated in June and July 1981. The transformation and isolation of these clones is described in Barton et al. (1983). All three mutant *Agrobacterium* strains are characterized by insertion of foreign DNA at the Hpa I site of Bam H1 fragment 14a in pTiT37 (see Fig. 1 for restriction map indicating the insertion site). HA21-34N is a non-transformed clone requiring cytokinin and auxin which has been isolated from pith cultures established in June 1979.

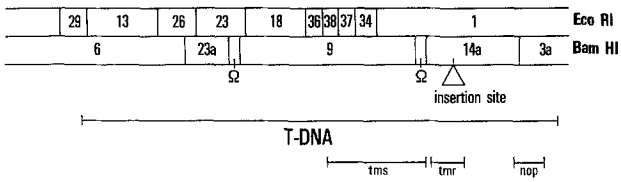

Fig. 1. Restriction map of pTiT37, indicating the tms, tmr and nopaline-synthesis loci and the site of DNA insertion used to generate pTiT37 14a/a and pTiADH (redrawn from Bevan and Chilton 1982, and Barton et al. 1983)

Stock lines were cultured in vials, 95 mm long, 25 mm diameter, on LS (Linsmaier and Skoog 1965) medium supplemented with either no hormones (HT37-8-10-25), 0.3 mg/l kinetin (6-furfurylamino purine) (HADH4–11, HADH2–3, HADH2–5, and H14a/a-41) or 2.0 mg/l α-naphthaleneacetic acid (NAA) and 0.3 mg/l kinetin (HA21-34N), and transferred every four weeks. All media were solidified with 9.0 g/l Bactoagar (Difco Laboratories, Detroit, Mich., USA) purified as described in Binns and Meins (1979). In growth experiments tissues were grown, one piece per flask, for two successive four-week transfers in 50-ml Erlenmeyer flasks containing 20 ml of medium. All tissues were grown at 25° C under constant light from cool-white fluorescent lamps (F40/CW; Sylvania, Danvers, Mass., USA; fluence 8.72 W m^{-2} of photosynthetically active light). Growth is expressed as $\frac{W-W_o}{W_o}$, where W is the fresh weight of the tissue four weeks after the second transfer and W_o is the initial fresh weight of the second transfer.

Primary explants of 30–40-cm-tall, greenhouse-grown tobacco plants (either from seed, cv. Havana 425, or regenerated from culture) were prepared for culturing by surface-sterilizing leaves (10–12 cm in length) and stems with a soap and water wash and then three repetitions of the following sequential washes: 1 l water with 50 mg detergent (Alconox, New York, N.Y., USA) and 7% (v/v) commercial bleach – water – 80% ethanol. After this the material was rinsed three times in sterile distilled water. Leaf explants were made as 5-mm^2 sections lacking major veins. Cortex explants were made by peeling the epidermis off the stem and excising the underlying cortex. These tissues were examined under a dissecting microscope to insure that they did not contain small amounts of vascular tissue. The initial fresh weight of cortex explants was approx. 10–15 mg.

Results

1. Cells transformed by pTiT37 14 a/a and pTiT37 ADH are auxin independent but cytokinin requiring. Matzke and Chilton (1981) observed that strains of *Agrobacterium tumefaciens* carrying pTiT37 with an insert of the kanamycin-resistance gene from Tn5 at the Hpa I site of BamH1 fragment 14a (Fig. 1) induced slow-growing tumors on sunflower and root-forming tumors on carrot discs. This strain, designated T37 14a/a, as well as strains obtained by the insertion of the yeast ADH gene at the same site (T37 ADH; Barton et al. 1983) would not induce tumors on intact tobacco plants. In order to obtain cell lines transformed by these

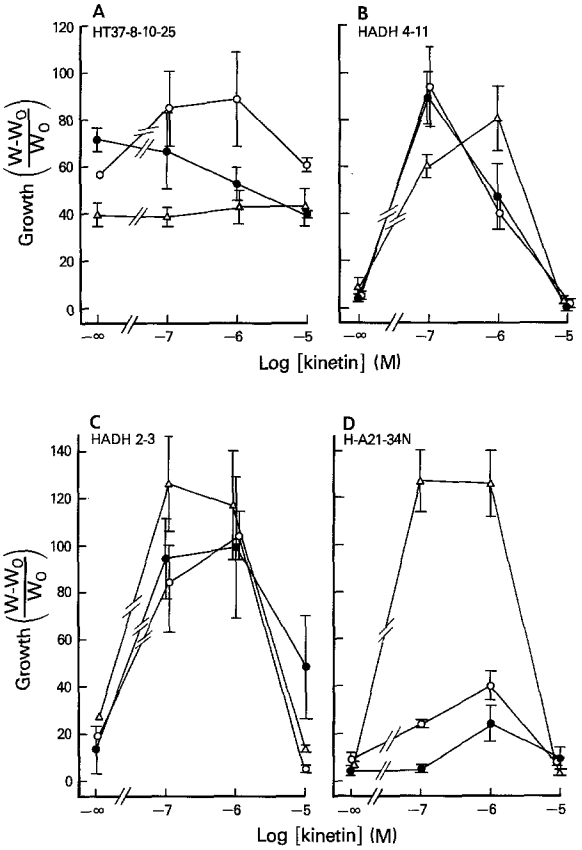

Fig. 2A–D. Kinetin dose response of various clones at (●) 0, (○) 10^{-7} M and (△) 10^{-6} M NAA. **A** HT37-8-10-25; **B** HADH4–11; **C** HADH2–3; **D** H-A21-34N. Bars = ±SE (N = 6). W_0 = 20 mg

strains it was necessary to infect stem segments in vitro and screen the resultant overgrowths for the presence of nopaline. When freed of bacteria these transformed tissues grew slowly on hormone-free medium unless roots were formed spontaneously. Such root formation occurred in most explants and stimulated growth considerably. Single-cell clones were isolated from these primary transformed tissues under non-selective conditions (i.e. the plating medium was supplemented with both auxin and cytokinin), and then screened for nopaline (Barton et al. 1983). Nopaline-positive clones did not grow on hormone free medium, and did not initiate roots as often as the noncloned transformants.

The hormone autonomy of nopaline-positive clones transformed by mutant Agrobacteria was compared to that of wild-type tumors by testing each line for growth at several auxin and cytokinin concentrations. Cloned tumor line HT37-8-10-25 (H425 tobacco transformed by the wild-type T37 strain) grew on hormone-free medium as a spontaneously shoot-forming teratoma. Some stimulation of growth occurred at low auxin and cytokinin levels (Fig. 2A). In contrast, clones derived from tobacco cells transformed by the mutant T37ADH required exogenously supplied cytokinin, but not auxin, for continuous growth (Fig. 2B, C). On auxin-free medium shoots were initiated by these lines at the highest kinetin concentrations (10^{-5}–10^{-6} M), and could have contributed to their auxin-independent phenotype (Binns et al. 1982). However, auxin-independent growth was demonstrated by the fact that at lower kinetin levels (10^{-7} M) the tissues grew vigorously in an unorganized fashion in the absence of auxin. Lines transformed by T37 14a/a strain showed the same type of hormone requirements (data not presented). In contrast, cloned non-transformed tobacco cells required auxin as well as cytokinin for continuous growth (Fig. 2D). We conclude from these experiments that the insertion of DNA at the HpaI site of Bam H1 fragment 14a in pTiT37 disrupts a gene that controls cytokinin autonomy in the transformed cells but does not affect auxin autonomy.

2. Auxin autonomy is suppressed in regenerated plants. Treatment of HADH2 and H14a/a clones with high levels of kinetin (10^{-5}–10^{-6} M) resulted in profuse bud initiation, mimicking the phenotype of T37-transformed clones. A striking difference, however, was that shoots from HADH2 and H14a/a clones formed roots when excised and placed into an appropriate medium. Complete fertile R_0 plants (i.e. plants regenerated from culture; Chaleff 1981, p. 94) carrying the T-DNA were obtained, and these were capable of passing the T-DNA on to their progeny (Barton et al. 1983). The question raised here is whether the cells of the regenerated plants retained the T-DNA in a biologically functional form.

Nopaline was present in all regenerated plants, indicating that the nopaline-synthesis locus of the T-DNA was active. We analyzed the tissues from the regenerated plants for hormone autonomy in order to determine (1) whether the auxin autonomy system(s) were active, and (2) how the developmental ("epigenetic") state of tissues from regenerated plants affected their phenotype when returned to culture. The Havana 425 cultivar of tobacco was particularly well suited for these experiments. First, previous studies on shoots derived from H425 cells transformed by T37 showed that auxin autonomy was suppressed and that there was tissue-specific variation in the conditions required for the reinitiation of this phenotype (Braun and Wood 1976; Binns et al. 1981). Tissues lacking vas-

Table 1. Growth of primary explants of various tissue types

Plant	Tissue	Growth $\frac{W-W_0}{W_0}$			
		Basal LS medium	LS +auxin[a]	LS +cytokinin[b]	LS +auxin[a] +cytokinin[b]
H425	cortex	0.5±0.1[c]	141.1±17.9	45.5 (1)[d] 0.1± 0.2 (7)	150.1±15.0
	leaf	1.5±0.5	1.6± 0.8	175.7±29.1[d]	224.1±23.7
HADH2–5 plant #2	cortex	3.9±2.9	18.4± 2.8	111.4±13.7 (3)[d] 3.9± 3.4 (5)	24.8± 6.8
	leaf	0.4±0.2	0.3± 0.1	138.5±38.2[d]	13.3± 2.9

[a] Supplemented with 10 μm NAA
[b] Supplemented with 1 μM kinetin
[c] Growth is measured as $\frac{W-W_0}{W_0}$ where W = FW after the 2nd of two four week transfers on the same medium, and W_0 is the inital FW of explants for the second transfer (20 mg); ±SE, $n=8$
[d] Tissue pieces showing shoot formation; calculated separately from tissue pieces without shoots (if any)

cular tissue (pith, cortex, epidermis) required auxin for growth and reinitiation of the auxin-independent phenotype, whereas tissues containing vascular tissue (leaf, stem) did not. Second, tissue cultures from normal, non-transformed H425 plants exhibited different stable states of cytokinin requirement depending on the tissue of origin (Meins and Lutz 1979): cultured cortex was cytokinin independent whereas cultured leaf tissue were cytokinin requiring. Thus, leaf and cortex cells are in different but stable developmental (epigenetic) states in relation to their cytokinin requirement. The question we asked is, Do these stable epigenetic states affect the phenotype of T-DNA containing cells?

A comparison of the conditions required for growth of primary explants from normal H425, and R_0 No. 2 from clone HADH2–5, indicated few differences (Table 1). The cortex of both plants was cytokinin habituated (i.e. grew on medium containing only auxin), confirming the observations of Meins and Lutz (1979). These tissues would not grow on either basal or kinetin-containing medium unless shoots formed indicating that the auxin autonomy system was suppressed. Leaf tissues responded quite differently: tissues from both plants grew and formed shoots profusely on kinetin-containing medium but did not grow on auxin-containing medium. A breakdown of the relative amount of shoot versus callus growth on kinetin-containing medium demonstrated the only real difference between H425 and HADH2–5 tissues. The shoots represented 75% of the fresh-weight increase of leaf explants from H425 whereas from such explants of HADH2–5 the callus was responsible for over 80% of fresh-weight increase. This relatively greater proliferation of callus in the case of HADH2–5 leaf tissue indicates that the auxin-autonomy system is active or reactivated early during the culture period. This conclusion is further supported by the fact that growth of such tissues was inhibited on medium containing both auxin and cytokinin, characteristic of hormone-independent cultures supplemented with supraoptimal levels of hormone. Explants of cortex and leaf tissues from R_0 plants of clone H14a/a-41 responded to the culture conditions tested in the same manner as the HADH2–5 tissues (data not shown).

3. Reinitiation of auxin autonomy in tissues from regenerated plants. The results of the experiments described above indicated that the primary cortex explants of plants from HADH2–5 were not auxin autonomous, but that leaf tissues might be auxin independent. In terms of auxin autonomy these observations were virtually identical to those made in previous experiments in which the hormone requirement of tissues from shoots regenerated from tobacco transformed by wild-type T37 were examined (Braun and Wood 1976; Binns et al. 1981). Cortex explants from these shoots required auxin (only) for growth whereas leaf tissues grew on hormone-free medium. An interesting feature of the

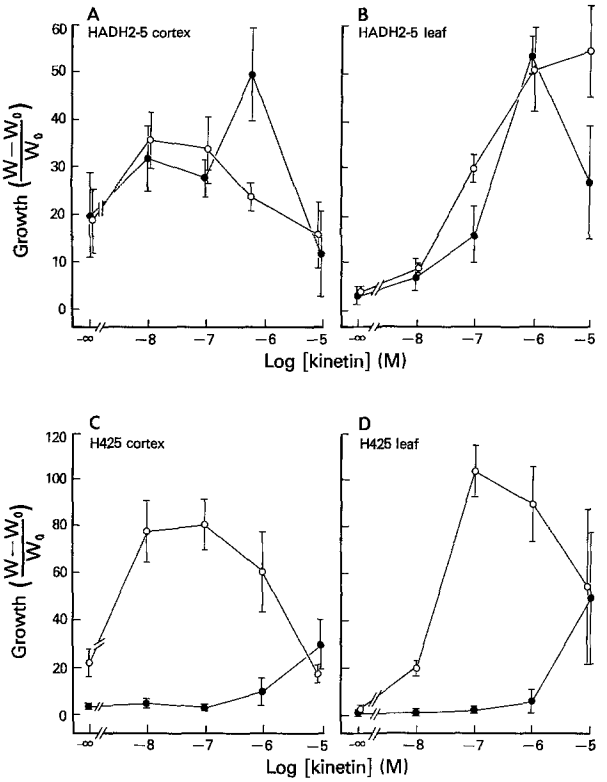

Fig. 3A–D. Kinetin dose response of various tissues at (●) 0 and (○) 10^{-6} M NAA tested after incubation of the indicated primary explants for four weeks on 10^{-5} M NAA (**A**, **C**) or 10^{-6} M kinetin (**B**, **D**). Bars = ±SE ($N=6$). $W_0 = 20$ mg

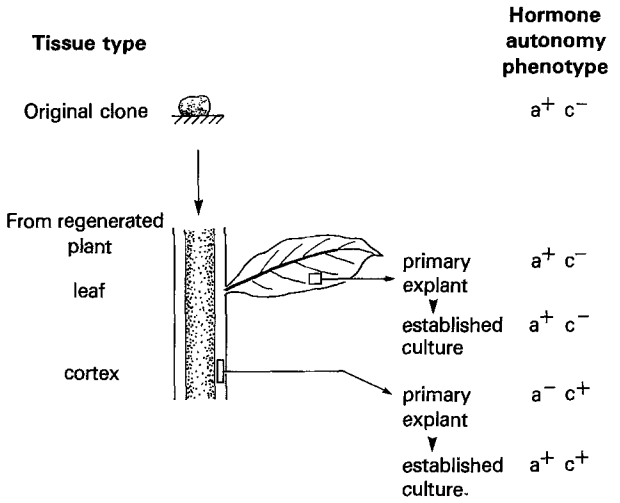

Fig. 4. Summary of tissue-specific variation of hormone autonomy in primary and established cultures from plants regenerated from cells transformed by T37 Agrobacteria carrying a mutation at the *tmr* locus in its Ti plasmid. a^+ indicates auxin autonomous; a^-, auxin requiring; c^+ cytokinin autonomous; and c^-, cytokinin requiring

cortex tissues from such plants was that after exposure to auxin the cells reinitiated the auxin-independent phenotype and thereafter grew on hormone-free medium.

To determine whether auxin autonomy was reinitiated in tissues taken from the regenerated HADH2–5 plants, primary explants of cortex and leaf were cultured on various media for four weeks and then tested for growth over a range of auxin and cytokinin concentrations. The results showed that in fact auxin autonomy was reinitiated in the cortex tissues after exposure to auxin containing medium. The tissues grew well at both zero and 10^{-6} M naphthaleneacetic acid over a range of cytokinin concentrations (Fig. 3A). Interestingly these tissues now grew continuously on hormone-free medium, but did so in an unorganized fashion. Thus, the cytokinin-habituated phenotype of the HADH2–5 cortex apparently compensated for the lack of cytokinin autonomy caused by the T-DNA mutation, but did not cause the tissue to exhibit the teratomatous phenotype of tobacco cells transformed by wild-type T37. This compensation was not a consequence of some peculiarity of the plant used for the primary explants because the leaf tissue from this plant gave the typical response of cells transformed by the mutant T-DNA (Fig. 3B). These were auxin independent but cytokinin requiring regardless of whether the primary explant was grown on medium containing only cytokinin (Fig. 3B) or on medium supplemented with both auxin and cytokinin (data not shown). A summary of these results is shown in Fig. 4. The responses of normal H425 tobacco tissues to the same experimental conditions are shown for comparison (Fig. 3C, D). These experiments clearly demonstrate that the epigenetic states of cells can profoundly affect the phenotype of cells carrying the same T-DNA.

Discussion

The use of cells transformed by T-DNA mutants of *Agrobacterium tumefaciens* facilitates analysis of the cellular and physiological functions of the mutant gene(s). In this study the effect of DNA inserts at the *tmr* locus of pTiT37 (Hpa I site of Bam fragment No. 14a; Matzke and Chilton 1981; Barton et al. 1983) on the tumorous phenotype has been examined in detail. Five conclusions can be drawn from these experiments: 1) Mutation at this locus virtually eliminates the cytokinin-independent phenotype noted in tobacco cells transformed by the wild type T37 strain; 2) in this system cyto-

kinin and auxin autonomy are not linked – the cells transformed by the mutant bacteria are completely auxin autonomous; 3) the auxin-independent phenotype of the transformed cells is suppressed in plants regenerated from them; 4) the auxin-independent phenotype can be reinitiated by exposure of explants to auxin in culture; and 5) the developmental state of plant cells carrying T-DNA can exert substantial influence over their hormone-autonomy phenotype.

The morphology of tumors initiated by *Agrobacterium tumefaciens* strains carrying mutations at the *tms* (shooty) or *tmr* (rooty) loci indicated that their hormone balance was altered in comparison to wild-type tumors (Ooms et al. 1981; Garfinkel et al 1981; Leemans et al. 1982). The shooty phenotype was thought to be based upon the result of a relative auxin deficiency and the rooty phenotype upon a relative cytokinin deficiency. In these studies I have demonstrated that plasmids pTiT37 14a/a, pTiT37 ADH2, and pTiT37 ADH4, all carrying an insert at their *tmr* locus, transform cells to a cytokinin-requiring but auxin-independent state. These results were unexpected because the wild-type plasmid, pTiT37, induces shooty tumors in tobacco, indicating a relative auxin deficiency. The cell lines transformed by the mutant plasmids did not exhibit such a deficiency. It should be noted, however, that pTiT37 transformed tobacco cells contain an RNA transcript homologous to the transcript of the *tms* locus of octopine-type plasmids (Bevan and Chilton 1983) albeit at a low abundance. Thus the hormone imbalance resulting in the shooty phenotype of T37-transformed cells may be caused by cytokinin overproduction (or hypersensitivity) rather than auxin deficiency (or insensitivity). This idea is consistent with the measurements of endogenous auxins and cytokinins in various tumor lines (Pengelly and Meins 1982; Amasino and Miller 1982; Akiyoshi et al. 1983). The locus mutated in the strains described here is apparently responsible for this postulated cytokinin overproduction.

The absence of direct linkage between hormone systems is demonstrated by the fact that cells containing mutant T-DNA require cytokinin but not auxin for growth. This contrasts with the usual observations on hormone habituation in cultured tobacco cells. In this case the cytokinin and auxin autonomy systems are activated sequentially: cytokinin independence always precedes auxin independence (Meins 1982). The results presented here demonstrate that in crown-gall tumors such sequential activation of autonomy systems is not in effect.

Auxin autonomy is suppressed in plants regenerated from cells transformed by the mutant bacteria. Reinitiation of this system in tissues taken from such plants follows the pattern observed in shoots derived from cells transformed by wild-type T37 bacteria. (Braun and Wood 1976; Binns et al. 1981). Tissues expected to have high endogenous auxin levels (leaf, vacular tissue) reinitiate the auxin-independent phenotype without exposure to exogenous auxin. In contrast, tissues expected to have low endogenous auxin levels (pith, cortex) require such a treatment for initial growth but thereafter can grow in the absence of auxin (Fig. 4). These results demonstrate that cytokinin autonomy controlled by the *tmr* locus is not involved in the expression, suppression or reinitiation of auxin autonomy in crown-gall tumor cells.

An intriguing result of these experiments was that the developmental state of tissues in the regenerated plants affected their hormone-independent phenotype when returned to culture. Cultured leaf tissues exhibited the original phenotype noted in the cloned transformed lines, whereas cultured cortical tissues, after exposure to auxin, grew continuously on hormone-free medium. Normal H425 leaf tissues are always hormone dependent in culture, while cortex tissues are cytokinin habituated, but auxin requiring (Meins and Lutz 1979). Cytokinin habituation of HADH2–5 cortex cells can apparently complement the cytokinin requiring phenotype associated with the mutation at the *tmr* locus. This result demonstrates that the epigenetic state of plant cells can play a crucial role in determining their transformed phenotype and indicates that such states must be considered during analysis of T-DNA gene function.

In conclusion, the *tmr* locus controls, either directly or indirectly, cytokinin autonomy in cells transformed by *Agrobacterium tumefaciens*. Because of developmental variation, however, cells carrying T-DNA mutated at the *tmr* locus may exhibit either the cytokinin-requiring or the cytokinin-independent phenotype. We are currently examining the activity of the T-DNA, in terms of both hormone autonomy and nopaline synthesis, in the progeny of plants regenerated from the HADH2 and H14a/a clones.

The expert technical assistance provided by Sara Piddington is gratefully acknowledged. Thanks also to Dr. Mary-Dell Chilton (Washington University, St. Louis, Mo., USA) and Dr. Ken Barton (Cetus Corp., Madison, Wis., USA) for stimulating discussions concerning the function(s) of T-DNA genes, and to Dr. Nicola Neff for critically reading an earlier version of this manuscript. This work was supported by National Science Foundation grant PCM-8104064.

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Received 21 February; accepted 4 April 1983

Erratum

Planta (1983) **157**, 371–375, paper by S.J. Neill, R. Horgan and J.K. Heald: Determination of the levels of abscisic acid-glucose ester in plants

Table 1, the column under the heading 'Solvent' should read:

3, 4, 5, 6