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Hairy roots are more sensitive to auxin than normal roots

(*Agrobacterium rhizogenes*/root elongation/proton excretion/transmembrane potential/1-naphthaleneacetic acid)

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ABSTRACT **Responses to auxin of *Lotus corniculatus* root tips or protoplasts transformed by *Agrobacterium rhizogenes* strains 15834 and 8196 were compared to those of their normal counterparts. Three different types of experiments were performed, involving long-term, medium-term, or short-term responses to a synthetic auxin, 1-naphthaleneacetic acid. Root tip elongation, proton excretion by root tips, and transmembrane electrical potential difference of root protoplasts were measured as a function of exogenous auxin concentration. The sensitivity of hairy root tips or protoplasts to exogenous auxin was found to be 100–1000 times higher than that of untransformed material.**

Agrobacterium rhizogenes is responsible for the hairy-root disease of plants, which results in abundant adventitious root formation at the site of inoculation in many dicots (1). Such roots can be grown *in vitro*, in the absence of the inciting bacterium (2), and they generally grow better than normal roots of the same species. Hairy-root cultures exhibit a typical phenotype whose most characteristic traits are lack of geotropism and high incidence of lateral branching as compared to normal roots (3). New compounds, named opines, are often found in hairy roots, for which they can constitute useful biochemical markers (4). These opines are specific for the strain that has incited the disease. The pathogenic determinants in *A. rhizogenes* are located on plasmids (5) that have been called Ri plasmids (2). The molecular basis for hairy-root disease is the transfer and expression into the plant genome of specific DNA sequence(s), called T-DNA regions, originally located on Ri plasmids (6, 7). In the related disease, crown gall, incited by *Agrobacterium tumefaciens* strains harboring Ti plasmids (for a review, see ref. 8), T-DNA genes expressed in transformed plant cells specify the synthesis of auxin and cytokinin, which are responsible for cell proliferation resulting in tumor growth (8).

The symptoms of hairy-root disease—abundant root formation at the point of inoculation and high incidence of secondary root formation—are suggestive of auxin effects. Indeed, similar symptoms are produced by *A. tumefaciens* strains with Ti plasmid T-DNA mutants affected in cytokinin biosynthesis (9–11). Since the latter are clearly due to T-DNA-encoded auxin synthesis and since some *A. rhizogenes* strains also carry auxin-synthesis genes homologous to those of crown gall T-DNA (12, 13) it was conceivable that Ri plasmids were spontaneous Ti plasmid mutants. Accordingly, the molecular basis for the hairy-root syndrome would simply be T-DNA-encoded auxin synthesis. However, recent studies on the role of Ri plasmid T-DNA auxin genes show that in hairy root this role is accessory (14, 15), and auxin assays show no higher content in *in vitro* cultured hairy roots than in normal roots (16). Because the same effects could theoretically be produced if hairy-root cells, instead of having a high auxin content, were highly sensitive to auxin, we compared auxin sensitivity of normal and transformed material.

This paper describes experiments showing that *Lotus corniculatus* hairy roots and the cells that compose them are 2–3 orders of magnitude more sensitive to the synthetic auxin 1-naphthaleneacetic acid (NAA) than those of normal plants. This higher sensitivity is not due to increased NAA uptake.

MATERIALS AND METHODS

Bacterial Strains. *A. rhizogenes* strains 15834 and 8196 were from our collection (4). Strain 15834 carries an agropine-type Ri plasmid with two T-DNA regions called T_L and T_R (12, 13, 17). The representative opine of 15834 is agropine, whose synthesis is encoded by T_R-DNA (17). No opine has been associated so far with 15834 T_L-DNA. Strain 8196 carries a mannopine-type Ri plasmid with a single T-DNA region, and its representative opine is mannopine.

Plant Material. Surface-sterilized seeds of the legume *L. corniculatus* (Etablissements Devaux-Chanu, Barran, France) were germinated and grown on agar plates of half-strength Monnier's medium containing mineral salts (18) and vitamins (19) but without sugar. Root explants taken from 10- to 12-day-old plantlets were cultured *in vitro* on the same medium supplemented with sucrose at 30 g/liter (Mo/2 medium). One culture that exhibited good growth under these conditions was selected as the source of clonal material for further experiments. Spontaneous shoot formation on cultured roots was observed after 6–7 weeks. Shoots, about 1 cm long, were cut and rooted on the same medium, and they were used as the source of untransformed material in subsequent experiments and for inoculations.

Inoculation, *in Vitro* Culture, and Opine Analysis. Stems of regenerated plants were wounded and inoculated. Hairy-root cultures were established on Mo/2 medium as described (20). The presence of opines was checked by high-voltage paper electrophoresis (4, 20).

DNA Preparation and Dot Blot Hybridization. Three to 8 grams of *in vitro* grown plant material was used to prepare DNA as described (21). The DNA was purified by isopycnic centrifugation as described (6). Purified DNAs were dissolved in 10 mM Tris/5 mM EDTA buffer, and concentrations were estimated by comparison with known amounts of bacteriophage λ DNA (Boehringer) after agarose gel electrophoresis and staining (22). For dot blot hybridizations, aliquots with the amount of DNA to be spotted (0.25–5 μg) were precipitated with ethanol, centrifuged, dried, and redissolved in 10 μl of Tris/EDTA buffer. DNA samples were

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Abbreviations: AVG, aminoethoxyvinylglycine; Btp, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NAA, 1-naphthaleneacetic acid; E_m, transmembrane electrical potential difference.

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spotted on GeneScreen*Plus* (New England Nuclear) membrane. Denaturation, hybridization, and washings were as recommended by the manufacturer. Hybridized blots were autoradiographed (22). The probe, plasmid pMP30 (23), which covers about 60% of the T_L-DNA of strain 15834, was labeled with ^{32}P to $\approx 3 \times 10^8$ dpm by random-primed complementary strand synthesis with a reagent kit (Amersham).

Effects of NAA on Root Elongation and Proton Excretion. *L. corniculatus* roots were cultured on Mo/2 agar medium in the dark for 15–20 days and then on liquid Mo/2 medium for another 15–20 days. Before root elongation was measured, excised root tips were stored for 30–60 min in a 144-mm Petri dish containing 75 ml of Mo/2 medium. Random lots of 20 segments were sorted, measured, and transferred to 90-mm Petri dishes containing 25 ml of Mo/2 medium supplemented with various concentrations of NAA. The dishes were incubated in the dark at 28°C. After 24 hr the segments were taken out of the dishes and measured.

For measurement of proton excretion, 1-cm-long root tips were preincubated for 1 hr in 0.5 mM $CaSO_4$/10 mM glucose under vigorous aeration. Random lots of 40–60 segments were then transferred to 1-ml portions of 1 mM 2-(*N*-morpholino)-ethanesulfonic acid (Mes)/1,3-bis[tris(hydroxymethyl)methylamino]propane (Btp) buffer (pH 6.0) containing 0.5 mM $CaSO_4$, 5 mM K_2SO_4, 10 mM glucose, and various concentrations of NAA. Proton excretion was measured by back-titrating the incubation medium to the initial pH.

Inhibition of Ethylene Biosynthesis. To test the possible involvement of ethylene in the growth response of root tips to NAA, the action of two inhibitors of ethylene biosynthesis, aminoethoxyvinylglycine (AVG) and Co^{2+} ions, on root tip elongation was tested according to Mulkey *et al.* (24). Root tips were preincubated for 1 hr in Mo/2 medium supplemented with 1 μM AVG and 100 μM $Co(NO_3)_2$ and then incubated for 24 hr under the same conditions in the presence of NAA. To test the action of the inhibitors of ethylene biosynthesis on proton excretion, root tips were preincubated for 1 hr in the presence of AVG and Co^{2+} ions. Three sets of combined concentrations of AVG and Co^{2+} were tested: 0.1 and 10 μM, 1 and 100 μM, and 10 and 1000 μM. Proton excretion was then measured during 1 hr in the presence of the inhibitors.

Effect of NAA on the Transmembrane Electrical Potential Difference (E_m) of Protoplasts. Protoplasts were prepared from cultured *L. corniculatus* roots by a method slightly modified from that used for potato root protoplast isolation (25). In brief, 2- to 3-cm-long apical root segments were incubated in 5 mM Mes/NaOH buffer (pH 5.6) containing 1.5% (wt/vol) cellulase Onozuka RS (Yakult), 0.05% (wt/vol) pectolyase Y-23 (Seishin Pharmaceutical), 0.4% (wt/vol) hemicellulase (Sigma), 0.55 M mannitol, and macroelements of Binding *et al.* (26) overnight at 26°C in the dark. The resulting suspension was filtered through nylon cloth (53-μm mesh size). Protoplasts were collected and washed twice by centrifugation at 55 × *g* for 5 min, first with 5 mM Mes/NaOH buffer (pH 5.6) containing 0.25 M NaCl and macroelements of Binding *et al.* and then with the measurement medium [5 mM Mes/NaOH buffer (pH 5.6) containing 0.5 M mannitol and macroelements of Binding *et al.*]. The final protoplast pellet was suspended in the measurement medium at 10^5 protoplasts per ml and stored at 4°C until used. About 5×10^5 protoplasts were routinely obtained from 1 g of fresh root tips. E_m was measured according to the procedure used by Ephritikine *et al.* (27) to study the influence of NAA on tobacco mesophyll protoplasts. Protoplasts were immobilized in a microholder and impaled with a glass microelectrode according to the method of Rona and Cornel (28). Measurements were carried out at room temperature on aliquots of the stock protoplast suspension immediately after dilution to 5×10^3 protoplasts per ml with the measurement medium.

Uptake of NAA by Root Tips. Fifteen to twenty 1-cm-long root tips were preincubated under agitation for 40 min at room temperature in 1 ml of 1 mM Mes/Btp buffer (pH 5.6) containing 0.5 mM $CaSO_4$, 5 mM K_2SO_4, and 10 mM glucose. [^{3}H]NAA (27 nCi; Centre Energie Atomique, Gif sur Yvette, France; 1 nCi = 37 Bq) was then added (final concentration 1 nM or 100 nM) and incubation was continued for 30 min. Root segments were collected by filtration on GFA filters (Whatman), washed twice with cold incubation medium, and homogenized in aqueous 80% ethanol. The radioactivity of the homogenate was measured by scintillation counting.

RESULTS

Transformation of *L. corniculatus*. Lotus plants were inoculated with *A. rhizogenes* 15834 and 8196 and hairy-root cultures were established. Opine analysis was performed on extracts from these cultures (data not shown). One opine-positive line of each type of transformant was then selected and propagated to constitute the source of transformed material used in experiments described below. Transformation was assessed by dot blot hybridization with the T_L-DNA probe (data not shown). Strong signals were obtained with DNAs from both transformed lines, demonstrating the presence of T_L-DNA in the 15834-transformed line and confirming its homology with that of pRi8196 (29). Control DNAs (normal *L. corniculatus* and heterologous DNAs) did not hybridize with the probe.

Effect of NAA on Root Elongation. The elongation of normal roots displayed the classical shape for dose–response curves of roots to auxin (see for example ref. 30). It was stimulated by NAA at 0.1–10 nM and inhibited at concentrations >10 nM (Fig. 1). In contrast, the dose–response curves of roots transformed by *A. rhizogenes* 15834 or 8196 exhibited only the auxin-dependent inhibition of elongation. The characteristic shapes of the dose–response curves (Fig. 1) were consistently observed and were independent of variations in the rates of elongation observed from experiment to experiment for control roots of the normal and transformed types (results not shown). Thus, judging from the exogenous concentration of auxin necessary to get the incipient inhibition of elongation (i.e., about 10 nM for normal roots and 0.01–0.1 nM for transformed roots), one may conclude that transformed roots are 100–1000 times more sensitive to NAA than normal ones. Incubation with 1 μM AVG and 100 μM

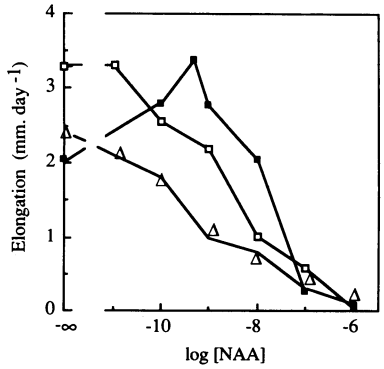

FIG. 1. Dose–response curves for elongation of apical root segments in the presence of NAA. Elongation was measured as the difference between the mean length of 20 root segments (initial length, about 1 cm) before and after 24 hr of incubation. The standard error for each point was <0.3 mm/day. Essentially identical results were obtained in three independent experiments. ■, Normal lotus roots; □, roots transformed by strain 8196; △, roots transformed by strain 15834.

Co^{2+} inhibited root tip elongation by 20–40% for both normal and 8196-transformed roots at all NAA concentrations tested (0.01 nM to 1 μM). Shapes of the dose–response curves were not significantly affected by this treatment (data not shown).

Effect of NAA on Proton Excretion. The net proton efflux from normal root tips was increased at low concentrations of NAA and was decreased at NAA concentrations >10 nM (Fig. 2). In contrast, proton excretion by both types of transformed roots was inhibited by NAA, even at the lowest concentration used. Thus, here again, judging from the threshold concentration above which proton excretion was inhibited (i.e, about 100 nM for normal roots and 0.1 nM for transformed roots), one may conclude that transformed roots are about 1000 times more sensitive to NAA than normal roots. Inhibition by 1 nM NAA of proton excretion by 8196-transformed root tips was not significantly affected when AVG and Co^{2+} were present in the incubation medium at, respectively, 0.1 and 10 μM, 1 and 100 μM, and 10 and 1000 μM (data not shown).

Effect of Fusicoccin on Proton Excretion. To test the specificity of the response of root tips to NAA in terms of proton excretion, the influence of fusicoccin was investigated (Fig. 3). The compound markedly stimulated the net proton excretion, as demonstrated for roots of various plant species (31–33). Over the range of concentration tested, no difference was observed between transformed and normal roots in their response to fusicoccin.

Effect of NAA on the E_m of Root Protoplasts. The protoplast populations appeared to be composed of three classes according to protoplast diameter, vacuolar size, and abundance of amyloplasts. The largest protoplasts were issued from root cortex cells. They exhibited larger vacuoles and fewer amyloplasts than root cap protoplasts. Their stability in the measurement medium at 4°C was good (<10% loss after 7 hr).

When cortex protoplasts were suspended in the measurement medium without NAA, only a small electrical polarization was observed, as described for other plant materials (27, 28, 34, 35). E_m values (mean ± SD) for independent protoplast populations prepared at different times were -2.6 ± 0.3 mV for normal protoplasts ($n = 5$ preparations) and $+1.5 \pm 0.3$ mV for transformed protoplasts ($n = 6$ preparations). For each preparation, the electrical polarization of protoplasts was reasonably stable. For example, the mean E_m value of a control protoplast population was -3.5 ± 0.7 mV at time zero and -3.0 ± 0.7 mV after 7 hr of storage at 4°C.

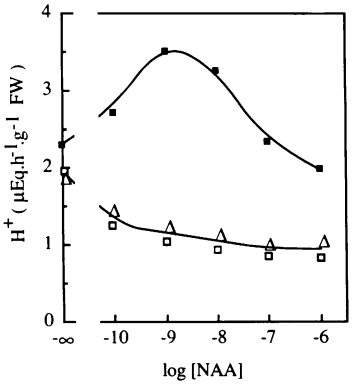

FIG. 2. Dose–response curves for proton excretion by apical root segments in the presence of NAA. Random lots of 40–60 segments were incubated as described in *Materials and Methods*. The pH of the medium was monitored with a pHM_{82} standard digital pH meter (Heito) with a combination glass microelectrode. The net flux of protons excreted during 1 hr was determined by titrating the incubation medium to the initial pH with 1 mM NaOH and is expressed as microequivalents (μEq) of protons excreted per hr per g of material fresh weight (FW). ■, Normal lotus roots; □, roots transformed by strain 8196; △, roots transformed by strain 15834.

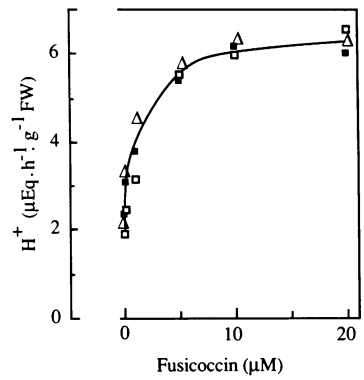

FIG. 3. Proton efflux of apical root segments in the presence of fusicoccin. Net proton efflux was measured and is expressed as in Fig. 2. However, the buffer concentration in the incubation medium was 2.5 mM Mes-Btp, and 10 mM NaOH was used for back-titration. ■, Normal lotus roots; □, roots transformed by strain 8196; △, roots transformed by strain 15834.

E_m values were consistently modified under the influence of NAA regardless of the intensity of the initial polarization of control protoplasts. For normal protoplasts, E_m was lowered when the medium was supplemented with NAA, with a maximal effect ($\Delta E_m = -4.0$ mV) between 10 and 100 nM NAA (Fig. 4). Increasing NAA concentrations reduced this hyperpolarization until it was canceled at 10 μM NAA. The modifications of E_m were established in less than 2 min after NAA addition and were stable during the time necessary for one series of experiments (about 40 min). NAA-induced E_m variations were reproducible from one experiment to another.

Protoplasts of transformed roots exhibited the same type of response in terms of timing, stability, intensity, and shape of the dose–response curve (Fig. 4). However, in comparison with normal roots, a remarkable shift in the dose–response curve was observed, as the maximal ΔE_m response was obtained for concentrations as low as 0.1 nM NAA. This response was completely canceled at 10 nM NAA. No difference was observed in the behavior of root protoplasts from the two transformed lines (Fig. 4).

Uptake of NAA by Normal and Transformed Root Tips. No detailed kinetic study of the uptake of NAA was done.

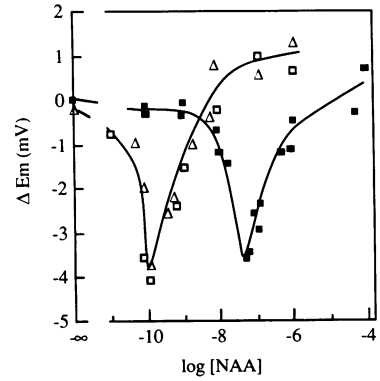

FIG. 4. Dose–response curves for E_m of root protoplasts in the presence of NAA. E_m measurements of 15 individual protoplasts were made for each condition. For each experiment a complete range of concentrations was tested on the same protoplast suspension. NAA-induced E_m variations (ΔEm, mV) were calculated from the reference value measured in the absence of the synthetic auxin. Standard errors were <0.3 mV. Identical results were obtained in three independent experiments. ■, Normal lotus roots; □, roots transformed by strain 8196; △, roots transformed by strain 15834.

Normal and 8196-transformed roots were simply compared in their ability to absorb NAA. Two NAA concentrations (1 nM and 100 nM) were selected according to the differential physiological responses they induce (Figs. 1–4). The duration of uptake experiments was 30 min, a period of time short enough to minimize auxin metabolization and long enough for differences in auxin sensitivity to be fully expressed. As a matter of fact, the difference in the intensity of NAA-induced proton excretion, routinely measured in 1-hr incubations (Fig. 2), is clearly expressed after only 20 min (results not shown). When incubated in the presence of 1 nM NAA, normal and transformed root tips absorbed respectively 3.29 ± 0.31 and 2.76 ± 0.41 pmol of NAA per g of fresh weight (n = 4 experiments). When the NAA concentration was increased to 100 nM, normal and transformed root tips absorbed respectively 316 ± 43 and 233 ± 14 pmol of NAA per g of fresh weight (n = 4 experiments). Thus the amounts of NAA absorbed by transformed roots were slightly lower than those absorbed by normal roots.

DISCUSSION

Experiments involving long-term response, such as root elongation measured after 24 hr, medium-term response, such as proton excretion measured after 1 hr, or short-term response, such as modification of E_m, which is established in less than 2 min, gave similar results. They demonstrate that transformed roots are 100- to 1000-fold more sensitive to exogenous auxin than normal roots (Figs. 1, 2, and 4). This suggests that the large differences observed in the sensitivity of transformed roots as compared to normal roots are due to the same early effect of exogenous auxin on root cell physiology. These results confirm that protoplasts, despite their very low initial electrical polarization compared to cells, react immediately to auxin by a modification of their E_m. The sensitivity of this response is fully correlated with the one displayed by the growth response of the organ or the cells they were isolated from. A similar correlation between electrical response of protoplasts and physiological response to auxin was observed previously for an auxin-resistant mutant of *Nicotiana tabacum* requiring about 10 times higher auxin concentrations for its cells to proliferate *in vitro*. Indeed, the auxin dose–E_m response curve of mesophyll protoplasts of this mutant was shifted by about 1 order of magnitude in comparison with protoplasts of a wild-type plant (27).

Several important questions are raised by the results presented here. They address the origin of increased sensitivity to auxin of transformed roots and the incidence of this increased sensitivity on the hairy-root phenomenon. Various hypotheses can account for our observations, such as (*i*) activation of hormone biosynthesis resulting in increased levels of endogenous auxins, (*ii*) modification of the uptake and accumulation of exogenous auxin, (*iii*) interference of ethylene with auxin effects, and (*iv*) modification of the reception–transduction system of the auxin signal.

According to the first hypothesis, one should expect a major role for T_R-DNA-borne auxin genes of agropine strains (e.g., 15834). However, these genes are certainly not responsible for the observations reported in this paper, as demonstrated by the identical behavior of the two transformed lines. In fact, auxin content of primary or *in vitro* cultured hairy roots was slightly lower than that of control roots (16).

The second hypothesis postulates that NAA uptake and intracellular accumulation should be much higher in transformed roots than in normal ones, resulting in the observed shift in dose–response curves. In fact, NAA uptake by transformed roots was slightly lower than that of normal roots. Thus, the increased sensitivity of normal roots cannot be due to a modification of their capacity to absorb and accumulate exogenous auxins.

Mulkey *et al.* (24) demonstrated that inhibition of ethylene production by AVG and Co^{2+} in maize roots results in a lower auxin sensitivity responsible for a shift of their growth-response curve to exogenous auxin. Lotus roots treated with the same inhibitors exhibited a behavior giving no support to the hypothesis of interference of ethylene in their response to auxin. AVG and Co^{2+} inhibited the elongation of lotus root tips, contrarily to their action on maize roots. Further, the inhibition of proton excretion induced by 1 nM NAA in transformed roots (Fig. 2) was not decreased by AVG/Co^{2+} treatment, suggesting that the differential sensitivity to NAA of proton excretion by normal and transformed roots was not dependent on a different ethylene status.

The most attractive hypothesis is certainly the one concerning a modification of the reception–transduction system in transformed roots. One of the merits of this hypothesis is to give a logical explanation for the fact that short-term and long-term responses show the same sensitivity ratio when normal and transformed roots are compared. Models in which transformed cells have an increased number of auxin receptors or an increased efficiency of the transduction system could account for our observations (36). Interestingly, fusicoccin-stimulated proton excretion was not modified by transformation (Fig. 3), whereas the effect of auxin on the same process was drastically affected (Fig. 2). This suggests that (*i*) the machinery responsible for proton excretion is not affected by transformation and (*ii*) the specific modification of the response to auxin concerns early events of the hormone action, such as reception or transduction.

The differential response to auxin that we observed relates to properties of differentiated and growing roots. We do not know how early the hypersensitivity to auxin is expressed, either as one of the first events after transformation or later on after the development of root primordia into hairy roots—i.e., whether it concerns both the induction of root formation and the characteristic responses of differentiated hairy roots to auxin studied here.

At first sight, it seems paradoxical that transformed roots could grow normally or even faster than normal roots with so high an auxin sensitivity and apparently normal auxin levels. In fact, dose–response curves for normal roots (Fig. 1) show that the same growth rate of 2 mm/day displayed by root tips in the presence of 10 nM NAA or the absence of NAA corresponds to very different physiological status. Even root tips treated with 1 nM NAA, which display a higher growth rate than controls, can be considered to be in a state of relative inhibition corresponding to a supraoptimal auxin level. The elongation curves for transformed roots (Fig. 1) suggest that in hairy roots, endogenous auxin levels are already above the concentration for optimal stimulation but nevertheless low enough to allow good growth and leave ample room for inhibition by exogenous auxin. The phenotype of hairy-root cultures can at least in part be accounted for by increased auxin sensitivity. For example, supposing that auxin is the limiting factor for secondary meristem initiation in normal roots, one can speculate that it is no longer limiting in transformed roots, where increased auxin sensitivity could be responsible for the high frequency of lateral root formation.

It is clear that the single T-DNA region of pRi8196 or the T_L-DNA of pRi15834 must somehow control auxin sensitivity. However, the role of T_R-DNA deserves further comment. Studies on agropine-type Ri plasmid mutants in which only the formation of primary roots was taken into account (37) have given the impression that the two T-DNAs of these plasmids are able to induce hairy root. However, the fact that roots formed from either normal cells or cells with T_R-DNA alone do not exhibit the hairy root phenotype (41) was

neglected. This points out the need to check that putative hairy roots carry hairy-root T-DNA, defined here as the single T-DNA region of pRi8196 or the T_L-DNA region of agropine-type Ri plasmids. Other criteria, such as the phenotype of *in vitro* cultivated roots or, when applicable, opine analysis, could be applied to fulfill this requirement.

Experiments in which T-DNA subfragments have been inserted into plant genomes, yielding phenotypes that are definitely related to the hairy root phenotype have been conducted in several laboratories (38–40). These experiments should make it possible to identify putative gene(s) responsible for increased auxin sensitivity.

The results presented here demonstrate a fundamental difference between crown gall and hairy root. In these two proliferative diseases, T-DNA genes responsible for cell proliferation act in radically different ways. In crown gall, phytohormone-biosynthetic genes are directly responsible for tumor growth. In contrast, hairy-root T-DNA appears to act in a more subtle way, by encoding a modified physiological response to hormone. In this respect the two systems would have evolved completely different mechanisms for achieving the same end, the triggering of cell proliferation. From this fundamental difference stems the interest of studies on hairy root. Whereas the crown-gall phenomenon is merely a problem of hormone synthesis and, therefore, has not provided better opportunities for understanding the hormone response than classical studies, the mode of action of hairy-root genes is clearly at a different level and may open new approaches to the study of auxin response.

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