

Single *rol* Genes from the *Agrobacterium rhizogenes* T_L-DNA Alter Some of the Cellular Responses to Auxin in *Nicotiana tabacum*¹

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ABSTRACT

Two kinds of cellular responses to auxin, the hyperpolarization of protoplasts and the division of protoplast-derived cells, were compared in *Nicotiana tabacum* plants transformed by different T-DNA fragments of *Agrobacterium rhizogenes* strain A4. Using transmembrane potential difference measurements to characterize hormonal sensitivity of mesophyll protoplasts, we found a 30-fold increase in sensitivity to auxin in protoplasts transformed by the whole Ri A4 T-DNA. Furthermore, the *rol* genes of the Ri A4 T_L-DNA, together or as single genes, were able to increase the sensitivity to auxin by factors up to 10⁴. The different effects of the single *rol* genes on the sensitivity of mesophyll protoplasts to auxin, *rolB* being the most powerful, were consistent with their respective rhizogenic effects on leaf fragments (A Spena, T Schm ulling, C Koncz, J Schell [1987] EMBO J 6: 3891–3899). No difference was seen concerning the effects of auxin on division of cells derived from normal or transformed protoplasts. These results suggest that only some cellular responses to auxin could be selectively altered by *rol* genes. They also show that *rol*-transformed tobaccos can be a model system to study auxin action in plants.

The soil bacterium *Agrobacterium rhizogenes* induces the hairy root disease on dicotyledonous plants by transferring a fragment of DNA (T-DNA) from its root inducing plasmid (pRi) into the plant cell (30). The proliferation of adventitious roots at the site of infection suggests auxin-like effects of the Ri T-DNA genes. Indeed, auxin biosynthetic genes have been identified on the T_R-DNA of agropine type strains but their presence is not required to induce the hairy root syndrome in tobacco (26). In addition, no clearcut change in the content of endogenous hormones was induced by the Ri transformation in various materials (6, 23; H. A. Van Onckelen, D. Chriqui, personal communication). Morphogenetic effects of *A. rhizogenes* have thus been recently reevaluated in terms of responsiveness of transformed cells to auxin (5). Hairy roots from different species (20, 21) as well as leaf fragments from

hairy root tobacco regenerants (23) were shown to be more sensitive to auxin than their normal counterparts. This feature suggests that Ri T-DNA genes induce the proliferation of transformed cells by a unique mechanism, as compared to *A. tumefaciens* oncogenes, which cause disease by encoding enzymes for hormone biosynthesis (30).

Recently, it was shown that a set of only a few genes of the pRiA4 T_L-DNA, namely the three genes *rolA*, *B*, and *C* (28), is able to induce the full hairy root syndrome in tobacco (4, 10, 24, 27). Moreover, each of these genes is able on its own to modify tobacco plant development (17, 18, 22, 24), *rolA* and *rolB* being able to induce transformed root formation (4, 24, 27).

In this paper we compared the auxin sensitivity of normal tobacco plants with sensitivity of plants transformed with the whole T-DNA of pRiA4 or the three genes *rolA*, *B*, and *C*. Auxin effects on the transmembrane potential difference of protoplasts and on cell division were investigated. According to the former test, single *rol* genes were found to increase the sensitivity of mesophyll protoplasts to auxin. However, the proliferation of both normal and transformed protoplasts-derived cells exhibited a similar dependence on auxin.

MATERIALS AND METHODS

Plant Materials

Transformation of tobacco, *Nicotiana tabacum* Petit Havana SR1, plants by different complements of the pRiA4 T-DNA was described elsewhere (18, 24). Transgenic plants were crossed with normal tobacco plants; seeds were germinated on a half-strength MS medium (15) complemented with 50 mg · l⁻¹ kanamycin. Kanamycin resistant plantlets were propagated *in vitro* and transferred to the greenhouse 1 month before protoplast isolation.

Mesophyll Protoplast Isolation

Leaf tissues were digested overnight as described by Caboche (2) in T₀ medium in the presence of 15 μM NAA³ and 5 μM BA. Protoplasts were washed twice in 0.3 M KCl, 5 mM CaCl₂, 1 mM Mes (pH 5.7), and resuspended in T₀ with no

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³ Abbreviations: NAA, 1-naphthaleneacetic acid; Em, transmembrane electrical potential difference; CaMV, cauliflower mosaic virus.

NAA ($2\text{--}5 \times 10^6$ protoplasts $\cdot\text{mL}^{-1}$). This stock solution was either plated for culture or stored at 4°C for electrophysiological measurements.

Effect of NAA on the Em of Protoplasts

Em was measured on freshly isolated protoplasts by the microelectrode technique as previously described (8). For each NAA concentration, an aliquot of the protoplast stock solution was diluted to 10^4 protoplasts $\cdot\text{mL}^{-1}$ in T_0 medium with the appropriate NAA dose. Fifteen to twenty individual measurements were carried out at room temperature during the following 30 min and a mean Em value was calculated.

Protoplast Culture at High Density

Axenicly prepared protoplasts were plated in T_0 medium with various concentrations of NAA at a density of 5×10^4 protoplasts $\cdot\text{mL}^{-1}$ and incubated in the dark at 26°C for 5 to 7 d. The occurrence of the first division was estimated by observation with an inverted microscope.

Protoplast-Derived Cell Culture at Low Density

The procedure followed was that described by Muller *et al.* (14). Briefly, protoplasts were cultured in the presence of $15 \mu\text{M}$ NAA for 7 d. Protoplast-derived cells were then washed three times in the culture medium C (14) depleted of auxin and plated in the medium C complemented with various doses of auxin at a density of 100 cells $\cdot\text{mL}^{-1}$. Developing colonies were counted after 1 month. Results were expressed as relative plating efficiency, *i.e.* the proportion of cells which developed colonies among the cells which had already divided at the time of low density plating.

RESULTS

Mesophyll Protoplasts Isolated from *A. rhizogenes* Transformed Tobacco Plants are More Sensitive to Auxin than Normal Ones

In the absence of exogenous auxin, mesophyll protoplasts isolated from normal tobacco plants exhibited a low basal Em, with a mean value ranging in three independent experiments from -7.3 to -8.1 mV (SE < 0.5 mV). Auxin effects on Em values were studied for NAA concentrations between 10^{-10} and 10^{-4} M (Fig. 1). As observed earlier with protoplasts of different plant materials (8, 20, 21), an auxin-induced hyperpolarization was detected for NAA doses up to the optimal concentration of 3×10^{-7} M ($\Delta\text{Em} = -2.5$ mV), with a relative depolarization for supraoptimal concentrations.

A similar approach was used for protoplasts isolated from tobacco plants regenerated from hairy roots induced by the *A. rhizogenes* agropine type strain A4. The obtained dose-response curve was comparable to that of normal protoplasts, according to its shape or the amplitude of the maximal response (Fig. 1). However, transformed protoplasts exhibited a maximal polarization at 10^{-8} M NAA and were thus 30 times more sensitive to auxin than normal protoplasts. A similar increase in sensitivity was observed in protoplasts transformed by the *A. rhizogenes* mannopine type strain 8196 (Table I).

The same type of result was obtained with protoplasts isolated from two independently transformed tobacco clones, transgenic for the three genes *rolA*, *B*, and *C*. These plants exhibited a typical hairy root syndrome, more pronounced than in the transformed plants studied above and comparable with the T' state described by Tepfer (25). Auxin treatment induced a hyperpolarization of *rolABC*-transformed protoplasts with a maximal amplitude at 3×10^{-10} M NAA thus indicating that these protoplasts were 1000 times more sensitive to auxin than normal protoplasts (Fig. 1).

Single Genes from the pRiA4 T-DNA Increase the Sensitivity of Mesophyll Protoplasts to Auxin

In order to further identify putative gene(s) responsible for the increased sensitivity to auxin of transformed protoplasts, plants transgenic for the single genes *rolA*, *rolB*, or *rolC* were analyzed through the auxin effects on the Em of mesophyll protoplasts. Examples of dose-response curves for NAA of such plants are given in Figure 2. For every *rol* gene, two independently transformed clones were tested (Table I). Each transformed clone exhibited an increased auxin sensitivity, which was reproducible when protoplasts from several plants were tested. Moreover, although a strong variability in the expression of transferred traits has been described for transgenic plants (29), no major discrepancy was found between protoplasts from independent clones transgenic for the same gene combination, except in the case of *rolA*. The extents of the induced increases were thus evaluated at 30 to 1,000, 3,000 to 10,000, and 10 for the *rolA*, *B*, and *C* genes, respectively.

Because the expression of the *rol* genes is regulated by the host plant cell (7, 19), we tested plants transgenic for *rolC* under the control of the strong and constitutively expressed 35S CaMV promoter. CaMV-*C* transformed protoplasts exhibited a maximal auxin-induced hyperpolarization at 10^{-9} M

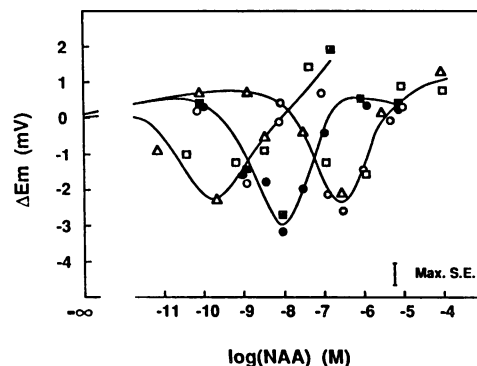


Figure 1. Effects of NAA on the Em of tobacco mesophyll protoplasts, normal (shaded symbols), transformed by the pRiA4 T-DNA (black symbols) (clone RSIII) or transformed by the *rolA* + *B* + *C* gene set (white symbols) (clone 1/27). Each Em value was calculated as the mean value of 15 to 20 individual measurements. Em value in the absence of NAA was taken as a reference. Em variations (ΔEm) from the reference value were plotted as a function of NAA concentration. Different symbols represent independent experiments, and the maximal standard error (Max SE) is indicated.

Table I. Modifications of the Sensitivity to Auxin of Tobacco Mesophyll Protoplasts Induced by Ri T-DNA Genes

The effects of auxin on the Em of protoplasts isolated from tobaccos transformed by different complements of the Ri T-DNA were studied as described in "Materials and Methods." Dose-response curves were established and the sensitivity to auxin of transformed protoplasts was expressed, by reference to the sensitivity of normal ones, as the ratio of the auxin concentrations inducing the maximal hyperpolarization in normal and transformed protoplasts, respectively. (Number in parentheses indicates the number of independent experiments for each clone).

Transferred Genes	Clone Reference	Relative Sensitivity to Auxin
None	SR1	1 (3)
T-DNA pRiA4	RS III	30 (3)
T-DNA pRi8196	VIII	30 (1)
ABC	1/27	1,000 (3)
	39	1,000 (1)
A	2I	1,000 (2)
	3I	30 (2)
B	1100,2B	10,000 (5)
	1100,2A	3,000 (2)
C	9a	10 (3)
	1c	10 (1)
CaMV-C	X	300 (2)

NAA (Table I) which corresponds to a 300-fold increased sensitivity to auxin.

With Respect to Cell Division, Normal and Transformed Protoplast-Derived Cells Exhibit Similar Sensitivities to Auxin

To further compare normal and transformed plants, the influence of auxin on the occurrence of the first division of cultured protoplasts was studied with NAA concentrations in the culture medium ranging from 0 to 10^{-4} M (Fig. 3A). In the absence of auxin, less than 1% of normal protoplasts had divided after 5 d. Auxin strongly stimulated the appearance of the first division with more than 20% protoplasts having divided after 5 d at the optimal concentration of 10^{-5} M. Higher concentrations revealed a toxic effect of auxin. Protoplasts transformed by the Ri T-DNA or the single *rolB* gene exhibited a similar behavior, the optimal concentration of 10^{-5} M inducing comparable division rates (20–40%).

When cultured at a rather high population density (5×10^4 cells·mL⁻¹), protoplasts and cells derived from them modify drastically auxin concentration in the medium (2, 3). The proliferation of protoplast-derived cells incubated at low density (100 cells·mL⁻¹) provides a system with conditions of minimal auxin degradation. For this, mesophyll protoplasts were cultured in the presence of 10^{-5} M NAA. After the first divisions were observed, microcolonies were diluted and their proliferation was studied as a function of auxin concentration in the culture medium (Fig. 3B). While protoplast-derived cells were unable to divide in the absence of auxin, their division was stimulated by low auxin concentrations up to 10^{-7} M where optimal plating efficiencies (20%) were reached. Higher auxin concentrations were found to be cytotoxic. Transformed materials needed similar auxin concentrations

to divide, the optimal concentration for cell division being 10^{-7} M as for normal cells.

DISCUSSION

Auxin effects on elongation and H⁺-extrusion of root tips allowed Shen *et al.* (20, 21) to show that hairy roots of a variety of plant species displayed an increased sensitivity to auxin. Such a rise in sensitivity was also displayed at the cellular level by hairy root protoplasts in their electrical response to auxin (20, 21). The auxin-induced hyperpolarizations measured here on tobacco mesophyll protoplasts, despite their small amplitude (2–3 mV), are reproducible and statistically significant (SE < 0.5 mV) (see, for example, Figs. 1 and 2). The rationale of the low Em values of protoplasts and the low amplitude of their response to auxin has been recently investigated by Shen *et al.* (21) on root protoplasts of *Catharantus roseus*. Reducing ion leakage from the microelectrode tip and the intensity of a chloride conductance at the plasmalemma increased the initial polarization of the protoplasts from +4 mV to -65 mV and the intensity of response to auxin from -5 mV to -20 mV. Interestingly, the shape of

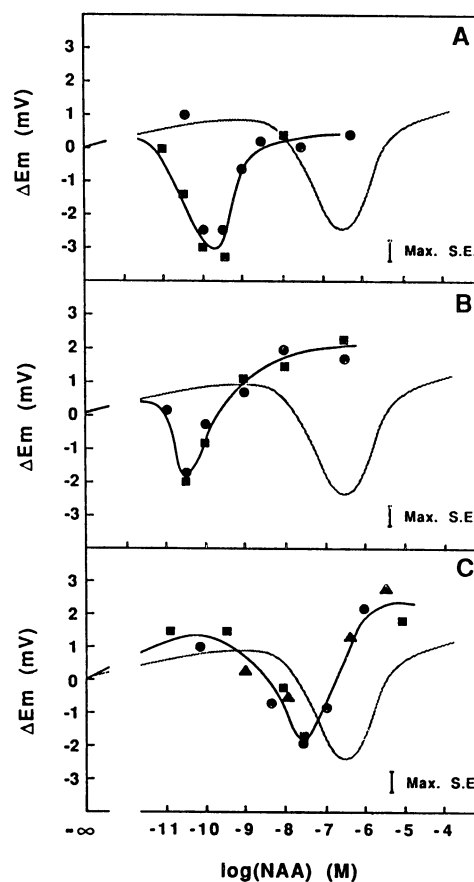


Figure 2. Effects of NAA on the Em of mesophyll protoplasts from *rolA*, *rolB*, or *rolC* transformed plants. A, *rolA* transformed tobacco (clone 2I); B, *rolB* transformed tobacco (clone B1100,2B); C, *rolC* transformed tobacco (clone 9a). The response of normal protoplasts (dashed line) is reported from Figure 1. For experimental details see legend of Figure 1 and "Materials and Methods."

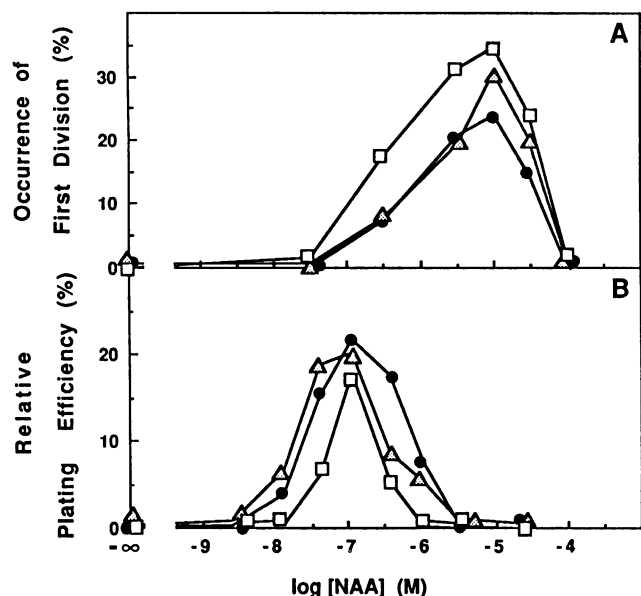


Figure 3. Effects of NAA on divisions of protoplasts and protoplast-derived cells from normal or transformed tobacco plants. A, Occurrence of first division in protoplast culture at high density; B, proliferation of colonies in protoplast-derived cell culture at low density. Black symbols, normal; white symbols, pRiA4 T-DNA transformed (clone RSIII); shaded symbols, *rolB*-transformed (clone B1100,2B).

the dose-response curve to auxin and, more specifically, the optimal concentration were independent of the conditions used for Em measurements. Using the same approach here, tobacco mesophyll protoplasts were impaled with microelectrodes filled by either 1 M KCl (standard condition) or 50 mM KCH_3CO_2 and basal Em values of -7.0 ± 0.4 mV and -23.6 ± 0.9 mV, respectively, were measured. In addition to this limited rise in the Em value, the estimated auxin-induced hyperpolarization was not amplified, whereas standard errors were increased. This result led us to use classical KCl 1 M-filled microelectrodes to characterize the hormonal sensitivity of normal and Ri transformed tobacco mesophyll protoplasts. In this paper we extend the observations of Shen *et al.* (20, 21) by showing that the increased hormonal sensitivity of the electrical response is not restricted to root protoplasts but is also strongly expressed in leaf protoplasts, and that the transformation by single *rol* genes, either *rolA*, *B*, or *C*, is sufficient in tobacco to induce such an increase.

Striking enhancements of adventitious rhizogenesis in tobacco by single *rol* genes have been reported by several authors (4, 24, 27). The respective efficiencies of these genes: $B > A > C$ have been evaluated by Spina *et al.* (24) through leaf disc inoculation experiments. Using the same constructs, an analogous classification has been determined here according to the *rol* genes effects on the sensitivity to auxin of the electrical response of mesophyll protoplasts. Such a correlation between rooting capacity and the sensitivity to auxin of the Em of protoplasts has already been described in the case of a nonrooting auxin-resistant mutant of *N. tabacum* (8). These data suggest that the increased sensitivity to auxin of *A. rhizogenes* transformed cells might be one of the determinants of their high rooting capacity.

The distinct quantitative effects of the *rol* genes on the sensitivity of protoplasts to auxin, as well as on rhizogenesis, might reflect differences in gene expression levels. Recent results on the regulation of the *rolB* promoter in tobacco mesophyll protoplasts have shown that our standard procedure for protoplast isolation, in the presence of 15 μM NAA, is optimal for *rolB* expression (13). Furthermore, the sensitivity to auxin of *rolB*-transformed protoplasts could be modulated in good correlation with *rolB* expression (C. Maurel, H. Barbier-Brygoo, M. Bouvier-Durand, J. Tempé, J. Guern, unpublished data). The importance of *rol* gene expression levels is also illustrated by the differences in auxin sensitivity found between protoplasts expressing the *rolC* protein under the control of the *rolC* or the 35S CaMV promoters. However, in both cases, hormonal sensitivity was lower than that conferred by *rolB* and, thus, the *rol* gene products have likely different potencies on auxin sensitivity.

The functional analogy exhibited by the three *rol* genes at the cellular level stands in contrast with their distinct effects on whole plant morphology. For instance, CaMV-*C* plants are dwarf with side shoot formation whereas CaMV-*B* plants exhibit leaf necrosis (18). Although the precise functions of the *rol* gene products remain unknown, we assume that pleiotropic effects of the *rol* genes on several cellular functions, one of them being the sensitivity to auxin, could account for the diversity of morphogenetic effects. The molecular bases of the increased sensitivity to auxin of Ri transformed cells are also poorly understood. Shen *et al.* (20) first suggested that early events of the reception and transduction of the auxin signal could be affected since short-term (<2 min) and long-term (24 h) auxin responses are both modified in hairy roots. Such an assumption is further sustained by the recent finding that Ri-transformed protoplasts might exhibit an increased number of plasmalemma sites recognized by an antibody raised against an auxin-binding protein from corn coleoptiles (1).

In strong contrast with their effects on the sensitivity to auxin of the electrical response of protoplasts, the whole Ri T-DNA, *rolB* alone or even the constitutively expressed CaMV-*C* gene (data not shown) did not modify the cell division response to the hormone. Auxin-controlled cell division is a long-term response and further work is needed to evaluate to what extent it depends on auxin metabolism. Normal and transformed cells should be compared in these respects. Nevertheless, the situation of Ri-transformed cells differs from that observed by Ephritikhine *et al.* (8) when comparing an auxin-resistant mutant to a wild-type tobacco. In this case, the auxin-induced hyperpolarization of protoplasts and the auxin-controlled cell division indicated for the mutant similar reduction in auxin sensitivity. Moreover, in this system, both responses exhibited the same specificity toward a set of auxin analogs (8). Such results led to the idea that the same auxin receptors could be involved in both the short-term cell-surface and the long-term cell division responses. On the contrary, the results obtained in the present work might suggest the existence of different reception-transduction pathways for auxin, controlling cell division on the one hand and cell elongation and rhizogenesis on the other hand. Such a possibility was already proposed by Nakamura *et al.* (16). As a matter of fact, a variety of binding sites for

auxin have been described (12) but the functional characterization of most of them is still lacking (9).

Though unexplained, the selectivity of *rol* gene effects on some auxin responses might participate in the intriguing compatibility of Ri transformation with whole plant regeneration and development. For instance, *rolB* increases by 10,000-fold the auxin sensitivity of protoplasts but *rolB*-transformed plants do develop normally at least for the major morphogenetic traits (24). The more pronounced phenotypic alterations exhibited by CaMV-*B* transformed tobacco show that the fine control by the plant of *rolB* gene expression (7, 13, 19) should be important. The viability of these plants can also be explained if the auxin-controlled cell division, essential for any morphogenetic process, is effectively insensitive to the *rolB* encoded function.

In conclusion, several plant genes have been genetically identified, whose mutation modifies the cellular responses to phytohormones (11), but none of these genes has been isolated yet. Here we have shown that single *rol* genes of *A. rhizogenes* T₁-DNA are able to increase the sensitivity of protoplasts to auxin. It is the first time to our knowledge that such kind of direct evidence has been presented in the field of phytohormone studies. These findings give the opportunity to study the specific modulation of some auxin responses in genetically defined model systems and *rolB*-transformed protoplasts are currently used in this respect.

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