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rol A modulates the sensitivity to auxin of the proton translocation catalyzed by the plasma membrane H^+-ATPase in transformed tobacco

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In order to investigate the effect of the *rol* A gene product on the plasma membrane response to auxin, a clone of *rol* A-transformed tobacco was prepared. Auxin sensitivity was measured by the auxin concentration which induced the highest stimulation of H^+-ATPase-mediated proton translocation on isolated plasma membrane vesicles. Both transformed and control plants exhibited identical auxin-sensitivity changes during vegetative and induction to flowering periods. However the sensitivity of flowering-transformed plants was 100-times higher than that of control plants. Consistent observations were also made when using *rol* A+B+C-transformed plants. These results suggest that the *rol* A gene product could either participate in or affect the reception/transduction pathway of auxin signal at the plasma membrane.

rol A gene; Auxin; Plasma membrane H^+-ATPase; Flowering

1. INTRODUCTION

Plants transformed by the T-DNA of *Agrobacterium rhizogenes* exhibit phenotypic abnormalities that are consistently observed in a variety of transformed species. As first described in tobacco, the predominant phenotypic features are: wrinkled leaves, decreased apical dominance, shortened stems, adventious root formation, modified root geotropism, alteration of flower morphology and reduced fertility [1,2]. A distinction has been made between the usual Ri phenotype (T), and an extreme form of the Ri phenotype, designated T′ [2]. Plants transformed by a group of three genes from the T_L-DNA, genes 10, 11 and 12, corresponding to the loci *rol* A, B and C [3], exhibit all these characteristics. The individual influences on plant development of genes 10, 11 and 12 have been described [4], but the most prominent aspects of the modified phenotype (wrinkled leaves and short stems) can be induced by the expression of gene 10 alone (our results; [4,5]). We have shown that the degree of phenotypic alteration is correlated with the level of expression of gene 10 (unpublished results). It has also been shown that reversion of the T′ phenotype to the less pronounced T phenotype, which is sometimes observed in transformed plants, is associated with decreased expression of gene 10 [5].

Recently, it has been shown in tobacco leaf protoplasts that expression of genes *rol* A+B+C enhances auxin sensitivity of stimulation of the transmembrane electrical potential difference [6]. In addition *rol* A, *rol* B and *rol* C can also enhance this response individually, *rol* B being the most effective and *rol* C the less effective one [6]. Beside these functional indications, almost nothing was known until very recently concerning the nature of *rol* genes-encoded proteins. In the case of *rol* C, however, direct biochemical data are now available [7] showing that the protein can hydrolyse cytokinin glucoside conjugates. For *rol* B, the gene product would hydrolyse indole glucosides [8]. Thus, the *rol* C and *rol* B gene products could be responsible for increased levels of free cytokinin and auxin, respectively, via hydrolysis of glucoside conjugates.

The function of the *rol* A gene remains at this time not known, and the mechanism of its effect on auxin sensitivity of membrane potential has not been established. It could involve modifications of the plasma membrane H^+-ATPase activity, as this enzyme is the main electrogenic pump of the plant plasmalemma [9], and is known to be responsive to auxin [10]. In fact it was recently shown that auxin sensitivity of proton translocation by tobacco leaf plasma membrane H^+-ATPase can be studied in vitro, and that ATPase sensitivity depends critically on plant development stage; whereas for vegetative or flowering plants the auxin sensitivity is relatively low, a transient 1000-fold increase takes place during floral induction [11,12].

In this work we purified plasma membrane vesicles

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from leaves of normal and transformed tobacco plants and compared the auxin sensitivity of H^+-ATPase-mediated proton translocation throughout plant development. It was observed that auxin sensitivity is specifically increased in *rol* A-transformed plants during the flowering stage, but not during other stages of plant development.

2. MATERIALS AND METHODS

Transformed tobacco plants (*Nicotiana tabacum* cv Xanthi) were obtained by leaf disc transformation according to ref. [13]. Transformed kanamycin resistant shoots were obtained on medium [14] containing 0.1 mg/l NAA (naphthalene acetic acid), 1 mg/l BA (benzyladenine), 50 mg/l kanamycin, 500 mg/l cefotaxime. Plant DNA was isolated according to ref. [15]. 10 μg of DNA were cleaved by appropriate enzymes, submitted to electrophoresis on agarose gels, transferred to nylon membrane and hybridized with ^{32}P-labelled probes as described elsewhere [16].

For the investigation of auxin effects, plants were grown from seed in a controlled chamber (20°C, 16000 lx, 16 h photoperiod). Measurement of the rate of leaf appearance on controls and on plants transformed with *rol* A or with the T_L DNA of *A. rhizogenes* showed that they exhibited identical developmental characteristics. Leaf plasma membrane vesicles were purified from a microsome suspension by phase-partitioning as described previously [11,12]. Biochemical characterization indicated that vesicles contained less than 10% of other contaminating membranes [11,12]. Proton translocation was monitored on native vesicles by the fluorescence-quenching rate of 9-amino-6-chloro-3-methoxy-acridine (ACMA) after addition of 250 μM ATP to start the reaction [11,12]. The assay medium (2 ml) contained 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-Tris buffer (pH 6.5), 2 μM ACMA, 600 μM $MgSO_4$, 50 mM KNO_3, 50 mM KBr and 25 μg of membranes. Auxin was added from a 100-fold concentrated solution in acetonitrile. Controls included 1% acetonitrile and were performed in quadruplicate for each plasma membrane sample (at the beginning, at the end and during the experiment). Mean variation coefficients for these controls amounted to 4.5% for control plants, and to 4.6% for *rol* A-transformed plants. For auxin concentrations inducing the highest stimulation of the proton translocation, measurements were repeated from two to four times. Mean variation coefficients for these points amounted to 3.5% for control plants, and to 3.6% for *rol* A-transformed plants.

3. RESULTS AND DISCUSSION

Two clones of transformed tobacco plants were used for these studies. The clone A4(10) is transformed by the wild-type A4 strain (D. Tepfer, unpublished). The extent of the transferred DNA is represented in Fig. 1. At least two copies of T_L-DNA are found in the genome of these plants, one shorter than the other. The T_R-DNA does not contain the *aux* 1 and *aux* 2 genes responsible for auxin biosynthesis, as no hybridization was detected with *Sal*I fragment 6. When *Sal*I fragment 8 was used as probe, only bands corresponding to border fragments were observed. With this probe, no bands corresponding to internal fragments have been detected for *Bam*HI, *Hin*dIII, *Sal*I and *Xho*I digests. The clone *rol* A(3) has been obtained after tobacco leaf disc transformation by the strain GV3101(pMP90, pMRK10) [17]. The only fragment transferred in these plants is the *Eco*RI(9075)–*Nru*I(10968) fragment from the T_L-DNA bearing gene 10, corresponding to the *rol* A locus. One or two copies of this fragment have been integrated in the genome, as deduced from the intensity of bands revealed on autography.

Addition of ATP to plasma membrane vesicles prepared from leaves of various types of tobacco led to quenching of ACMA fluorescence which was reversed upon further addition of nigericine (not shown). This indicated that the plasma membrane H^+-ATPase forced protons to accumulate inside the vesicles in agreement with previous results obtained with comparable tobacco leaf plasma membrane vesicles [11,12]. No clear difference concerning the rates of quenching was observed between the materials. This suggested that the proton translocation itself was not really dependent on the genotype used. In the presence of IAA, a concentration-dependent biphasic response was observed for *rol* A-transformed plants as well as for control plants. With

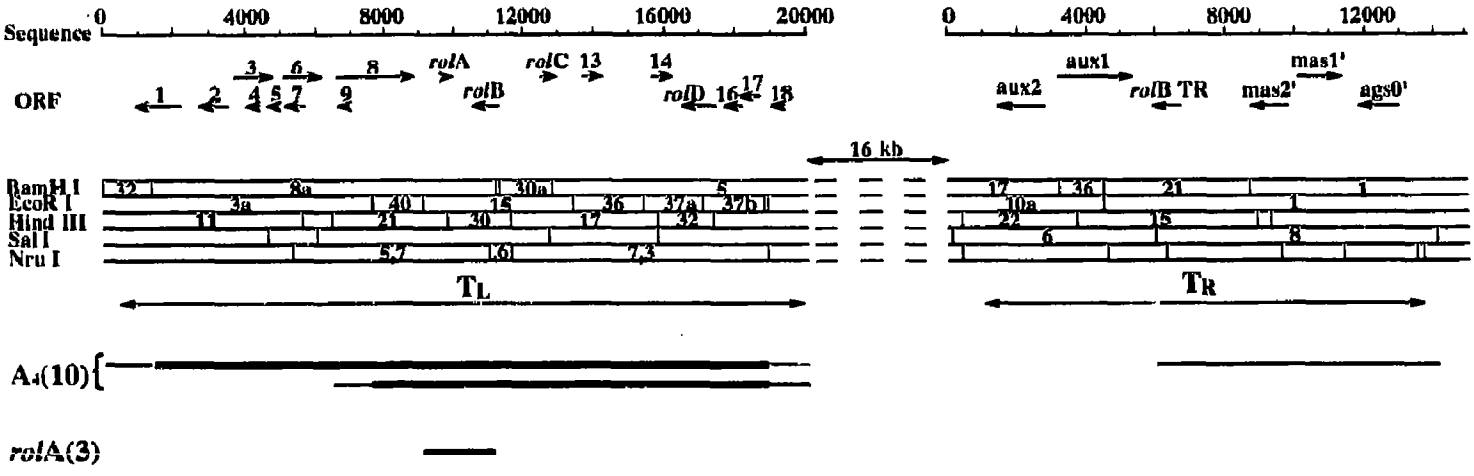

Fig. 1. Structure of the T-DNA in tobacco plants A4(10) and *rol* A(3). From top to bottom are shown successively: sequence coordinates, deduced open reading frames, restriction map, and the extent of the T-DNA determined by DNA blot hybridization, in which internal fragments are indicated by thick lines and border fragments by thin lines.

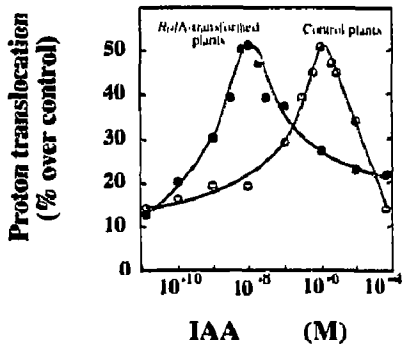

Fig. 2. Auxin stimulation of H^+-ATPase-mediated proton translocation in plasma membrane vesicles from normal and *rol* A-transformed tobacco leaf. 14-week-old plants were used for membrane purification. Proton translocation was measured by the initial fluorescence quenching rate of ACMA. For normal plants a maximum of 59 ± 5 (n = 3) % quenching · min^{-1} · mg^{-1} was reached in the presence of 10^{-6} M IAA (the control activity, in the absence of IAA, amounted to 39 ± 3 (n = 4) % quenching · min^{-1} · mg^{-1}). For *rol* A-transformed plants, the corresponding activities were of 90 ± 4 (n = 2) and 59 ± 4 (n = 4) % quenching · min^{-1} · mg^{-1}, respectively, in the presence of 10^{-8} M IAA and in the absence of auxin.

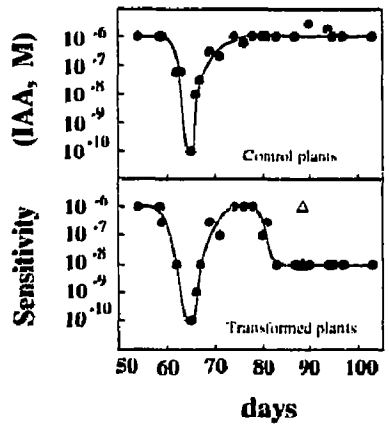

Fig. 3. Auxin sensitivity changes during plant development. Sensitivity (taken as the auxin concentration inducing the highest stimulation in proton translocation experiments) is plotted vs. the number of days after sowing. Data are from two complete experiments with ca. 60 tobacco plants each for each type of plant material. For each point all leaves from a few plants were harvested and processed for plasmalemma purification. Upper panel: normal tobacco plants. Lower panel: *rol* A-transformed plants; triangles show sensitivity values for 88-day-old tobacco transformed with the T_L-DNA of *A. rhizogenes* with (full triangle) T′ phenotype or (empty triangle) reverted T phenotype.

increasing IAA concentration, the H^+-ATPase mediated proton translocation was at first stimulated (Fig. 2), but beyond a certain concentration further addition of auxin led to a decrease of the stimulation. For 14-week-old plants (Fig. 2), the auxin concentration inducing the largest stimulation was two orders of magnitude lower for plasma membrane vesicles from transformed plants than for the control plants. Such biphasic dose–response curves are typical for tobacco plasma membrane vesicles [11,12]. As they are also observed here with *rol* A transformed plants (Fig. 2), it can be concluded that *rol* A does not modify the nature of the auxin response but that it could change the ATPase sensitivity to auxin.

As auxin concentrations inducing the highest stimulation (termed auxin sensitivity) have been previously shown to change during plant development [11,12], a systematic investigation was performed on the two types of plants. For control tobacco plants (Fig. 3, upper panel) quite high auxin concentrations (10^{-6} M) were necessary for ATPase stimulation when plants were vegetative (up to ca. 60 days). The sensitivity dramatically increased thereafter during a period of a few days which has been shown to be related to floral induction [12]. Thereafter, sensitivity then decreased and returned to ca. 10^{-6} M for flowering plants (the mean sensitivity for control plants between days 75 and 110 was of 1.3 10^{-6} ± 0.7 10^{-6} M, n = 14). These sensitivity changes are identical to those previously observed, although the sensitivity is slightly higher here [11,12]. This difference could be due to the use of a new batch of seeds. For *rol* A-transformed plants (Fig. 3, lower panel) an identical pattern was observed up to ca. 75 days. However, the sensitivity increased then, in one week, to 10^{-8} M IAA (the mean sensitivity for *rol* A-transformed plants between days 83 and 110 was of 1.0 10^{-8} ± 0.1 10^{-8} M, n = 9). At this stage *rol* A expressing plants were therefore 100 times more sensitive to auxin than controls. In complementary experiments the auxin sensitivity was determined for plants transformed with the T_L-DNA of *A. rhizogenes* which either exhibited the extreme T′ phenotype or moderate T phenotype. At the same developmental stage, the proton translocation for the T′ plants was stimulated by 10^{-8} M IAA (Fig. 3, lower panel, full triangle), whereas for the T plants the auxin sensitivity was of 10^{-6} M IAA (Fig. 3, lower panel, empty triangle).

It appears therefore that plants which exhibit pronounced phenotype modifications (due to transformation with either *rol* A or the T_L-DNA of *A. rhizogenes*) are 100 times more sensitive to auxin than plants exhibiting either the normal phenotype or the moderate T phenotype. This suggests that the increased sensitivity of transformed plants should not be due to a particular insertion position of the *rol* A gene in the plant genome, but rather reflects the effect(s) of the *rol* A gene product. This product is still unknown. However, effects reported here were observed in vitro with purified plasma membrane vesicles and two hypothesis can be therefore proposed: (i) first the *rol* A gene product could participate directly to the reception/transduction pathway of auxin signal at the plasma membrane; (ii) alternatively the *rol* A gene product could indirectly affect the synthesis or maturation of some element(s) of the reception/transduction pathway.

In conclusion it could be emphasized that the differential sensitivity to auxin of plasma membrane vesicles from flowering normal or transformed plants offers a convenient system which is suitable for comparative

biochemical analysis. Such analysis should allow us to discriminate between a direct effect of the *rol* A gene product at the plasma membrane level and an action elsewhere in the cell.

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