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Alterations of Auxin Perception in *rolB*-Transformed Tobacco Protoplasts[1]

Time Course of *rolB* mRNA Expression and Increase in Auxin Sensitivity Reveal Multiple Control by Auxin

Christophe Maurel[2], Nathalie Leblanc, Hélène Barbier-Brygoo*, Catherine Perrot-Rechenmann, Michelle Bouvier-Durand, and Jean Guern

Institut des Sciences Végétales, Centre National de la Recherche Scientifique, F-91198 Gif-sur-Yvette Cedex, France

Expression and physiological effects of the root-inducing *rolB* gene of *Agrobacterium rhizogenes* T-DNA were studied simultaneously in tobacco (*Nicotiana tabacum*) mesophyll protoplasts. The kinetic study of the expression of *rolB* mRNA following exogenous auxin application showed that auxin transiently stimulated *rolB* expression, with mRNA levels starting to accumulate 6 to 9 h after auxin was supplied and increasing 300-fold after 12 to 18 h. The parallel study of the auxin sensitivity of *rolB*-transformed protoplasts, as assayed by their electrical response to the hormone, showed that the auxin treatment generated an increase in sensitivity by a factor of up to 100,000, whereas in untransformed protoplasts the same auxin treatment induced an increase in auxin sensitivity that never exceeded 30- to 50-fold. This reflects a strong cooperative effect of auxin and *rolB* in transformed protoplasts. Surprisingly, the maximal increase in sensitivity was observed several hours before the maximal accumulation of *rolB* mRNA, suggesting that the dramatic control of auxin sensitivity by auxin in *rolB*-transformed protoplasts requires only low levels of *rolB* expression. Antibodies directed against ZmER-abp1, the major auxin-binding protein from maize, differentially altered the auxin sensitivity of the electrical response of *rolB*-transformed and normal protoplasts. This suggests that alterations of the auxin reception-transduction pathway at the plasma membrane of *rolB*-transformed protoplasts may account for their increased auxin sensitivity.

Auxin has been implicated in the control of growth and developmental processes at the cellular, tissue, and plant levels (reviewed by Davies, 1987). The molecular mechanisms by which this phytohormone affects so many different responses are not yet known. In the last few years a large number of auxin-binding proteins have been reported, with much discussion as to their possible role as auxin receptors (Venis and Napier, 1992). One of these, maize ZmER-abp1, has been characterized in detail and has been used to explore the perception of auxins at the plasma membrane of different plant cells. Several lines of electrophysiological evidence have shown, for example, that antibodies directed against ZmER-abp1 alter the auxin response of the plasma membrane of tobacco (*Nicotiana tabacum* L.) mesophyll protoplasts (Barbier-Brygoo et al., 1989, 1991) and of maize coleoptile protoplasts (Rück et al., 1993), and specific fragments of this protein modify the electrical properties of the plasma membrane of *Vicia faba* guard cells (Thiel et al., 1993).

Further insight into the mechanisms of auxin perception at the plasma membrane can be provided by the characterization of plant systems altered in their responses to auxin. Protoplasts from tobacco plants transformed by *Agrobacterium rhizogenes* T-DNA, especially by the single *rolB* gene, exhibit an increased sensitivity of their electrical response to auxin (Maurel et al., 1991b). This finding is in agreement with a number of reports in which an increased sensitivity to auxin was described for excised organs, isolated protoplasts, or membrane vesicles from various plant materials containing the whole pRi T-DNA or some of its subfragments (Shen et al., 1988, 1990; Spano et al., 1988; Barbier-Brygoo et al., 1991; Vansuyt et al., 1992). These findings suggested that morphogenetic effects of pRi T-DNA transformation might involve changes in hormone responsiveness and/or hormone content. Biochemical characterization of the RolB protein allowed Estruch et al. (1991) to show that RolB displays a β-glucosidase activity. However, recent experimental evidence from the same group (Spena et al., 1993) and others (Nilsson et al., 1993; Delbarre et al., 1994) invalidate the early proposal that this activity is involved in the direct release of free auxin from β-glucoside conjugates. Thus, the function of the RolB protein in transformed cells remains unknown.

At present, the increased auxin sensitivity of the membrane response of transformed tobacco protoplasts remains the only identified cellular effect of the *rolB* gene. As such, it deserves

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* Corresponding author; fax 33-1-69-82-37-68.

Abbreviations: E_m, transmembrane electrical potential difference; GUS, β-glucuronidase; NAA, 1-naphthaleneacetic acid; T-DNA, transferred DNA.

to be analyzed in more detail to obtain additional information on *rolB* action and to allow additional use of *rolB*-transformed plants in auxin perception studies. In the present work, taking into account the auxin dependence of *rolB* promoter expression (Maurel et al., 1990; Capone et al., 1991), we investigated the questions of how fast *rolB* mRNA expression was stimulated in *rolB*-transformed protoplasts by the application of exogenous auxin, and how this stimulation was associated with alterations in the sensitivity of their membrane response to auxin. We also explored changes at the plasma membrane of *rolB*-transformed protoplasts that could be associated with the changes in their sensitivity to auxins. For this we used IgGs directed against the maize protein ZmER-abp1 as a probe for immunologically related proteins at the surface of tobacco protoplasts.

MATERIALS AND METHODS

Bacteria and Plasmid Manipulations

Bacterial strains and recombinant DNA techniques were as previously described (Maurel et al., 1990). For construction of pCMB-B:GUS, pBSE15, a BlueScript M13+ vector (Stratagene) carrying the *Eco*RI 15 fragment of pRi1855 was used to isolate a 1377-bp *Sal*I-*Bam*HI fragment containing the first 4 bp of the *rolB* coding sequence and 5′ flanking, noncoding sequences. This fragment was cloned in the corresponding unique restriction sites of pBI101 (Jefferson et al., 1987), a plant transformation vector carrying the GUS coding sequence with the nopaline synthase polyadenylation elements, giving rise to pBGUS10. A 2593-bp *Hpa*I-*Sma*I fragment encompassing the *rolB* gene with 1177 bp of the 5′ and 646 bp of the 3′ noncoding sequences was also isolated from pBSE15 and cloned in the *Sma*I site of BlueScript M13+. The resulting plasmid was linearized by digestion with *Nae*I and cloned in pBGUS10 at the unique *Hpa*I restriction site located 1177 bp upstream of the *rolB* start codon, thus giving rise to a construct carrying a *rolB* gene and a *rolB*:GUS gene fusion in tandem.

Plant Transformation and Growth

DNA constructs were introduced into *Nicotiana tabacum* cv Xanthi via *Agrobacterium tumefaciens* transformation in leaf disc inoculations according to Horsch et al. (1985). The transgenic plants selected for further study were grown in a greenhouse (22°C; 9 h of light per d) and corresponded to either in vitro-propagated primary transformants or to their progeny obtained after selfing.

Southern Blot Analysis

Plant DNA was purified from leaves as described (Chilton et al., 1982), digested with the restriction enzymes indicated, subjected to electrophoresis on a 1% agarose gel (10 μg of DNA per lane), and blotted on a GeneScreenPlus membrane (New England Nuclear). DNA blotting, hybridization to DNA probes, and membrane washings were performed according to the manufacturer's manual. DNA fragments of PCR amplification were subjected to electrophoresis on a 2.5% Nusieve agarose gel (FMC, Rockland, ME) and blotted on Hybond N+ membrane (Amersham) in alkaline conditions. ^{32}P-labeled probes (1×10^9 to 2×10^9 dpm/μg DNA) were obtained by random priming using a labeling kit (Amersham or Boehringer Mannheim).

Mesophyll Protoplast Isolation and Culture

Leaf tissues were digested over a 13- to 15-h period in To medium (Caboche, 1980) with 0.1% cellulase R10 (Yakult), 0.02% macerozyme R10 (Yakult), 0.05% driselase (Sigma), 5×10^{-6} M benzylaminopurine, and no auxin. After digestion, protoplasts were washed twice at 4°C in 0.3 M KCl, 5 mM $CaCl_2$, 1 mM Mes, pH 5.7. Protoplasts were then resuspended in To medium without auxin, at a density of 5×10^6 protoplasts/mL, and stored at 4°C until use within an 8-h period. In all kinetic experiments, isolated protoplasts were cultured at 21°C for the indicated time in To medium (5×10^5 protoplasts/mL) in the absence or the presence of 10^{-5} M NAA; protoplasts were washed twice in the absence of auxin prior to electrophysiological measurements. During the culture period used in these experiments (up to 24 h), *rolB*-transformed and untransformed protoplasts behaved similarly, and auxin-treated protoplasts did not differ from control protoplasts. As revealed by calcofluor staining, all preparations underwent wall regeneration, starting a few hours after isolation. When protoplast culture was pursued over a few days, cell division started between the 2nd and 3rd d, but only when auxin was present in the culture medium.

For immunological investigations, the protoplasts were prepared with NAA present during digestion.

mRNA Isolation and Amplification of *rolB* cDNA

Total RNA was prepared from about 1.5×10^7 protoplasts as described by Han et al. (1987). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT)-cellulose (Pharmacia) according to the standard method (Aviv and Leder, 1972).

For cDNA first-strand synthesis, 1 μg of poly(A)$^+$ RNA was used with 0.5 μg of oligo(dT)$_{12-18}$ as a primer and Moloney murine leukemia virus reverse transcriptase (Gibco BRL). After an incubation period of 90 min at 42°C, one-third of the reaction volume was used to carry out the amplification procedure. A pair of 24-mer primers corresponding to *rolB* sequences 5′-TGGCGACAACGATTCAACCATATC-3′ and 5′-GTGCCGCAAGCTACAACATCATAG-3′ was used for PCR amplification of a 255-bp fragment from *rolB* cDNA. After a first cycle with a heating denaturation of 2 min, a PCR of 35 cycles (each with 95°C, 50 s; 53°C, 1 min; 72°C, 1 min) was performed in 50 μL of the reaction mixture containing 200 μM each of 2′-deoxynucleoside 5′-triphosphates, 2 μM each of the primers, and 1 unit of Taq polymerase (Promega) in the corresponding buffer.

Electrical Response of Mesophyll Protoplasts to Auxin

The E_m of protoplasts was measured under the microscope by the microelectrode technique as described previously (Ephritikhine et al., 1987; Barbier-Brygoo et al., 1991). For each NAA concentration tested, 15 to 20 individual measurements were performed at room temperature on an aliquot of

the protoplast stock solution diluted to 5×10^4 protoplasts/mL in To medium with the appropriate NAA dose, and a mean E_m value was calculated. ΔE_m represents E_m variations from the mean E_m value measured in the absence of auxin. For each tobacco clone and for each protocol of protoplast preparation, the dose-response curve to auxin of the electrical response of protoplasts was established in at least two independent experiments.

Inactivation of the Electrical Response to Auxin by Anti-ZmER-abp1 Antibodies

The polyclonal IgG raised against ZmER-abp1, an auxin-binding protein from maize coleoptiles (Hesse et al., 1989) expressed in *Escherichia coli*, was affinity purified with homogenous ZmER-abp1 coupled to a BrCN-Sepharose column (K. Palme, unpublished data) and were kindly provided by Dr. K. Palme (Köln, Germany). The cross-reactivity to tobacco proteins of this anti-ZmER-abp1 antibody was assayed on leaf microsomal proteins that were partially purified, subjected to SDS-PAGE, and immunoblotted as described by Venis et al. (1992).

Protoplasts were incubated in To medium at a density of 10^4 protoplasts/mL for 5 min at 4°C in the presence or the absence of the indicated IgG dose. Auxin was added and E_m measurements were performed over the next 20 min as described above. The effects of one IgG concentration on the auxin response of a protoplast preparation were studied as follows: two E_m dose-response curves to auxin were simultaneously determined in the presence and in the absence of IgG. For the control IgG, we used rabbit IgG from pooled sera of unimmunized animals (Zymed Laboratories, San Francisco, CA).

RESULTS

Introduction into Tobacco of B-B:GUS, a Construction with Both the *rolB* Gene and a *rolB*:GUS Fusion

We constructed pCMB-B:GUS, a bacterial plasmid suitable for gene transfer to plants via *A. tumefaciens* and carrying a tandem gene construct named B-B:GUS. Figure 1 shows that the B-B:GUS construction carries a chimeric gene with a *rolB* promoter fragment up to −1177 bp, the coding sequence of the GUS reporter gene (Jefferson et al., 1987), and the nopaline synthase terminator. The construct also carries a functional *rolB* gene with identical upstream regulatory elements and its own polyadenylation sequences.

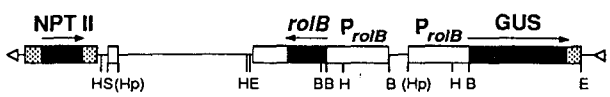

Figure 1. Physical map of the T-region of pCMB-B:GUS. The coding regions of the *rolB*, the GUS, and neomycin phosphotransferase II (NPT II) genes are indicated by solid bars; arrows show the direction of translation. Open and gray bars represent untranslated regulatory sequences from the *rolB* and the nopaline synthase genes, respectively, and P_{rolB} corresponds to the *rolB* promoter directing either *rolB* or GUS expression and oriented according to the corresponding gene. Triangles indicate the T-region borders. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sal*I. Brackets indicate restriction sites deleted due to cloning.

B-B:GUS was introduced into tobacco by *A. tumefaciens*-mediated transformation of leaf discs. Forty to 50% of the kanamycin-resistant plants displayed GUS activity in histochemical assays. Some of them displayed slight developmental alterations, including reduced size, lanceolate leaves, and early flowering. A primary transformant (clone BBGUS6) was chosen for further characterization. T-DNA structures were investigated by Southern analysis using the *rolB* promoter as a probe. For three restriction enzymes tested (*Eco*RI, *Hin*dIII, *Bam*HI), bands were detected at the expected positions for internal T-DNA fragments, showing that at least some of the T-DNA inserts were colinear with the construction (data not shown).

Time Course of Auxin Effects on *rolB* Gene Expression in *rolB*-Transformed Protoplasts

The effects of exogenous auxin on *rolB* promoter expression were first investigated by monitoring GUS enzymatic activity on protoplasts isolated from BBGUS6 plants prepared in the absence of auxin and then cultured in the absence or in the presence of a range of auxin concentrations. Auxin stimulated GUS expression in a dose-dependent manner, with a maximal stimulation at 10^{-5} M NAA (data not shown).

In additional experiments, BBGUS6 protoplasts were thus cultured in the absence or presence of 10^{-5} M NAA, and poly(A)$^+$ RNA was purified after various times of culture. A PCR procedure, via cDNA synthesis, was performed to allow the amplification of a 255-bp fragment from *rolB* sequences present in protoplast mRNAs. This fragment was detected by hybridization of PCR products with a radiolabeled *rolB* probe. The autoradiograms in Figure 2, A and B, show that *rolB* mRNA was barely detectable in freshly isolated protoplasts. In protoplasts cultured in the absence of auxin (Fig. 2A), the RNAs accumulated as early as 15 min after the beginning of the protoplast culture, reached a peak accumulation after 1 h, and then decreased to the initial level. The quantification of hybridization signals in three independent experiments confirmed that *rolB* mRNA levels were transiently increased by 50- to 100-fold after 0.5 to 2 h of culture (Fig. 2C). In protoplasts cultured in the presence of auxin, a similar transient accumulation of *rolB* mRNA occurred in the early hours of the culture, followed by a decrease of *rolB* mRNA to the initial level after 6 h of culture (Fig. 2B). However, a second increase in *rolB* mRNA levels was triggered specifically by the presence of auxin. *rolB* mRNA levels were maximal after 12 to 18 h of culture, with an accumulation of 300- to 500-fold over the initial level (Fig. 2D).

Time Course of Auxin Effects on Auxin Sensitivity of *rolB*-Transformed Protoplasts

The auxin-induced hyperpolarization was used to characterize the auxin sensitivity of BBGUS6 protoplasts under experimental conditions corresponding to those used for the mRNA expression studies.

We characterized the auxin-induced E_m variations of BBGUS6 protoplasts cultured in the absence or in the pres-

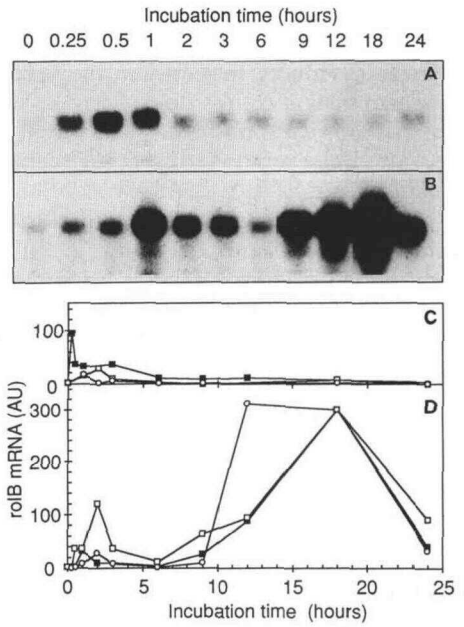

Figure 2. Time course of *rolB* mRNA levels in tobacco mesophyll protoplasts cultured in the presence or in the absence of 10^{-5} M NAA. A and B, PCR products obtained after amplification of *rolB* cDNA from BBGUS6 protoplasts cultured for the indicated times in the absence (A) or in the presence (B) of 10^{-5} M NAA were hybridized with a radiolabeled full-length *rolB* probe. The Southern blot is from a representative experiment. No hybridization signal was detected when the same procedure was performed using untransformed protoplasts. C and D, Quantitative analysis of *rolB* expression. Slot-blot hybridization signals corresponding to serial dilutions of PCR products from three independent kinetic experiments were quantified by a Bio Image Analyzer System (Millipore). The intensity of each hybridization spot is represented in arbitrary units for the various incubation times in the absence (C) or in the presence (D) of auxin. For each independent experiment, the highest value for protoplasts cultured in the absence or in the presence of auxin was taken as the internal reference of 300 arbitrary units.

ence of auxin (10^{-5} M NAA) over time periods up to 12 h. In each case, a dose-response curve to auxin was established and auxin sensitivity was deduced from the auxin concentration inducing the maximal hyperpolarization. Examples of dose-response curves corresponding to different time periods of culture in the absence or presence of auxin are shown in Figure 3. BBGUS6 protoplasts cultured in the absence of auxin exhibited a fairly constant auxin sensitivity over the time of culture, with maximal hyperpolarization induced by 10^{-6} to 10^{-7} M NAA, close to the sensitivity observed on freshly isolated protoplasts (Fig. 3C). In the presence of auxin during protoplast culture, the maximal hyperpolarization was successively obtained for 3×10^{-9} M (Fig. 3A), 3×10^{-10} M (Fig. 3B), and 10^{-11} M NAA (Fig. 3C) after 1, 2, and 5 h of auxin treatment, respectively; beyond this period, the auxin sensitivity of the response remained stable (Fig. 3D).

Data from these experiments and others are collected in Figure 4, which shows the auxin sensitivity of the membrane response as a function of the incubation time of protoplasts in the absence or presence of auxin. In contrast to what was observed in the absence of auxin, the auxin sensitivity of BBGUS6 protoplasts was raised sharply over the first hours of culture in the presence of auxin. The maximal sensitivity level was reached after 5 to 6 h of auxin treatment and corresponded to a 10,000-fold increase over the initial level. The same increase in sensitivity was observed when BBGUS6 protoplasts were prepared by overnight digestion of leaf tissues in the presence of 10^{-5} M NAA (data not shown).

We also performed similar experiments using untransformed protoplasts. Freshly isolated protoplasts or protoplasts cultured in the absence of auxin exhibited a maximal hyperpolarization at a concentration of 10^{-4} M NAA. After 1 h of incubation in the presence of auxin in the culture medium,

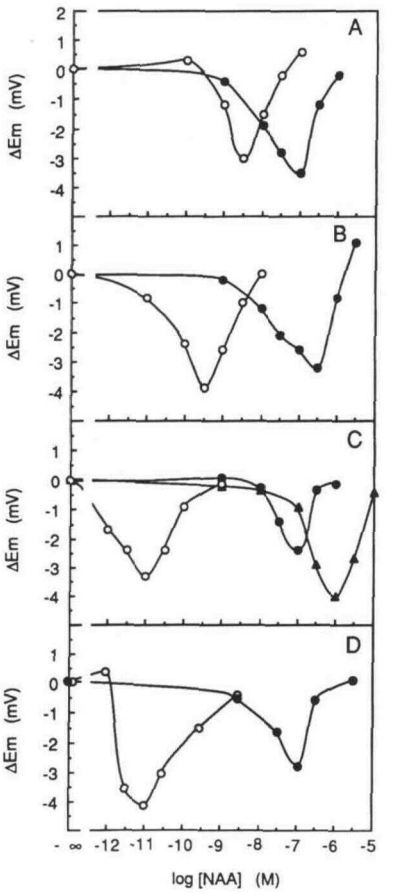

Figure 3. Effects of auxin treatment on the auxin sensitivity of the membrane response of protoplasts isolated from BBGUS6 plants. Protoplasts were isolated by overnight digestion of leaf tissues in the absence of auxin (freshly isolated protoplasts, ▲). Aliquots from the same protoplast preparation were then cultured for different time periods in the absence (●) or the presence (○) of 10^{-5} M NAA. The E_m was measured and dose-response curves to auxin were established as described in "Materials and Methods." ΔE_m indicates E_m variations from the reference value estimated in the absence of exogenous auxin. Each point in the curves corresponds to the mean value from 15 to 20 individual measurements, and maximal SE did not exceed 0.5 mV. Data from one representative experiment are presented for protoplasts cultured for 1 h (A), 2 h (B), 5 h (C), and 12 h (D).

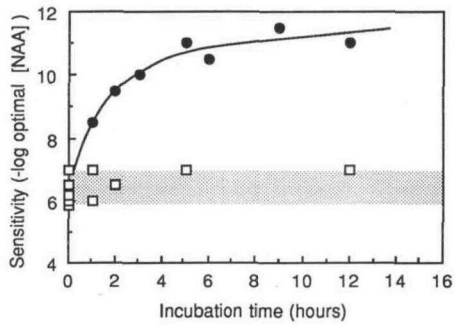

Figure 4. Evolution with time of the auxin sensitivity of BBGUS6 protoplasts cultured in the presence (●) or in the absence (□) of 10^{-5} M NAA. Protoplasts were isolated by overnight digestion of BBGUS6 leaf tissues in the absence of auxin and subsequently cultured in To medium in the presence or absence of 10^{-5} M NAA for the indicated times. Auxin sensitivity was studied using the electrical response of protoplasts to the hormone and estimated by the NAA concentration inducing the maximal hyperpolarization (−log optimal [NAA] in molar concentration). The gray area corresponds to the maximal range of sensitivities observed in freshly isolated protoplasts.

the maximal hyperpolarization of untransformed protoplasts was observed at 3×10^{-6} M NAA, which corresponded to an increase in auxin sensitivity by a factor of 10- to 30-fold (data not shown). For longer incubation times, no further change in the sensitivity of untransformed protoplasts to auxin was observed. These results show that auxin was able to induce a large amplitude shift in auxin sensitivity only in transformed protoplasts.

Responsiveness of *rolB*-Transformed Protoplasts to IgGs Directed against ZmER-abp1

IgGs raised against the maize protein ZmER-abp1 produced in *E. coli* were assayed for their ability to recognize abp1 homologs in tobacco leaves. In proteins partially purified from microsomal fractions, anti-ZmER-abp1 IgGs specifically detected an abp1 homolog of 22 kD (Fig. 5A). The effects of these IgGs on the dose-response curves of NAA-induced E_m variations in untransformed and *rolB*-expressing BBGUS6 protoplasts were evaluated on freshly isolated protoplasts prepared by digestion of leaves in the presence of 2×10^{-6} M NAA. Figure 5B shows the auxin sensitivity of the response as a function of anti-ZmER-abp1 IgG concentration in the external medium. Untransformed protoplasts in the absence of the antibody exhibited a basal sensitivity with a maximal hyperpolarization observed for 1×10^{-6} to 3×10^{-6} M NAA. Their incubation in the presence of IgGs shifted the dose-response curve toward higher auxin concentrations, i.e. they reduced the auxin sensitivity. In the presence of 5×10^{-10} M IgG, the maximal hyperpolarization was observed at 10^{-4} M NAA, corresponding to a reduction in sensitivity by a factor of 30 to 50. The basal auxin sensitivity of BBGUS6 protoplasts (maximal hyperpolarization at 10^{-12} M to 10^{-11} M NAA) was also reduced by the IgG treatment, but it was necessary to apply at least 100 times more IgGs (10^{-7} M) to observe a reduction by a factor of 30 (Fig. 5B). Pooled rabbit IgG from unimmunized animals used at the highest concentration tested for anti-ZmER-abp1 IgG (10^{-7} M) had no effect on the electrical response of either type of protoplast to auxin.

DISCUSSION

The *rolB* gene from *Agrobacterium rhizogenes* has been recognized for almost 10 years to play a pivotal role in the development of the hairy root disease (White et al., 1985). However, thus far no one has been able to detect the presence of the RolB protein in transformed plants, although anti-RolB antibodies have been described (Trovato et al., 1990). The only report on the presence of the *rolB* mRNA in plants has shown that when *rolB* expression was directed by its own promoter, massive amounts of mRNA were needed, in the most favorable case of tobacco stem tissues, to detect hybridization signals in northern blots (Schmülling et al., 1988). The present work provides data concerning the expression of a full *rolB* gene in tobacco mesophyll protoplasts. This expression could be detected with the use of a high-sensitivity detection method via PCR amplification. We characterized here the levels of *rolB* mRNA in freshly isolated *rolB*-transformed protoplasts and their change upon protoplast culture in the absence or in the presence of auxin. The transient increase in *rolB* mRNA levels during the early hours of the protoplast culture was independent of the presence of auxin and may result from either protoplast preparation or handling prior to culture, suggesting that stimuli other than exogenous auxin are able to induce the expression of *rolB*.

On the other hand, auxin elicited a specific and massive

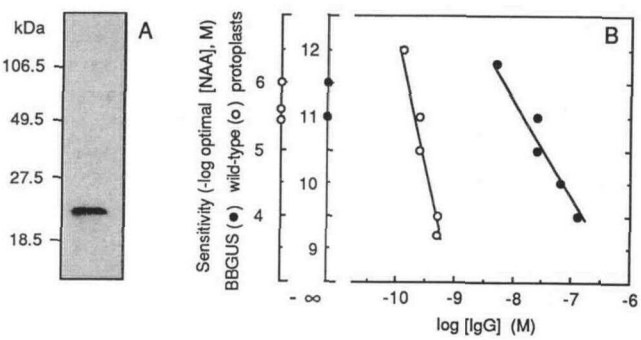

Figure 5. Activity of anti-ZmER-abp1 IgG in tobacco. A, Blot of partially purified proteins from leaf microsomal fraction (wild-type genotype) probed with anti-ZmER-abp1 antibodies, showing a tobacco abp1 homolog of 22 kD. B, Effects of anti-ZmER-abp1 IgG on the electrical response of wild-type (O) and BBGUS6 (●) protoplasts to auxin. Protoplasts were prepared by overnight digestion of leaf tissues in the presence of 2×10^{-6} M NAA. Aliquots of the protoplast stock suspension were either pretreated or not pretreated with various concentrations of anti-ZmER-abp1 IgG for 5 min at 0°C, and dose-response curves of E_m to auxin were established at room temperature as described in "Materials and Methods" and in the legend to Figure 2. For each curve, sensitivity was estimated as the NAA concentration inducing the maximal hyperpolarization (−log optimal [NAA] in molar concentration) and plotted as a function of IgG concentration. In all the experiments, anti-ZmER-abp1 IgG had no significant effect on the reference E_m value in the absence of auxin or on the amplitude of the auxin response.

rolB mRNA accumulation starting 6 to 9 h after auxin was supplied to the protoplasts. The time course of this effect indicates that *rolB* is not an early auxin-regulated gene, and is consistent with the time course of GUS accumulation in *rolB*:GUS protoplasts that could be detected after 6 h of auxin treatment (Maurel et al., 1990). However, *rolB* mRNA levels declined after 18 h of culture in the presence of auxin (Fig. 2, B and D), whereas GUS activity was stable up to 24 h (Maurel et al., 1990). This may reflect regulation processes of *rolB* mRNA synthesis and/or stability that could not be detected using the stable GUS reporter enzyme.

The parallel study of the auxin sensitivity of *rolB*-transformed protoplasts, as assayed by their electrical response to the hormone, showed first that the auxin treatment generates an increase in auxin sensitivity of large amplitude long before the massive auxin-induced increase in *rolB* mRNA levels (Figs. 2 and 4). Our results indicate that in protoplasts there is no simple relationship between the levels of *rolB* mRNA expression and auxin sensitivity because similar levels of *rolB* expression can be associated with different levels of auxin sensitivity. In the absence of auxin, the transient 50- to 100-fold increase in *rolB* mRNA levels induced no significant change in the sensitivity of the membrane response, whereas in the presence of auxin a similar mRNA increase was associated with a rise in sensitivity by a factor of 1,000 to 10,000 within 3 h (Fig. 4). These results reflect the critical role of auxin in controlling the auxin sensitivity of transformed protoplasts. In wild-type protoplasts, auxin treatment also induced an increase in auxin sensitivity. However, this increase never exceeded 30- to 50-fold. From these control experiments we conclude that auxin and *rolB* strongly cooperate in increasing the hormonal sensitivity of transformed protoplasts.

In the present work, we used tobacco protoplasts to demonstrate a multiple control by auxin of *rolB* gene expression on one hand and auxin sensitivity on the other hand. Such control may underlie more integrated processes such as auxin-controlled rhizogenesis in various transformed materials (Cardarelli et al., 1987; Capone et al., 1989; Maurel et al., 1991a). In transformed protoplasts, the levels of *rolB* mRNA seem to be nonlimiting for enhancement of auxin effects. In transformed plant tissues, prolonged induction of *rolB* by auxin may be necessary to mediate morphogenetic effects of *rolB*, possibly involving cellular effects other than the modulation of auxin sensitivity.

Three independent laboratories have now shown that the RolB protein is not acting through a release of auxin from auxin Glc esters (Nilsson et al., 1993; Spena et al., 1993; Delbarre et al., 1994). Interestingly, Delbarre et al. (1994), working on the same biological material that was used in this study, namely transformed protoplasts from the BBGUS6 clone, have shown that normal and transformed protoplasts do not differ in their ability to accumulate and metabolize exogenous auxins. Under the conditions used to measure auxin sensitivity, no difference could be observed either in the intracellular or in the extracellular auxin concentrations between the two types of protoplast. These data support the idea that the increased auxin sensitivity of *rolB*-transformed protoplasts could follow from alterations in the reception-transduction of the auxin signal.

Here we have shown that protoplasts expressing the increased sensitivity to auxin quantitatively differ from normal protoplasts in their responsiveness to IgGs raised to ZmER-abp1, a maize auxin-binding protein. We demonstrated previously that these IgGs are able to block the electrical response of tobacco protoplasts, indicating that proteins involved in auxin recognition (tARPs) and sharing common epitopes with ZmER-abp1 are present at the surface of the protoplasts. We have also shown that about 100- to 1000-fold more IgGs were needed to reduce by 30-fold the sensitivity of *rolB*-transformed protoplasts compared to normal ones. This large difference in the immunoreactivity of the surface of *rolB* protoplasts could indicate that the perception of the auxin signal or its transduction has been altered (for instance, the number of tARPs may be increased or their efficiency modified), with such changes somehow participating in the shift in auxin sensitivity. The fact that *rolB* protoplasts show the same increase in sensitivity to the agonist antibody D16 directed against a peptide from ZmER-abp1 and to auxin (Venis et al., 1992) further supports the idea that these protoplasts are altered in the perception step of the signal at the outer face of the plasma membrane. The mechanisms by which the RolB protein could alter or interact with the auxin perception chain at the plasma membrane are still entirely unknown. Nevertheless, the present work demonstrates that tobacco protoplasts transformed by *rolB* provide a relevant and unique cellular system in which to study the mechanisms of auxin perception.

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