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An antibody raised to a maize auxin-binding protein has inhibitory effects on cell division of tobacco mesophyll protoplasts

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Abstract An antibody raised to the auxin-binding protein Zm-ERabp1 from maize coleoptiles (anti-abp1 IgG) was tested for its effect on cell division of protoplasts prepared from tobacco (*Nicotiana tabacum*, cv. Xanthi) mesophyll. It was formerly demonstrated that this antibody is able to interact with an auxin-perception unit located at the plasma membrane and involved in the early electrical response of protoplasts to auxin. In different situations where auxin stimulates cell division, dual inhibitory effects by anti-abp1 IgGs were observed on occurrence of first cell division, an early event, and on size of protoplast-derived microcolonies, a delayed response. This latter response is likely associated in its origin and mechanisms to the inhibition of first division.

Key words Auxin-dependent response, protoplast-derived cells, signalling-pathway, plasma membrane, *Nicotiana tabacum.*

Abbreviations NAA, 1-naphthaleneacetic acid.

INTRODUCTION

A variety of plant cell responses to auxin have been described but the corresponding signalling pathways are far from being identified (Jones, 1994; Barbier-Brygoo, 1995). Tobacco leaf protoplasts constitute a widely used biological system to study auxin controlled responses such as reinitiation of cell division, gene expression and modifications of the electrical properties of the plasma membrane. The auxin-dependent expression of specific genes encoding for stress proteins (Fleck *et al.*, 1982; Grosset *et al.*, 1990) or proteins appearing concomitantly with the transition from G0 to S phase (Takahashi *et al.*, 1989, 1991; Takahashi and Nagata, 1992) has been described. Tobacco protoplasts have also been used as a biological system to study the transient expression of auxin-regulated genes (Walden *et al.*, 1993). Leaf and root tobacco protoplasts display a fast hyperpolarization of the plasma membrane when treated with auxin (Ephritikhine *et al.*, 1987; Barbier-Brygoo, 1995) and the perception elements involved in this response have been investigated. The most extensively described putative auxin receptor is the major auxin-binding protein from maize coleoptile (Zm-ERabp1), a protein located in the endoplasmic reticulum. A set of evidence indicates that plasma membrane located abp1 or abp1-related molecules are involved in the regulation by auxin of ionic exchanges and electrical properties of the plasma membrane in different systems (Barbier-Brygoo *et al.*, 1991; Rück *et al.*, 1993; Thiel *et al.*, 1993). In particular, it has been demonstrated on tobacco mesophyll protoplasts that the electrical response to auxin is inhibited by polyclonal antibodies raised to Zm-ERabp1 (anti-abp1 IgGs) (Barbier-Brygoo *et al.*, 1989, 1991). In contrast, a polyclonal antibody raised to a synthetic peptide (D16 antibody) reproducing the putative auxin-binding domain of abp1 exhibited auxin agonist activity on the membrane response (Venis *et al.*, 1992).

The question is raised of the possible involvement of auxin perception at the cell surface in more integrated responses such as cell enlargment or cell division. A few evidences suggest that it may mediate elongation responses (Löbler and Klämbt 1985; Venis *et al.*, 1990), but no data are available concerning its possible involvement in cell division. Contradictory evidence exist as to the hypothesis that the cell surface receptors responsible for the auxin-induced

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hyperpolarization could be involved in the regulation of cell division. For example, the fact that cell division and membrane hyperpolarization of tobacco protoplasts displayed the same pattern of specificity to auxin and auxin analogs (Caboche *et al.*, 1987; Barbier-Brygoo *et al.*, 1992) favours this hypothesis. Moreover, a tobacco mutant selected for its resistance to auxin exhibited the same shift in sensitivity to auxin of the two responses, plating efficiency and membrane hyperpolarization (Caboche *et al.*, 1987; Ephritikhine *et al.*, 1987). At the opposite, protoplasts isolated from *rolB*-transformed plants exhibited a very high sensitivity to auxin as to the electrical response but a normal sensitivity as concerns cell division (Maurel *et al.*, 1991) suggesting that these two responses do not share the same transduction chain.

The aim of this work has been to bring new arguments concerning the possible involvement of auxin perception at the cell surface in the regulation of cell division by investigating the effects of antibodies directed against abp1 on the division response of cells derived from tobacco mesophyll protoplasts.

RESULTS

Occurrence of first cell division

Cell division was studied over a 4 day period as a function of auxin concentration in the culture medium on two protoplast populations, one prepared in the presence of auxin during the leaf digestion (aux +), the other in the absence of auxin (aux –) (*fig.* 1). Whatever the digestion conditions, no cell division was observed after 1 day of culture. First division occurred between 1 and 2 days and continued for 2 days more. At day 3, cell populations were composed of a mixture of state 2 cells (first division) and state 4 cells (second division) in the ratio 2.5 to 1. Next divisions appeared after 3 days.

Cell division of protoplasts exhibited a bell-shaped dose-response curve as a function of the auxin concentration in the culture medium. Maximal cell division (50 to 60%) was reached after 3 to 4 days over a range of NAA concentrations between 10^{-6} and 3×10^{-5} M. Cell division was strongly decreased in the presence of 10^{-4} M NAA and was totally inhibited with 3×10^{-4} M. The only difference revealed by the comparison of (aux +) and (aux –) protoplast preparations was the ability of (aux +) protoplasts to divide in the absence of exogenous auxin.

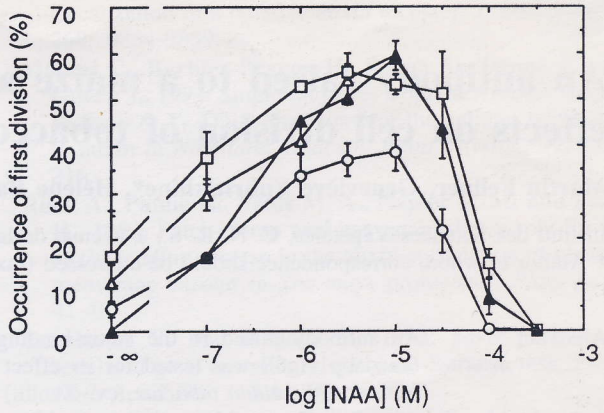

Figure 1. *Dose-response curves to auxin for cell division of mesophyll protoplasts isolated from tobacco leaves.* Protoplasts were prepared in the presence of 15 μM NAA (open symbols) or in the absence of NAA (black symbols, ▲). Occurrence of cell division, expressed in % of total protoplasts, was measured during the culture period after 2 days (○), 3 days (△) and 4 days (□). Mean values and standard errors were calculated from 4 independent experiments except at 4 days ($n = 3$).

Effects of antibodies directed against the maize auxin-binding protein, Zm-ERabp1, on cell division

The activity of the anti-abp1 antibody was mainly explored at the concentration of 2×10^{-9} M IgG which was already shown to inhibit by 90% the maximal electrical response to auxin of mesophyll protoplasts (Barbier-Brygoo *et al.*, 1991). Due to limitations in antibody availability, ten fold higher concentrations were tested in a few selected conditions. Freshly isolated protoplasts (aux +) and (aux –) were treated for 30 min by the antibody preparation and were then cultured for 2 weeks in the presence of the antibody and 10^{-5} M NAA, the optimal concentration for cell division (see *fig.* 1).

Effects of anti-abp1 antibodies on occurrence of cell division

While the non-immune IgG preparation was not effective on cell division (*fig.* 2), the anti-abp1 IgGs displayed in every experiment an inhibitory effect on that response of protoplast-derived cells. The amplitude of the inhibition was dependent on the antibody concentration (*fig.* 2), varying from 15% in the presence of 2×10^{-9} M anti-abp1 to more than 30% with a ten times higher concentration. Protoplasts cultivated in the presence of NAA exhibited the same behaviour whatever the digestion conditions (aux + or

aux –, *fig.* 2). Even in the extreme conditions where cell division was strongly limited by auxin availability, protoplasts (aux +) cultivated without auxin, the IgG-induced inhibition of first cell division was clearly observable (– 18% of control with 2×10^{-9} M anti-abp1) (data not shown). The maximal inhibitory effects were almost reached after 2 days of culture in the presence of the antibody (data not shown). Furthermore, we observed that the anti-abp1 IgGs did not affect significantly cell viability.

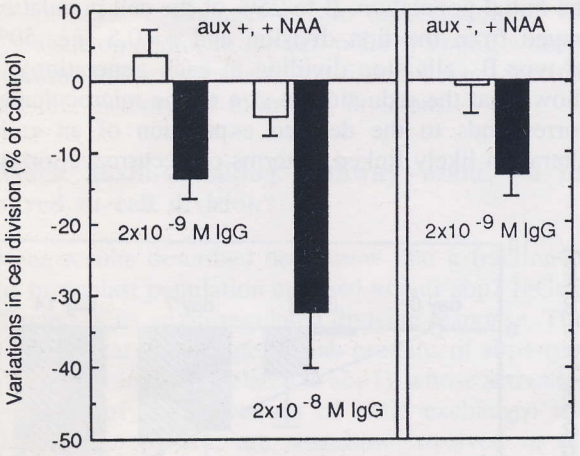

Figure 2. *Effect of antibodies raised to the auxin-binding protein Zm-ERabp1 on cell division of tobacco protoplasts.* Mesophyll protoplasts prepared without (aux –) or with 15 μM auxin (aux +) were cultivated in the presence of NAA (10^{-5} M) and non-immune IgG preparations (white columns) or anti-abp1 IgGs (black columns). Variations in cell division, expressed in % of control population not treated by antibodies, were measured after 4 days of culture. For (aux +, + NAA) protoplasts, mean values and standard errors were calculated from 11 experiments (non-immune IgGs, 2×10^{-9} M), 8 experiments (anti-abp1 IgGs, 2×10^{-9} M) and 2 experiments (non-immune and anti-abp1 IgGs, 2×10^{-8} M). For (aux –, + NAA) protoplasts, mean values and standard errors were calculated from 5 experiments.

Effects of anti-abp1 antibodies on the size of microcolonies

After 4 days of culture, when protoplast-derived cells were evolving to microcolonies, it became almost impossible to measure growth by counting the cells. Consequently, the activity of anti-abp1 IgGs was quantified by measurement of the size of the protoplast-derived cells or microcolonies at regular intervals (4, 7 and 14 days). This parameter gives a global estimation of the growth of cell colonies as resulting from both successive cell divisions and cell enlargement. We scored independently the size of individual cells, state-2 and -4 cells issued from the first and second divisions and microcolonies. Significant modifications of the size were observed only in this latter category. Mean values from four independent experiments testing the anti-abp1 IgG effects on the size of microcolonies originated from protoplasts (aux +, + NAA) or (aux –, + NAA) are presented on figure 3. The inhibitory effect of the antibody became apparent at 7 days, anti-abp1 IgGs inducing a decrease by about 15% of the size of the microcolonies. This effect was developing with time to reach 20-25% reduction in size after 14 days. Here again, even in the extreme conditions where cell division was strongly limited by auxin availability, protoplasts (aux +) cultivated without auxin, the size of microcolonies was also clearly reduced by the IgG treatment ($-14\% \pm 1.4$ at 7 days, $n = 4$) (data not shown). Finally, after 3 to 4 weeks of culture, as a consequence of the decreased size of the microcolonies, the cell suspensions issued from protoplasts treated by anti-abp1 IgGs were more finely granulated and less dense than the control ones (data not shown).

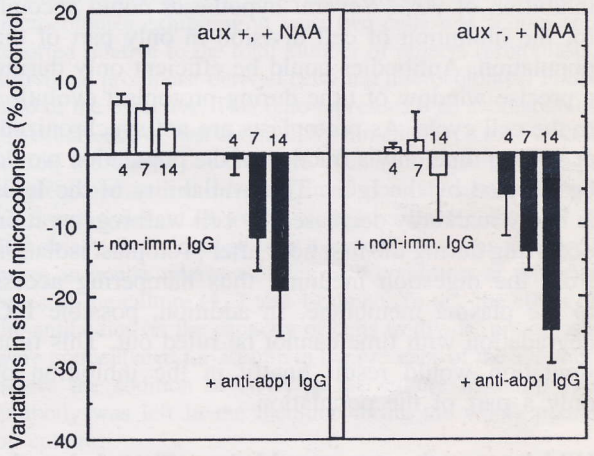

Figure 3. *Effect of antibodies raised to the auxin-binding protein Zm-ERabp1 on the size of microcolonies derived from tobacco protoplasts.* Mesophyll protoplasts prepared without (aux –) or with 15 μM auxin (aux +) were cultivated in the presence of NAA (10^{-5} M) and 2×10^{-9} M of a non-immune IgG preparation (white columns) or anti-abp1 IgGs (black columns). The variations in the size of microcolonies after 4 days (4), 7 days (7) and 14 days (14) were calculated as % of the size of a control population, *i.e.* not treated by antibodies. Mean values and standard errors were calculated from 4 independent experiments.

DISCUSSION

In order to test the possible involvement of abp1 or abp1-related proteins in cell division response,

we investigated the effects of anti-abp1 IgGs on cell division. We show that cell division is clearly affected by the anti-abp1 IgGs. An inhibitory effect was visible on occurrence of first divisions (*fig.* 2) and, later on, on growth of protoplast-derived microcolonies (*fig.* 3). These effects were specific as revealed by the non-immune controls and occurred independently of the procedure of protoplast preparation. Even in the extreme conditions of protoplasts prepared in the presence of auxin but cultivated in its absence, the dual effects of the IgGs on occurrence of first cell division and size of microcolonies were observed.

Why the inhibitory effect would be partial?

The inhibition of occurrence of first division only affects a fraction of the protoplast population, this fraction being about one third of the total population at the highest IgG concentration tested. This is in contrast with the fact that anti-abp1 IgGs at a ten fold lower concentration inhibits the short term electrical response of all protoplasts tested, with a mean inhibition of 90%. Several hypotheses could account for the inhibition of cell division in only part of the population. Antibodies could be efficient only during a precise window of time during protoplast evolution to the cell cycle. As protoplasts are not synchronized, at a given time only a fraction of the population would be affected by the IgGs. The availability of the IgGs is likely markedly decreased by cell wall regeneration occurring during the first hour after protoplast isolation from the digestion medium, thus hampering access to the plasma membrane. In addition, possible IgG degradation with time cannot be ruled out. This time limitation would result finally in the inhibition of only a part of the population.

Which growth step would be affected by the anti-abp1 antibodies?

The two inhibitory effects of anti-abp1 IgGs observed here appear in a different time scale: the decrease by anti-abp1 IgGs of occurrence of first cell division is a relatively early event, almost maximally expressed after two days of culture, whereas the reduction in the size of microcolonies is observable after 7 days. The fact that the anti-abp1 did not modify the size of the cells during the first divisions suggests that the reduction in size of the microcolonies would correspond to an alteration of cell division and not to a significant change in individual cell size. This second delayed effect is apparently in contradiction with the hypothesis raised above that antibodies are only active during an early and short period of time. In fact, the reduction in size of microcolonies can be highly linked to the inhibition of first division if, aside those protoplast-derived cells whose division is totally blocked (fraction A), other cells (fraction B) do perform the first division but are altered in their properties in such a way that the probability p that they undertake a further division is strongly decreased. This model nicely accounts for the experimental data (solid line of *fig.* 4) with fraction A corresponding to 15% of the initial population, B to 25% of the cell population issued from the first division and $p = 0.5$ (*i.e.* 50% of type B cells stop dividing at each generation). It shows that the reduction in size of the microcolonies corresponds to the delayed expression of an early alteration likely linked in terms of occurrence, origin

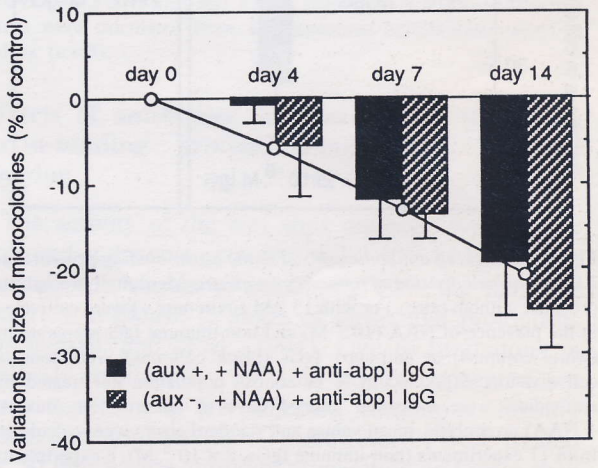

Figure 4. *Modification of the size of protoplast-derived microcolonies following from an alteration of the cell division probability in cell populations derived from tobacco protoplasts treated by Zm-ERabp1 antibodies.* Experimental results concerning the colonies derived from protoplasts prepared without (aux +) or with 15 μM auxin (aux +) and cultivated in the presence of NAA (10^{-5} M) and 2×10^{-9} M of anti-abp1 IgGs are replotted from figure 3. Variations in the size of the microcolonies after 4, 7 and 14 days have been calculated (open circles, solid line) as % of the size of control microcolonies not treated by antibodies, according to the assumption that the IgG-treated cell populations are composed of cells totally blocked in first division (fraction A, 15% of the initial population), cells which do undertake the first division but are altered in the following divisions (fraction B, 25% of the population) and non-affected cells (fraction C, 60% of the population). B cells are altered in such a way that 50% of them stop dividing at each generation ($p = 0.5$), consequently, the total number of cells (N) increases according to the relation: $N = N_{0C} \cdot e^{t.(Ln2/Tc)} + N_{0B} \cdot e^{t.(Ln\,(1+p)/Tc)}$ with N_{0C} and N_{0B} = number of cells at $T = 0$ belonging to fractions C and B, respectively and generation time, $Tc = 2$ days.

and mechanisms to the blockade of the first division in cells of fraction A. It also accounts for the progressive increase in the intensity of inhibition as the result of an amplification at each generation. Most importantly, it shows that the fraction of the initial protoplast population affected by the anti-abp1 IgGs treatment (about 40% for $p=0.5$ but higher values if $0.5<p<1$) may be much larger than that simply exhibiting a block of the first division. The nature of the alterations leading to modifications of the cell division behaviour at the second and following generations is a matter of speculation. Changes in the organization of the cytoskeleton and/or in the cell polarity could, without blocking the occurrence of the first division, alter the cell fate in terms of following divisions.

Which auxin-signalling pathway would be involved in cell division?

The results described here show that a fraction of the protoplast population exposed to anti-abp1 IgGs is affected in its auxin-regulated division response. This suggests that plasma membrane proteins of abp1-type (or immunologically related to abp1), whose activation triggers early modifications of ionic exchanges and electrical properties, are somehow involved in the regulation of division in protoplast-derived cells. This does not mean that this auxin response is mediated through a unique pathway governed by cell surface receptors. More complex models (Jones, 1994) where membrane hyperpolarization and cell division may be mediated by two distinct primary receptors, the one involved in the division response being possibly an intracellular receptor, have to be considered. Cross-talks between the two transduction pathways would render the functioning of one pathway (cell division) sensitive to the inhibition of the other pathway (membrane response). Further insights in the architecture of the signalling pathways involved in auxin-stimulation of cell division and their connection with other auxin transduction pathways can be expected from the study of auxin-regulated gene expression.

METHODS

Plant material. Mesophyll protoplasts from *Nicotiana tabacum*, cv Xanthi were isolated as previously described by Caboche (1980) in the presence of 15 μM NAA. In some experiments NAA was omitted during the enzymatic digestion procedure. The washed protoplasts were resuspended in medium T_0 (Caboche, 1980), depleted of NAA but with 5 μM 6-benzyladenine.

IgG preparation. The polyclonal antibodies raised to Zm-ERabp1, an auxin-binding protein from maize coleoptiles (Hesse *et al.*, 1989) expressed in *Escherichia coli*, were affinity-purified with homogenous Zm-ERabp1 coupled to a BrCN-Sepharose column (K. Palme, unpublished data) and were kindly provided by Dr K. Palme (Köln, Germany). Rabbit IgGs from pooled sera of unimmunized animals were used as control IgGs (Zymed Laboratories, San Francisco, CA).

Protoplast culture. Aliquots of a freshly isolated protoplast suspension were incubated in 24-wells microplates in the culture medium T_0, in the absence or the presence of various auxin concentrations (NAA from 10^{-7} to 3×10^{-4} M). Protoplasts were cultivated in a final volume of 300 μl, at the density 5×10^4 protoplasts ml^{-1}, in darkness and at 26°C. Cell division capacity of the protoplast-derived cells was quantified by two parameters, the occurrence of first cell division in the first days of culture and, later on, the size of the microcolonies. Protoplast-derived cells were observed under an inverted microscope and the number of microcolonies exhibiting at least two cells was scored and reported relative to the total number of objects (protoplasts, individual protoplast-derived cells and microcolonies) in the field of the objective. This ratio represented an estimation of the occurrence of first division. Systematically, a total of 200 to 250 individual objects were counted for each condition in one experiment at different times along the culture period (2, 3 and 4 days). As to the second parameter, the size of 100 objects, individual cells or microcolonies, was measured using an ocular micrometer for each condition, at different times of the culture (4, 7 and 14 days). To test the effect of the antibodies on the capacity of cells to divide, protoplasts were preincubated for 30 min in the presence of the antibody before the addition of NAA in the culture medium. The antibody was left in the medium during the whole period of culture.

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