The auxin-binding protein Nt-ERabp1 alone activates an auxin-like transduction pathway

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Abstract Hyperpolarization of tobacco protoplasts is amongst the earliest auxin responses described. It has been proposed that the auxin-binding protein, ABP1, or a related protein could be involved in the first step of auxin perception at the plasma membrane. Using for the first time homologous conditions for interaction between the protein Nt-ERabp1 or a synthetic peptide corresponding to the C-terminus and tobacco protoplasts, we have demonstrated that both can induce the hyperpolarization response. The results show that Nt-ERabp1 or the C-terminal peptide alone activates the auxin pathway from the outer face of the plasma membrane.

Key words: Auxin-binding protein; ABP1; Auxin; Tobacco protoplast; Hyperpolarization

1. Introduction

The plant hormone auxin plays an important role in a large variety of plant growth and developmental processes [1]. Although auxin effects have been described and studied at the whole plant and cellular levels, the molecular mechanisms of auxin action have yet to be elucidated. Biochemical approaches as well as characterization of early auxin regulated genes have implicated diverse signaling pathways in auxin responses which give a complex picture of auxin action [2,3]. Within the last 10 years, the search for auxin receptors has led to the identification of a number of soluble and membrane-associated proteins that bind auxin [4]. Their functional role in auxin signaling is still unclear and even the function of the most studied auxin-binding protein, ABP1, which is the best candidate for an auxin receptor, remains uncertain. ABP1 is a soluble protein first isolated from maize coleoptiles [5,6]. Genomic or cDNA clones of *ABP1* have been isolated from different plant species such as maize [6–8], *Arabidopsis* [9,10], tobacco [11,12], strawberry [13], red pepper [14] or apple tree [15]. All of the deduced amino acid ABP1 sequences possess a leader peptide at the N-terminus of the protein and a KDEL (Lys-Asp-Glu-Leu) motif at the C-terminus. These features are characteristic of endoplasmic reticulum resident proteins, which is consistent with the biochemical characterization of the ABP1 protein in maize (Zm-ERabp1) [4,5,16]. However, by using polyclonal antibodies to Zm-ERabp1, Diekmann and coworkers [17] have visualized the protein at the surface of maize protoplasts suggesting that a fraction of the protein could escape the ER and reach the plasma membrane. Electrophysiological responses to auxin have also provided some evidence in favor of an involvement of ABP1 or an immunologically related protein in early electrical responses at the plasma membrane [18–20]. In addition, a synthetic peptide comprising the last 12 residues of the C-terminus of Zm-ERabp1 was demonstrated to rapidly and reversibly modulate the K^+ currents of *Vicia faba* guard cells mimicking the effect of supraoptimal auxin concentrations [21,22]. The same maize peptide was also shown to induce the hyperpolarization of tobacco mesophyll protoplasts in the absence of auxin [23] and to provoke a cytosolic pH alkalinization of *Paphiopedilum tonsum* L. guard cells and consequently stomatal closure [24]. Considering these results, it is not clear whether the activity of the C-terminal peptide reflects that of the entire protein and if the peptide is acting at the plasma membrane or inside of the cells. The results from electrophysiological experiments have contributed to assign a possible role of auxin receptor to ABP1. However, the different data have been obtained by using heterologous tools on different electrical measurements and the involvement of ABP1 is still questionable [25]. Recently, interest in ABP1 was brought up to date again by work in which transgenic plants overexpressing ABP1 were shown to exhibit an increased capacity for auxin-mediated cell expansion [26].

We report here on the biological activity of synthetic peptides comprising the C-terminus of the tobacco auxin-binding protein 1 (Nt-ERabp1) and of Nt-ERabp1 itself on the electrical membrane response of tobacco mesophyll protoplasts. For the first time, such an investigation was undertaken using homologous conditions of interaction between peptides or protein and the plasma membrane. Furthermore, protoplasts expressing the *rolB* gene from *Agrobacterium rhizogenes*, which show increased sensitivity to auxin [27,28] were used to assess the physiological relevance of the responses induced by Nt-ERabp1 and the C-terminal peptides.

2. Materials and methods

2.1. Materials

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) from the wild-type clone XHFD8 and *rolB*-transformed plants (named BBGus plants, [28]) were grown from seeds in a greenhouse (22°C, 9 h photoperiod). Mesophyll protoplasts were isolated from young tobacco leaves using the procedure described in [29].

2.2. Peptide synthesis and ABP1 protein production

Synthetic peptides corresponding to the C-terminal regions of the maize and tobacco ABP1 proteins, designated pz(152–163), Nt-C12 (from D156 to L167) and Nt-C15 (from W153 to L167) (see Fig. 1), were prepared by the laboratory of J. Igolen (Pasteur Institute, Paris). The products were purified by HPLC and analyzed by sequencing. The recombinant tobacco protein, Nt-ERabp1, was produced in *Es-*

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cherichia coli and was purified as described by Leblanc and coworkers [11]. Zm-ERabp1 was purified from microsomal fractions of maize coleoptiles as described previously [30] and was kindly provided by R.M. Napier and M.A. Venis (Wellesbourne, UK).

2.3. Measurement of the electrical response of tobacco protoplasts

For each experiment, 100 μl aliquots of the protoplast suspension (5×10^4 protoplasts/ml) were incubated with different concentrations of peptide (or protein) for 5 min at room temperature. The transmembrane E_m of these protoplasts was then measured by the microelectrode technique as described in [31]. Membrane potential variations (ΔE_m) reported for each experimental condition correspond to the average of 15–20 individual measurements. Dose-response curves were established by plotting ΔE_m values as a function of peptide or protein concentrations.

3. Results and discussion

3.1. Activity of C-terminal peptides from maize and tobacco ABP1 in the electrical membrane response of tobacco mesophyll protoplasts

The comparison of ABP1 C-terminal regions issuing from different species reveals that their sequences are rather divergent (Fig. 1). Only eight amino acids out of 15 are conserved. These eight residues include the three amino acids Trp, Asp and Glu, the third cysteine of ABP1 sequences and finally the C-terminal tetrapeptide KDEL (Lys, Asp, Glu, Leu). In contrast, the nature and the number of amino acids located between the cysteine residue and the KDEL tetrapeptide vary from one protein to the other. Given the reported electrical effects of a synthetic peptide corresponding to the C-terminus of maize ABP1 [21,23] together with the poor conservation of C-terminal sequences between the different ABP1 proteins, we investigated the effect of the C-terminal region of tobacco ABP1 on the electrical response of tobacco protoplasts. Two distinct peptides were synthesized. The peptide Nt-C12 has the same length than the maize peptide and corresponds to the last 12 amino acids of tobacco ABP1 (from D156 to L167). The Nt-C15 peptide reproduces the last 15 amino acids of tobacco ABP1 (from W153 to L167), including the three highly conserved amino acids Trp-153, Asp-154 and Glu-155.

The incubation of tobacco protoplasts with the maize peptide, pz(152–163), in the absence of auxin, resulted in plasma membrane hyperpolarization within one minute. The dose-response curve shows that a maximal hyperpolarization of −4.5 mV was obtained for concentrations of 10^{-7} M and over (Fig.

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Nt-ERabp1     YWDEECYQTTSW.KDEL
Fr-ERabp      YWDEGCLELEPPPKDEL
At-ERabp      YWDEQCIQ.ESQ.KDEL
Zm-ERabp1     VWDEDCFEAA...KDEL

Consensus      WDE.C.......KDEL

pz(152-163)     DEDCFEAA...KDEL
Nt-C15        WDEECYQTTSW.KDEL
Nt-C12           ECYQTTSW.KDEL
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Fig. 1. Comparison of the C-terminal amino acid sequences of ABP1 previously identified from maize (Zm-ERabp1) [7], strawberry (Fr-ERabp1) [13], tobacco (Nt-ERabp1) [11] and *A. thaliana* (At-ERabp1) [9]. Conserved residues are indicated in bold type. The sequences of synthetic peptides Nt-C12, Nt-C15 and pz(152–163) are also indicated.

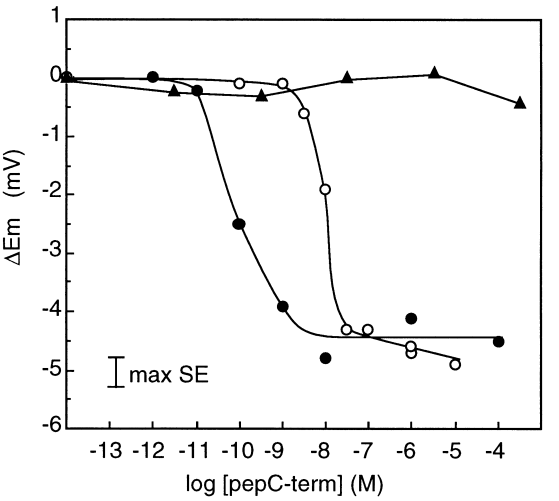

Fig. 2. Effects of synthetic peptides on the transmembrane potential difference (ΔE_m) of tobacco mesophyll protoplasts. Protoplasts were incubated either with pz(152–163) (○), Nt-C15 (●) or Nt-C12 (▲); peptides corresponding to the C-terminal regions of maize (open symbols) and tobacco ABP1 (full symbols). The dose-response curves were established by plotting ΔE_m values as a function of peptide concentration. Data are given for one representative experiment among four independent experiments. The mean E_m variation induced in these experiments by the optimal auxin concentration (3 μM, 1-NAA) was −4.3 mV. Maximal standard error (max SE) is indicated in the figure.

2) [23]. When protoplasts were incubated with the tobacco peptide Nt-C12, no hyperpolarization could be detected (Fig. 2). In contrast, Nt-C15 induced hyperpolarization with a dose-response curve similar in shape and in amplitude to that of pz(152–163), but shifted to lower concentrations. Peptide concentrations inducing half of the maximal response (EC_{50}) were 10^{-10} M and 10^{-8} M for Nt-C15 and pz(152–163), respectively. The tobacco peptide is thus 100-fold more active than the maize peptide when used to induce the response on tobacco protoplasts.

Despite the weak identity between the C-terminal domains of maize and tobacco ABP1, synthetic peptides corresponding to these domains were both able to induce the hyperpolarization of tobacco protoplasts. The higher efficiency of the tobacco peptide Nt-C15 reveals the relative importance of the whole C-terminal sequence, including divergent residues, as well as the possible involvement of the conserved Trp-153 residue which is missing in the maize synthetic peptide. The absence of effect of the 12 amino acids long tobacco peptide suggests that the Trp, Asp and Glu residues play a key role in the activation of the electrical response by such synthetic peptides. However, each change within a synthetic peptide of this length generates significant changes in the net charge and also in the structure of the peptide which could result in the loss of effect. Gehring and co-workers [24] have reported that a truncated C-terminal peptide, Pz(152–159), lacking the KDEL sequence, had no effect on pHi or stomatal movement of *Paphiopedilum tonsum* L. whereas the 12 amino acids long peptide, pz(152–163) induced guard-cell alkalinization and consequently stomatal closure. On the basis of these data, it is difficult to conclude on the relative importance of the different amino acids of the synthetic peptides. The obtained results set the C-terminal sequence as an interacting domain able to

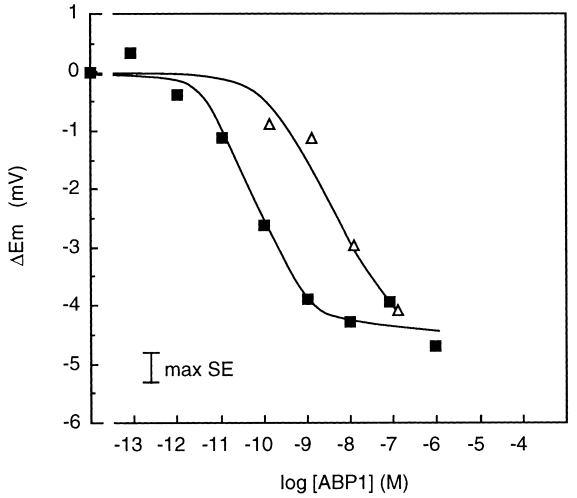

Fig. 3. Electrical membrane response of tobacco mesophyll protoplasts to Nt-ERabp1 (■) and to Zm-ERabp1 (Δ). The curves were established by plotting ΔE_m values as a function of protein concentration. For each genotype, data are given for one representative experiment among three independent experiments. The mean E_m variation induced in these experiments by the optimal auxin concentration (3 μM, 1-NAA) was −4 mV. Maximal standard error (max SE) is indicated in the figure.

initiate membrane polarization or pHi changes but the site of action of these peptides as well as the significance of the observed effects remain to be established.

3.2. *Tobacco and maize ABP1 induce the same response in tobacco protoplasts as their active C-terminal peptides*

Does the effect of the C-terminal peptides on the protoplast hyperpolarization reflect ABP1 action at the plasma membrane or do the peptides activate a distinct pathway? To address this question, we investigated the effects of tobacco and maize auxin-binding proteins, recombinant Nt-ERabp1 produced in *E. coli* and Zm-ERabp1 extracted from coleoptiles, on the electrical membrane response of tobacco protoplasts. As shown in Fig. 3, tobacco and maize ABP1 were both able to induce a membrane hyperpolarization of tobacco mesophyll protoplasts. This response resulted in a monotonous curve with a maximal hyperpolarization of −4.5 mV as described for the peptides. The EC_{50} for tobacco ABP1 is 10^{-10} M, whereas the EC50 for Zm-ERabp1 is about 3.10^{-9} M. The tobacco ABP1 protein is thus about 30-fold more active than the maize protein when used to induce the electrical membrane response of tobacco protoplasts.

As observed with pz(152–163) and Nt-C15, the activation of the electrical response was obtained with lower amounts of protein using homologous conditions of interaction (i.e. Nt-ERabp1/tobacco protoplasts). Interestingly, the active tobacco peptide, Nt-C15, and the recombinant Nt-ERabp1 protein exhibited the same efficiency in the hyperpolarization response of tobacco protoplasts and the shapes of the dose-response curves were identical (Figs. 2 and 3). Similar observations can be done with pz(152–163) and the maize protein. The whole Zm-ERabp1 protein was slightly more effective than the maize peptide to induce the same hyperpolarization. This could reflect that in heterologous conditions, other domains of the protein could facilitate or stabilize the interaction of the C-terminal domain with the tobacco plasma membrane. As the Trp residue is absent in the maize peptide, but present in Nt-C15, the difference of reactivity could also suggest that the Trp residue is involved in the interaction.

We have shown that tobacco and maize ABP1 proteins induce the same effect as their corresponding C-terminal peptides on the electrical response of tobacco protoplasts suggesting that both act in a similar way at the plasma membrane to activate this response. The addition of purified Nt-ERabp1 or Nt-C15 provokes maximal hyperpolarization of protoplasts for concentrations above 10^{-9} M whereas the auxin-induced electrical response of tobacco protoplasts results in a bell-shaped dose response curve [18]. With auxin, the maximal hyperpolarization is obtained with 3×10^{-6} M NAA and is followed by a relative depolarization for higher concentrations. From this point of view, ABP1 proteins and C-terminal peptides mimic the activating effect of auxin on the hyperpolarization but not the relative deactivation of supraoptimal auxin concentrations.

3.3. *RolB-expressing protoplasts are more sensitive to Nt-ERabp1 and Nt-C15 than untransformed protoplasts*

To determine if the electrical membrane response induced by Nt-ERabp1 or Nt-C15 was related to the biological activity of auxin, we analyzed their effects on protoplasts issued from *rolB*-transformed plants, named BBGus [28]. The *rolB* gene, from *A. rhizogenes*, has been shown to play an important role in the development of the hairy root disease [32]. In addition, *rolB*-transformed protoplasts exhibit a 10 000 to 100 000-fold increased sensitivity to auxin of the electrical response compared to untransformed protoplasts [27,28]. Fig. 4 shows the effects of Nt-ERabp1 and the two C-terminal peptides addition to *rolB*-transformed protoplasts. Both Nt-ERabp1 and Nt-C15 induced the hyperpolarization of BBGus protoplasts whereas Nt-C12 has no effect, as described with the untransformed protoplasts (Figs. 2 and 3). The dose-re-

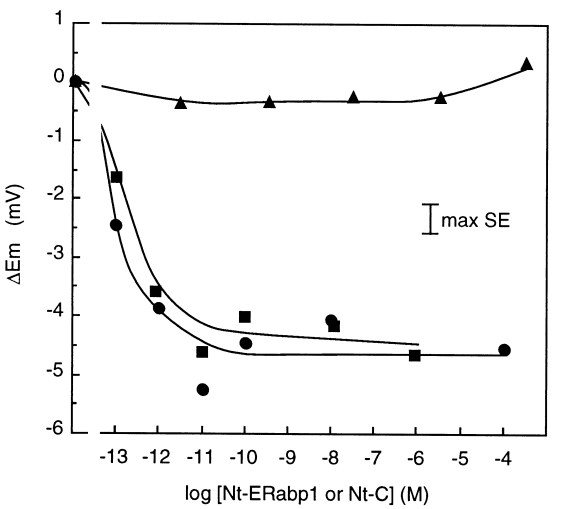

Fig. 4. Effects of Nt-ERabp1 (■) and its corresponding C-terminal peptides Nt-C15 (●) and Nt-C12 (▲) on the transmembrane potential of *rolB*-transformed tobacco protoplasts. The dose-response curves were established by plotting ΔE_m values as a function of peptide or protein concentration. Data are given for one representative experiment among four independent experiments. In this experiment, the different peptides were assayed on the same protoplast preparation. The mean E_m variation induced in these experiments by the optimal auxin concentration (10 pM, 1-NAA) was −4.3 mV. Maximal standard error (max SE) is indicated in the figure.

sponse curves of BBGus protoplasts to Nt-ERabp1 and Nt-C15 were shifted towards lower concentrations of protein and peptide. In both cases, the EC_{50} are observed for 10^{-13} M compared to 10^{-10} M in the untransformed background. Thus, in these experimental conditions about 1000-fold less protein or peptide are needed to induce the electrical membrane response of BBGus protoplasts. A similar shift in sensitivity was observed when comparing the response of BBGus and untransformed protoplasts to maize ABP1 and its C-terminal peptide (data not shown).

The shift in sensitivity observed with *rolB*-transformed protoplasts indicates a relationship between the peptide or ABP1 and the auxin responses. The results indicate that *rolB* expression potentiates the electrical response capacity of the protoplasts to auxin [28] and to Nt-ERabp1 itself. These data provide good evidence that the auxin and Nt-ERabp1 responses share the same pathway which may be modulated by RolB. Fillipini and co-workers [33] have described that a recombinant RolB protein, expressed in *E. coli*, exhibits a tyrosine phosphatase activity. Such phosphatase activity could play a role in the regulation of the auxin transduction pathway initiated at the plasma membrane; however, the precise function of *rolB* and its relation with auxin still have to be elucidated.

To conclude, the present report shows a panel of results obtained under homologous conditions, namely tobacco ABP1 protein and C-terminal peptides in a functional assay based on the electrical response of tobacco mesophyll protoplasts. A significant gain of efficiency is obtained under these conditions compared to the use of heterologous protein or peptide. The ABP1 C-terminal peptide and the whole ABP1 protein have been shown to induce a hyperpolarization of tobacco protoplasts, consistent with the idea that ABP1 interacts with plasma membrane components and that the C-terminal domain of the ABP1 protein is involved. To further analyze the relative importance of specific residues within the C-terminal domain of Nt-ERabp1 in the activation of the response, site-directed mutagenesis is in progress. In addition, we have shown that ABP1 protein and C-terminal peptides mimic the effect of optimal and infra-optimal concentrations of auxin on untransformed and *rolB*-transformed protoplasts. This provides direct evidence that ABP1 and auxin activate the same pathway. Our results highlight the need for a strict control of the levels of ABP1 present at the cell surface as, over a critical concentration, ABP1 alone is able to activate the auxin pathway originating from the plasma membrane.

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