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Indoleacetic acid pretreatment of tobacco plants in vivo increases the in vitro sensitivity to auxin of the plasma membrane H^+-ATPase from leaves and modifies the polypeptide composition of the membrane

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The sensitivity to auxin of the H^+-ATPase-mediated proton translocation was investigated in vitro using purified plasma membrane vesicles from tobacco leaves. In vivo pretreatment of tobacco plants with auxin promotes a 100-fold increase of the in vitro sensitivity to auxin. This effect is specific for biologically active auxins and is dose- and time-dependent. In addition, pretreatment with auxin induces the accumulation of several polypeptides in the plasma membrane. These polypeptides constitute the first set of hormone-responsive polypeptides evidenced in plant membranes.

Auxin; Plasma membrane H^+-ATPase; Proton translocation; Auxin-responsive polypeptides

1. INTRODUCTION

Auxins constitute the group of plant hormones for which arguments supporting the hypothesis of plasma membrane-located effects in their mechanisms of action are the most convincing. It is known that auxin is able to hyperpolarize the plasma membrane by stimulating an H^+-ATPase [1]. Stimulation of the H^+-ATPase-mediated proton translocation was demonstrated in vitro on plasma membrane vesicles [2] and on protoplasts by patch-clamp technique [3]. As auxin does not stimulate directly the H^+-ATPase [4] the existence of a reception and transduction pathway leading to ATPase stimulation should be assumed. However, no element of this pathway has been clearly identified yet.

Recently, the auxin effect on the proton translocation catalyzed by the H^+-ATPase was shown to depend on the development state of plants [2,5]. For instance, the proton translocation in vitro in plasma membrane vesicles from tobacco leaves was stimulated during the floral induction by auxin concentrations 1000 times lower than those which were effective for vegetative, induced or flowering plants. On the other hand, differences in the sensitivity to auxin were observed for induced plants and flowering plants between normal tobacco and *rol* A transformed plants [6]. Finally the comparison of two lines of Petunia showed that the overall sensitivity pattern was similar to that described for tobacco, but that for one line the responsiveness disappeared for induced or flowering plants [7]. In principle, this set of functional data offers opportunities to select well-defined systems, i.e. purified plasma membrane vesicles, suitable for a comparative biochemistry analysis. Such an approach could be an alternative way to identify the proteins involved in the response to auxin of the H^+-ATPase. However, in all the above situations, potential biochemical differences between plasma membrane vesicles differing by their sensitivity to auxin could be related as well to the sensitivity differences themself as to developmental or genetic differences.

In the present work, we took advantage of the plasticity of the response to auxin observed with induced or flowering plants [6,7] to try to modify, by an auxin pretreatment of plants, the response to the hormone. By this way, plasma membrane vesicles differing by their sensitivity to auxin were prepared from plants in the same developmental state. The sensitivity shift was shown to be specifically promoted by biologically active auxins. Quantitative comparison of plasma membrane polypeptides patterns, by two-dimensional gel electrophoresis and image analysis, allowed to evidence polypeptides which were systematically accumulated in the plasma membrane after plant pretreatment.

2. MATERIALS AND METHODS

Tobacco plants (*Nicotiana tabacum* cv Xanthi) were grown from seed in a controlled chamber (20°C, 70% relative hygrometry, 16 h photoperiod, 16,000 lx) as previously described [2] and cultivated in 3 liters pots containing a mixture of peat and gravel. Plants were used between 15 and 20 days after floral induction. At this state the sensitivity to auxin was nearly constant [5]. For auxin pretreatment, 150 ml of an hormone aqueous solution at the chosen concentration were

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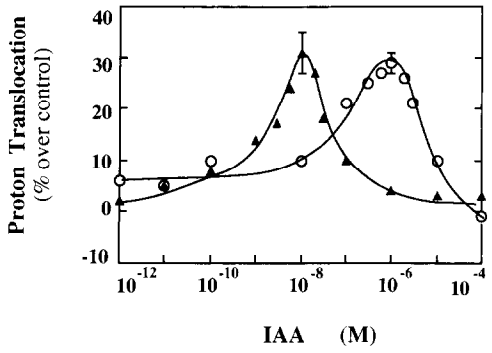

Fig. 1. Auxin effects on the proton translocation in plasma membrane vesicles. Proton translocation was measured by the initial fluorescence quenching rate of ACMA using plasma membrane vesicles purified 24 h after pretreatment of plants with water (control plants, circles) or with 150 nmol IAA per pot (triangles). Proton translocation activity in the absence of auxin amounted respectively to 105 ± 1 and 103 ± 3% of quenching · min^{-1} · mg^{-1} for control and pretreated plants.

added to each pot 2 h after the beginning of the light period. Control plants were treated with 150 ml water. Leaves were harvested the following days 2 h after the beginning of the light period and immediately processed for membrane purification.

Plasma membrane vesicles were purified from a microsome suspension by phase partitioning as previously [2,5]. Proton translocation was monitored on native vesicles by the fluorescence quenching rate of 9-amino-6-chloro-3-methoxy-acridine (ACMA) using the procedure described elsewhere [2,5–7]. The assay medium contained 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-Tris buffer at pH 6.5, 2 μM ACMA, 600 μM $MgSO_4$, 50 mM KNO_3, 50 mM KBr and 25 μg of membranes. Reaction was started by the addition of 250 μM ATP. Proton translocation was measured for a set of auxin concentration ranging between 10^{-12} M and 10^{-4} M IAA (indoleacetic acid). Auxin was added from a 100-fold concentrated solution in acetonitrile. Controls included 1% acetonitrile and were performed in quadruplicate (at the beginning, at the end and during the experiment). Mean variation coefficients for controls amounted to 5.6%. Bell-shaped dose–response curves were obtained and the sensitivity to auxin was determined as the auxin concentration inducing the highest proton translocation activity [2,5–7]. For optimal concentrations, measurements were repeated from two to four times. Mean variations coefficients of fluorescence quenching rate at optimal concentrations amounted to 3.5%.

Two-dimensional gel electrophoresis was performed according to O'Farrell [8] using pH 3.5–10 ampholytes for isoelectric focalisation and a 10 % acrylamide/bisacrylamide gel in the second dimension. After silver staining of proteins [9] the intensity of spots was quantified according to reference 10 [10] using an image analysis system (SAMBA 2005, Alcatel-TITN, Grenoble, France) fitted out with a black-and-white CCD camera and an image processing card (4 × 512 × 512 × 8 bits, Matrox MVP/AT). Quantification was performed in two steps. First, grey levels, from 0 to 256, of visually detected spots (G) and of background (G_0), and hence optical density (log G_0/G), were computed for ca 50 spots which were systematically analyzed. Five spots, the optical density of which was found to correspond to a constant fraction of the sum of optical densities of the 50 spots in all gels, were taken as internal references. In a second step, selected spots were compared to these references and their relative amount was expressed as the percentage of their optical density over the sum of optical densities of references.

3. RESULTS AND DISCUSSION

The ATPase-catalyzed proton translocation in plasma membrane vesicles from control plants was stimulated in a biphasic way according to auxin concentration (Fig. 1). In agreement with previous results for plants in the same developmental state [6], the highest proton translocation activity occurred in the presence of micromolar IAA (1.9×10^{-6} M ± $3.0\ 10^{-6}$ M, $n = 9$). On the other hand, when plants were irrigated with IAA solutions ranging between 10^{-10} M (15 pmol IAA per pot) and 10^{-4} M (15 μmol IAA per pot), the sensitivity of plasma membrane vesicles from leaves was found to increase in a way depending on both the hormone pretreatment (Figs. 1 and 2) and time (Fig. 3). In optimal conditions, the highest proton translocation activity in vitro occurred in the presence of ca. 10 nM IAA (9.3×10^{-9} M ± $1.5\ 10^{-9}$ M, $n = 8$). This higher sensitivity was observed 24 h after pretreatment (Fig. 2) for plants irrigated with 10^{-6} M IAA (150 nmol IAA per pot) or with the same amount of the biologically active analog indolebutyric acid (IBA), but not with the biologically inactive analog indolecarboxylic acid (ICA). The sensitivity increase was observed during 4 days, but disappeared 8 days after the pretreatment (Fig. 3). Finally, the pretreatment was repeated either 24 h or 24 h and 48 h after the first pretreatment and leaves were harvested 24 h after the last pretreatment. In all situations (data not shown), the proton translocation across plasma membrane vesicles was found to be stimulated by ca. 10 nM IAA as it was observed for a single pretreatment. All these results extend previous observations showing that the sensitivity to auxin of plants induced to flowering exhibits some plasticity [6,7]. The nature of the mechanism allowing for the modulation of the sensitivity remains however not clear. No investigation was performed here concerning the evolution of the auxin added. Therefore, although the effect is clearly saturable with respect to both time (Fig. 3) and dose (Fig. 2), the sensitivity shift cannot be rationalized in

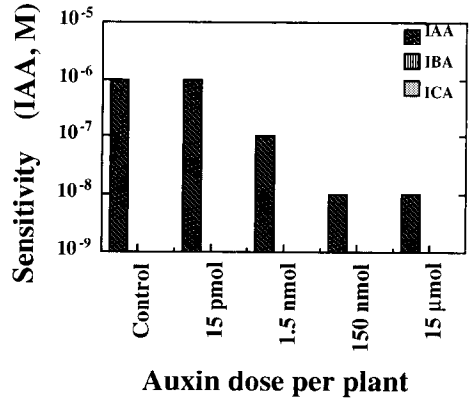

Fig. 2. Changes of the sensitivity to auxin of the plasma membrane according to pretreatment. The sensitivity to IAA of the plasma membrane H^+-ATPase was measured on membrane vesicles purified from plant leaves 24 h after pretreatment. Plants were pot irrigated with 150 ml of water (control), indoleacetic acid (IAA), indolebutyric acid (IBA) or indolecarboxylic acid (ICA) at concentrations ranging between 10^{-10} M and 10^{-4} M.

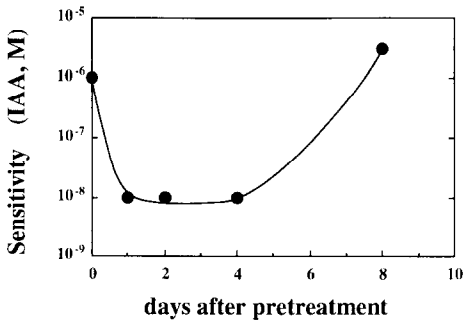

Fig. 3. Persistency of the pretreatment effect on the sensitivity to auxin of the plasma membrane H^+-ATPase. Plants were pot irrigated with 10^{-6} M IAA (150 nmol) and leaves were harvested from control plants (day 0) or treated plants at various time intervals.

terms of pretreatment concentration. However, new opportunities arise from the observation that the sensitivity to auxin at the membrane level, as assessed in vitro by the hormone effect on proton translocation, depends on the hormonal history of the plant. For instance, the manipulation of hormonal conditions allows one to obtain plasma membrane vesicles taken at the same developmental state and exhibiting a differential sensitivity suitable for comparative biochemistry.

To compare the plasma membrane polypeptide compositions of control plants to that of plants pretreated in conditions which induce the largest sensitivity shift (24 h after irrigation with 150 nmol IAA per pot), plasma membrane proteins were submitted to two-dimensional gel electrophoresis (Fig. 4A). About 150 polypeptides were eye-detected after silver nitrate staining and the largest spots were quantified by image analysis. No dramatic modification of the polypeptide pattern was observed between the two conditions. However, the relative abundance of several polypeptides appeared to change upon auxin pretreatment of plants. For some polypeptides the changes were quite moderate: ca. +50% for the polypeptide n°51 and ca. +70% for the polypeptide no. 52 (Table I). A larger variation was observed in the case of polypeptide no. 11 which increased of ca. +300% (Table I). As no systematic investigation, using membrane treatment at various ionic strengths or with detergents, was performed here, these polypeptides could a priori correspond as well to integral as to superficial proteins. In first analysis therefore, they should be taken only as polypeptides which remained associated with sedimentable material after all steps of differential centrifugation, phase partitioning and washing of phases. However, the 3 polypeptides constitute systematically major components of proteins in plasma membrane fractions from tobacco leaves or from cultured tobacco cells. In this latter material they have been shown to be present in plasma membrane fractions washed at high ionic strength (0.5 M KBr) or with the detergent Triton X-100 [11]. This suggests that the 3 polypeptides behave as integral proteins, at least in cell suspensions. Another possibility of artefact is

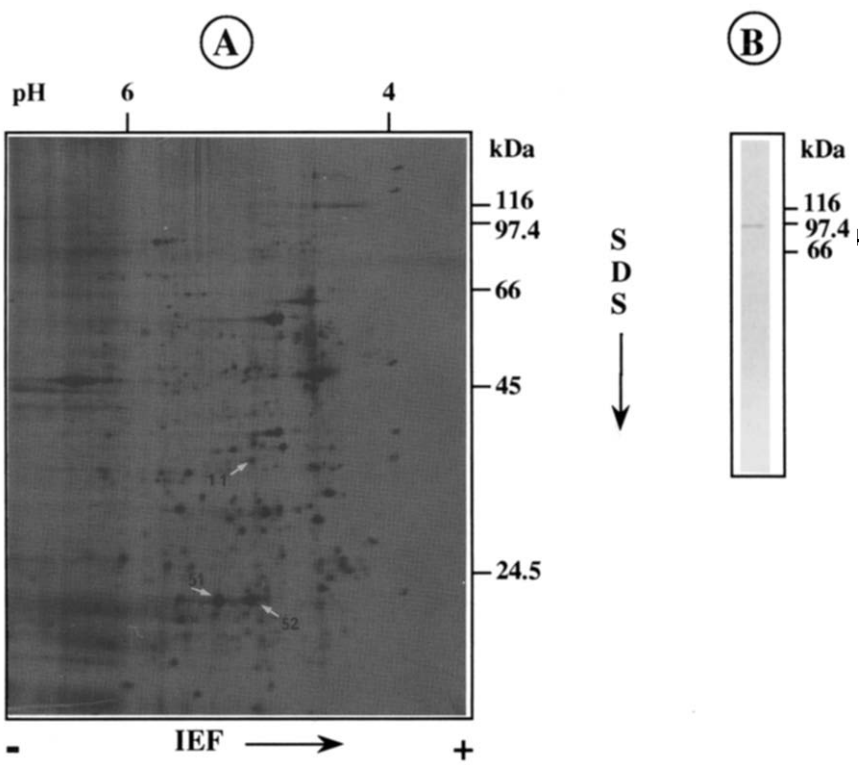

Fig. 4. Gel electrophoresis of plasma membrane proteins. (A) Proteins (40 μg) were separated by IEF in the first dimension and by SDS-PAGE in the second one. Arrows indicate the label of spots found to vary 24 h after pretreatment of plants with 150 nmol IAA. (B) Immunological detection of the plasma membrane H^+-ATPase after SDS-PAGE separation of proteins (10 μg plasma membrane proteins).

Table I

Characterization of auxin-responsive polypeptides in the plasma membrane from tobacco leaves

	Polypeptide n°		
	11	51	52
Polypeptide characteristics			
MW_r (kDa)	36.3 ± 2.0	23.2 ± 0.3	23.3 ± 0.3
pI	4.92 ± 0.15	5.29 ± 0.07	5.01 ± 0.10
Amount in plasma membrane vesicles			
Control	100 ± 20	100 ± 7	100 ± 11
+ 150 nmol IAA (24 h)	413 ± 57	151 ± 8	172 ± 30

that these proteins could be soluble proteins trapped in the internal volume of vesicles. Two facts argue against this hypothesis. Firstly, the ratio of internal vesicle volume over grinding buffer volume is extremely low (less than 0.001) whereas spots no. 11, 51 and 52 correspond to major polypeptides. Secondly, by using membrane preparations highly enriched in either inside-out vesicles or right-side-out vesicles, we observed that the above polypeptides are major polypeptides of both kinds of vesicles [12]. On this basis, it could be proposed that the 3 polypeptides correspond in fact to integral proteins of the plasma membrane from tobacco leaf.

The accumulation of proteins or corresponding transcripts in response to auxin has been described already in a few cases for soluble proteins [13,14]. Regarding membrane proteins, it was suggested on an immunological basis that the H^+-ATPase synthesis increased in response to auxin [15]. On the basis of its MW and pI, the H^+-ATPase was not clearly identified on 2D gels after silver staining. However, a single response corresponding to a polypeptide of ca. 97 kDa was evidenced by Western blot using antibodies raised against the plasma membrane H^+-ATPase from corn root (Fig. 4B). This indicates that polypeptides 11, 51 and 52 do not represent degradation products of the H^+-ATPase. As no systematic investigation concerning the plasma membrane H^+-ATPase was performed here according to auxin pretreatment, the present data evidence that, beside previous results concerning this enzyme [15], several plasma membrane polypeptides could be responsive to auxin. Both the nature of polypeptides and the process leading to their accumulation in the plasma membrane are unknown. The fact that they were resolved by two-dimensional gel electrophoresis offers now opportunities to prepare tools allowing to answer these questions.

In conclusion, the present data show that some plasticity can be evidenced in the plant plasma membrane at both a functional level and a structural level. These variations could constitute in plants a new way to investigate the mode of action of auxin and to identify new plasma membrane proteins and genes.

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