Proton pump and auxin effect in *Arabidopsis thaliana* leaves during the development

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Abstract

The plasma membrane from Arabidopsis thaliana leaves (wild-type and F mutant) has been purified by two-phase partitioning and the H+-transport activity was tested in vitro in the presence of different concentrations of IAA. While 1 µM of IAA had no effect, higher concentrations (10 µM and 100 µM) were inhibitory in 25 short days grown wild-type plants. However, the activity was increased by about 16 % after 10 supplementary short days of growth (from 25 to 35 SD) in the represence of 10 µM of IAA. No significant sensitivity to the tested concentrations of auxin was observed during the development in short days (30, 38, 42 SD) or even the plants were induced by a 24 h of continuous light. The variability was not observed for a given developmental stage, from one experiment to another, but it was also observed after light induction of F mutant plants.

Reference


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PROTON PUMP AND AUXIN EFFECT IN *Arabidopsis thaliana* LEAVES DURING THE DEVELOPMENT

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SUMMARY

The plasma membrane from *Arabidopsis thaliana* leaves (wild-type and F mutant) has been purified by two-phase partitioning and the H⁺-transport activity was tested *in vitro* in the presence of different concentrations of IAA. While 1 μM of IAA had no effect, higher concentrations (10 μM and 100 μM) were inhibitory in 25 short days grown wild-type plants. However, the activity was increased by about 16% after 10 supplementary short days of growth (from 25 to 35 SD) in the presence of 10 μM of IAA. No significant sensitivity to the tested concentrations of auxin was observed during the development in short days (30, 38, 42 SD) or even the plants were induced by a 24 h of continuous light. The variability was not observed for a given developmental stage, from one experiment to another, but it was also observed after light induction of F mutant plants.

Key words: *Arabidopsis thaliana*, H⁺-transport activity, vegetative and reproductive development, life cycle of higher plant

INTRODUCTION

The transition from vegetative to reproductive development is a crucial phase in the life cycle of higher plants. This suggests an understanding of the physiological, biochemical and molecular aspects of reproductive development. The plant development is controlled both by exogenous and endogenous signals and by genetic factors, but the mechanisms involved in the recognition of these different signals and their target are still not well understood. Studies underwent on spinach demonstrated the possibility to induce or to inhibit the flowering by different chemical and physical ways. The effect of these treatments has been confirmed by ultrastructural analysis of the apical meristem (1). Several markers, and especially those concerning the plasma membrane, have been found to change early during induction (critical photoperiod: 12 h of continuous light), and just after in the apex (2). It was shown also that the plasma membrane H⁺ATPase could be stimulated *in vitro* by exogenous auxin (3). The stage of development of the plant and the treatments that it could have been received before
plasma membrane extraction, had an important influence on the in vitro sensitivity of the H^+ATPase to IAA. The change in this sensitivity was also shown in other plant species during development (4). Interestingly, the changes of the sensitivity to auxin were correlated to the induction time of flowering (4) and seems to be due to the variation of the plasma membrane H^+ATPase content (5,6) or to the auxin-binding sites content at the plasma membrane level (7).

In this paper, we described the in vitro effect of exogenous IAA on H^+ accumulation in the purified plasma membrane vesicles extracted from *Arabidopsis thaliana* leaves. It is a plant with separated vegetative and reproductive phases. The transformation of the vegetative shoot meristem into a reproductive inflorescence meristem becomes apparent by changes in the structure and in the pattern of mitotic activity of the shoot apex (8). Flowering is promoted by long day photoperiods and low temperatures (vernalization), but neither requirement is absolute. *Arabidopsis thaliana* plant was chosen because, with a short generation time, a small genome size and a great number of available mutants, it is most attractive for future genetic studies, especially those related to the flowering process. Obtaining a similar sensitivity to IAA during flowering induction as in spinach leaves (3) would lead us to investigate genetic studies in *A. thaliana* to understand the molecular mechanism(s) leading to the change in the in vitro sensitivity in relation to the flowering induction.

**MATERIAL AND METHODS**

**Plant Material.** Seeds of Ler ecotype of *Arabidopsis thaliana* and seeds of a late-flowering mutant of Est ecotype (F mutant) were kindly provided from Dr. Maarteen Koornneef (9). Plants were cultivated in soil (Teramax Belflor, Bachman S. A., Chevroux, Switzerland) and grown in a phytotron for 4 weeks under non inductive short days of 8 h light (8:00 am to 4:00 pm) and 16 h dark (4:00 pm to 8:00 am ). The temperature was set at 20 ± 1°C and the relative humidity at 80 ± 5% during the light period and 60 ± 5% during the darkness. The light was provided by white fluorescent tubes (40 W, 244332, 400 µmol m^-2 s^-1, Sylvania daylight, USA).

**Preparation of microsomal vesicles.** Crude microsomal vesicles were prepared as described by Olivari et al. (10). Leaves (20 g) were harvested and homogenized at 4°C in a medium (4 ml per g fresh weight) containing 250 mM sucrose, 10 mM EGTA, 2 mM MgSO4, 2 mM Na2 ATP, 1 mM PMSF, 2 mM DTT, 0.5 % (w/v) BSA, 25 mM BTP-MES, adjusted to pH 7.6 in the presence of polyclar (1 g/20 g fresh weight). The homogenate was filtered and centrifuged at 6,000 x g (7,000 rpm, Sorvall, SS-34 rotor) for 10 min at 4°C. The resulting supernatant was filtered through one layer of Miracloth (475855 Calbiochem) and centrifuged at 30,000 x g for 30 min at 4°C. The pellet (crude microsomes) was resuspended in phosphate buffer (K2HPO4, Na2HPO4, 5 mM, pH 7.8) and plasmalemma was purified by phase system containing 6.2 % (w/w) Polyethylene glycol 3350, 6.2 % (w/w) Dextran (T500), 0.44 mM
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potassium phosphate buffer pH 7.8, 3 mM KCl and 0.22 M sucrose (11). Upper phases U3 and U'3 were combined and plasma membrane vesicles were recovered by centrifugation at 30,000 x g for 30 min at 4°C, washed in BTP-Mes 25 mM pH 6.7 additioned with 10 mM EGTA, resuspended in the same medium without EGTA and used immediately. For the test of the H+ pumping activity, the plasma membrane vesicles recovered by centrifugation were washed in the resuspension medium (250 mM sucrose, 10 % glycerol, 1 mM DTT, 0.2 % BSA, 1 mM PMSF, 2.5 mM BTP-MES pH 7.0) additioned with 10 mM EGTA, resuspended in the same medium without EGTA, and stored at -80°C until used.

Proton pumping activity. The initial rate of quinacrine fluorescence quenching was utilized to measure the plasmalemma proton translocation activity as described by Bennett and Spanswick (12). This activity was expressed in arbitrary unit (AU) min⁻¹ mg⁻¹ protein. Fluorescence was measured with an Aminco Bowmann spectrofluorimeter at the excitation/emission wavelengths of 420/500 nm. Thawed membrane vesicles (20-30 µg proteins) were incubated at room temperature for 10 min. in 1 ml of 25 mM BTP-Mes pH 6.7, 2 µM quinacrine, 0.25 µM valinomycin, and 50 mM KBr. The reaction was started by addition of 1.5 mM Mg ATP. The quenching of quinacrine fluorescence was completely reversed by addition of 10 µl of 1 mM monensin. The reaction could also be started by addition of 1.5 mM Na₂ ATP, in this case the reaction medium contained 5 mM MgSO₄.

Protein determination. Membrane aliquot (30 µl) was diluted in 1 ml of cold water and centrifuged at 96,100 x g (Beckman, T-50 rotor) for 30 min at 5°C. The pellet was resuspended in cold water (160 µl) and proteins was determined using Bio-Rad solution. 50 µl of protein suspension were diluted in 750 µl of water and 200 µl of Bio-Rad solution were added. The obtained solution was mixed and incubated at room temperature for at least 5 min. The OD at 595 nm was measured and proteins were quantified using BSA as standard.

Reproducibility of the data. In all the cases the H⁺ pumping activity from the same plasma membrane preparation was tested in the absence of auxin (control) and was repeated three times. It was expressed in arbitrary units (AU) min⁻¹ mg⁻¹ prot ± SD (see captions of figures). Some times the activity was also repeated three times when it was tested in the presence of a concentration of IAA to see whether the stimulation was significative.

RESULTS AND DISCUSSION

Arabidopsis thaliana (early ecotype Landsberg erecta) is a quantitative long day plant. It flowers within 28 long days (CL) of 24 h after sowing, or within 70 short days (SD) of 8 h after sowing. When the plants were grown in SD for 4 weeks after sowing and then transferred to continuous light of 24 h, it flower 15 days after transfert.

The plasma membrane was phase-partitioning purified from 4 weeks-old A. thaliana leaves (Ler) and characterized by biochemical markers as described by Bellamine and Greppin (13). The H⁺ transport activity of the plasma membrane vesicles extracted from 25 days-old leaves of A. thaliana grown in SD, was tested in vitro, in the presence of different concentrations of IAA. At this stage of plant development, auxin 1 µM had no effect on the in
vitro H⁺ATPase activity. Higher concentrations of IAA (10 μM and 100 μM) were inhibitory (fig. 1 A). Acetic acid, a substance devoid of biological activity, stimulated weakly the enzymatic activity as function of the concentrations used (data not shown), showing that the inhibition was specific for the auxin. However, when the growing time was extended to 30 SD, the plasma membrane H⁺ATPase activity exhibited no sensitivity to the tested concentrations of IAA (fig. 1 B). This IAA dose-response was also observed when the growing time was extended further to 38 or 42 SD or even the 4 weeks-old plants were transferred to an inductive long day of 24 h (fig. 1 D-E). However, a weak stimulation of the activity was observed in the presence of 10 μM IAA when the plants were grown during 35 SD (fig. 1 C).

Same experiments were done with a late flowering mutant of A. thaliana (F mutant). This mutant does not flower under short days and has a growth and flowering behavior as the wild type in long days. The in vitro H⁺ transport activity of the plasma membrane vesicles extracted from either 25 days-old leaves or 32 days-old leaves of this mutant grown in SD, was also inhibited in the presence of the tested concentrations of IAA (fig. 2). However, when the 4 weeks-old plants were transferred to an inductive long day of 24 h, the plasma membrane H⁺ATPase activity exhibited, in this case also, no sensitivity to the tested concentrations of IAA (fig. 2). The concentration of 1 μM IAA seemed to be stimulatory (ca. 10%).

The effect of IAA on the H⁺ATPase activity obtained in A. thaliana was different to that obtained in spinach (3). In the vegetative state, 10 μM and 100 μM IAA were inhibitory in A. thaliana, whereas, they had no effect in spinach. After light induction, the sensitivity at 1 μM IAA obtained in spinach, and which had been correlated to the acquisition of the flowering state, was absent in A. thaliana. After 35 SD, there is a weak stimulation (ca. + 8%) in the presence of 10 μM IAA of the H⁺ transport activity. This means that the activity was stimulated by about 16 % after 10 supplementary SD of growth (from 25 to 35 SD) (fig. 1 A, 1 C). This variability was never observed for a given developmental stage, from one experiment to another. However, it was also observed after light induction of F mutant plants (fig. 2).

Growth of both wild-type and mutant plants in SD for 25 days led to an inhibitory effect of high concentrations of IAA (10 μM and 100 μM) on the plasma membrane H⁺ATPase. This effect was also reported on the primary-root elongation in wild-type and auxin response mutants of A. thaliana (14), and emphasized once more the role of the H⁺ATPase in plant growth (15). After flowering induction by 24 h of continuous light, the plasma membrane H⁺ATPase was not inhibited by high concentrations of IAA (10 μM and 100 μM), in wild-type and F mutant of A. thaliana. This was observed since 30 SD in wild-type but still absent until 32 SD in the late flowering mutant (fig.s. 1 B, 2). It could so be taken as a marker of the flowering state in A. thaliana.
Fig. 1. Effect of IAA on the plasma membrane-H\textsuperscript{+}ATPase activity. Plasma membrane was purified from *Arabidopsis thaliana* (Ler) leaves grown in short days (SD) during (A) 25 days (control: 43.66 ± 0.90 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot), (B) 30 days (control: 44.52 ± 0.83 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot), (C) 35 days (control: 21.60 ± 0.67 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot), (D) 38 days (control: 38.15 ± 0.75 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot) or (E) 42 days (control: 23.33 ± 0.38 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot). (E) plants of 4 weeks-old were transferred to continuous light for 24 h (IND) (control: 85.19 ± 1.31 AU min\textsuperscript{-1} mg\textsuperscript{-1} prot). The plasma membrane vesicles were incubated in the reaction medium in the absence (control) or in the presence of the indicated IAA concentrations for 10 min. The reaction was started by addition of 1.5 mM Mg-ATP. The specific activity was expressed as a percent stimulation over the control level. Experiments were repeated two or three times with a new plasma membrane preparation.
Fig. 2. Effect of IAA on the plasma membrane-H\(^+\)ATPase activity. Plasma membrane was purified from F mutant of *Arabidopsis thaliana* leaves grown in short days (SD) during (□) 25 days (control: 48 ± 0.83 AU min\(^{-1}\) mg\(^{-1}\) prot) or (●) 32 days (control: 62.5 ± 0.20 AU min\(^{-1}\) mg\(^{-1}\) prot). (□) plants of 4 weeks-old plants were transferred to continuous light for 24 h (IND) (control: 51.4 ± 2.1 AU min\(^{-1}\) mg\(^{-1}\) prot). The plasma membrane vesicles were incubated in the reaction medium in the absence (control) or in the presence of the indicated IAA concentrations for 10 min. The reaction was started by addition of 1.5 mM Mg-ATP. The specific activity was expressed as a percent stimulation over the control level.

Finally, except a weak inhibitory effect (not exceed 10 %) of 10 μM and 100 μM IAA on the enzyme activity in 25 days-old wild type and 25 and 32 days-old F mutant, and a weak stimulatory effect (not exceed 10 %) of 10 μM IAA in 35 days-old wild type, no sensitivity to the tested concentrations of IAA was observed during plant development and after flowering induction by light. Our aims for the future is to emphasize these results by others obtained from other late and early-flowering mutants of *A. thaliana*. At this moment, we can not decide to investigate genetic studies to elucidate the molecular mechanism(s) leading to flowering in relation with the plasma membrane signalling.

REFERENCES


