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Effect of photoperiod and gibberellic acid on the plasma membrane H^+ATPase activity of spinach leaves

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Abstract

Plasma membrane H^+ATPase extracted from leaves of spinach plants induced to flower by gibberellic acid treatments or by a transfer to a photoperiod of 24 h had a lower Km_{app} than that from vegetative plants grown in short days. The Km_{app} obtained after inhibition by vanadate was decreased in vegetative plants and increased in induced ones showing a differential effect of this inhibitor on the kinetic properties of the enzyme between vegetative and induced plants. The phospholipid fatty acid analysis of the purified plasma membrane showed an increase of C18:1/C18:2 fatty acid ratio upon induction by light or by gibberellic acid treatments, whereas the saturated to unsaturated fatty acid ratio was kept constant. The decrease in the Km_{app} observed after induction may be thus interpreted in terms of the observed changes in lipid environment.

Introduction

Light plays a critical role in growth and development, and it was shown to affect gene expression and regulate enzyme activities in plants (Kristie and Jolliffe 1986, Casal *et al.* 1987, Ranjeva and Boudet 1987, Bernier 1988, Thompson 1991). Exogenous application of chemical compounds such as gibberellic acid (GA_3), a plant hormone, is also known to play a role in plant growth and development and to provoke or to favour induction of flowering in some species (Metraux 1987). Primary events occurring within the leaf cells upon exposure to inductive photoperiodic conditions or treatment with GA_3 could be transmitted from cell to cell within the whole plant. The transmission of a stimulus could involve the plasmalemma of leaf cells (Greppin *et al.* 1978). This hypothesis prompted us to undertake a systematic study of biochemical composition and properties of leaf cell membranes especially those of the plasma membrane. It has been demonstrated that light treatment of spinach leaves induced plasmalemma thickening (Crespi *et al.* 1989) and quantitative and qualitative modifications of plasmalemma sterol content (Crespi *et al.* 1993).

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The same modifications were also observed after a 72 h GA_3 treatment of plants maintained under short day. Karege *et al.* (1982) reported an activity modulation by light of spinach leaf peroxidases. Red light inhibited and far red light stimulated peroxidase activity in plants kept in short days. However, in plants induced by 24 h of continuous white light, or by 72 h GA_3 treatment, red light stimulated and far red light inhibited peroxidase activity. The direct *in vitro* activation of H^+ATPase by auxin has now been reported for several different plant species using plasma membrane isolated by two-phase partitioning (Santoni *et al.* 1991, François *et al.* 1992, Bellamine *et al.* 1993). The common characteristics of these experiments was that they were conducted with plasma membrane prepared from green leaves. In many cases the sensitivity of plasma membrane H^+ATPase towards auxin concentration depended closely on the developmental stage of plants used to prepare plasma membrane (Santoni *et al.* 1991, François *et al.* 1992).

In the case of spinach, this sensitivity appeared to be greatly influenced by treatments that induce the plant to flower (Bellamine *et al.* 1993). Vegetative spinach H^+ATPase exhibited a maximum sensitivity at low concentrations of indole-3-acetic acid (IAA), whereas spinach induced to flower by an inductive photoperiod of 24 h, or, in short day, by GA_3 during 72 h, showed a second peak of sensitivity for higher IAA concentrations. Such a change in sensitivity to a major plant growth regulator appeared to be largely independent of the synthesis of new specific proteins (Lefort *et al.* 1991).

In this paper we describe the activity of the H^+ATPase of purified plasma membrane vesicles (Km_{app} determination and vanadate inhibition). Fatty acid content of the plasmalemma preparation is also reported after light and GA_3 treatment.

Materials and methods

Plant material: Spinach (*Spinacia oleracea*, cv. Nobel) plants at the vegetative stage were grown in a phytotron for 4 weeks under non inductive short days (SD) of 8 h light (8.00 to 16.00, 400 µmol m^{-2} s^{-1}) and 16 h dark. Light was provided by white fluorescent tubes (40 W, *244332 Sylvania,* Danvers, USA). The temperature was set at 20 ± 1°C and the relative humidity at 80 ± 5 and 60 ± 5 % during the light period and in darkness, respectively.

Floral induction was initiated by 2 ways: (*1*) plants grown in SD for 4 weeks were exposed to continuous light (CL) for 24 h (400 µmol m^{-2} s^{-1}) as described by Auderset *et al.* (1986), or (*2*) plants grown in SD were sprayed by 1 mM GA_3 for consecutive 3 d as described by Crespi *et al.* (1989).

Preparation of microsomal vesicles: Crude microsomal vesicles were prepared as described by Bellamine *et al.* (1993). Plasmalemma was purified in an aqueous polymer two-phase system as described by Kjellbom and Larsson (1984). Upper phases U_2 and U'_2 were combined and plasma membrane vesicles were recovered by centrifugation at 30 000 *g* for 30 min at 4 °C, washed in the resuspension medium (250 mM saccharose, 10 % glycerol, 1 mM DDT, 0.2 % BSA, 2.5 mM BTP-MES,

pH 7.0), and resuspended in the same medium. The plasma membranes were stored at -80 °C until used.

ATPase activity: The plasmalemma ATPase activity (30 µg protein) was measured at 37 °C during 30 min in the presence of 50 mM KCl, 100 mM KNO_3, 1 mM sodium molybdate, 5 mM sodium azide and 50 mM Hepes-Tris, pH 6.7. The reaction was started by addition of 1.5 mM Mg ATP. The membrane vesicles were pelleted at 12 000 rpm and the inorganic phosphate released by ATP hydrolysis was determined by adding 0.2 cm^3 of the resultant supernatant to 0.5 cm^3 of 0.24 % (m/v) ammoniummolybdate (in 0.5 M H_2SO_4), 0.96 % (m/v) ascorbic acid and 0.8 % trichloroacetic acid. After incubation at 36 °C for 10 min the absorbance at 660 nm was measured (Dieter and Marmé 1980).

Proton translocation assay: The initial rate of quinacrine fluorescence quenching was utilized to measure the plasmalemma proton translocation activity as described by Bennet and Spanswick (1983). Fluorescence was measured with an *Aminco Bowmann* spectrofluorimeter (*Amerilan Instrument Company*, Maryland, USA) at the excitation/emission wavelengths of 420/500 nm. Thawed membrane vesicles (20 - 30 µg proteins) were incubated at room temperature in 1 cm^3 of 50 mM Hepes-Tris 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 0.01 cm^3 of 1 mM monensin.

Fatty acid analysis: Total lipids were extracted from purified plasma membrane vesicles with chloroform-methanol solution (2:1), and fatty acid methyl esters were prepared by transesterification with 10 % BF_3 containing anhydrous methanol at 75 -80 °C for 30 min as described by Chapman and Barber (1987) with some modifications. The aqueous phase was removed and organic solution was dried by addition of some crystals of anhydrous $MgSO_4$.

The solution was filtered and concentrated with a rotavapor. Then, it was dried again with some crystals of $MgSO_4$ and filtered. The solution was dried under nitrogen.

The fatty acid methyl esters obtained were then analysed by gas chromatography on an *Intersmat* chromatograph (*Intersmat Instruments*, Courtry, France) with *Nukol* (*Supelco*, Gland, Switzerland) fused silica capillary column (15 m × 0.53 mm ID) at 160 °C. Injection and flame ionization detector (FID) temperatures were about 220 °C. Signal output was quantified with *Varian* integrator (*Varian*, USA). Retention time and response factors were determined using pentadecanoic (C15:0) methylester as an internal reference.

Protein determination: Membrane aliquots were diluted 20 fold with cold water and centrifuged at 96 100 *g* for 30 min at 5 °C. The pellet was resuspended in cold water (0.16 cm^3) and proteins were determined using *Bio-Rad* solution (*Bio-Rad*, Glattbrugg, Switzerland). 0.05 cm^3 of protein suspension were diluted in 0.75 cm^3 of water and 0.2 cm^3 of *Bio-Rad* solution were added. The obtained solution was mixed

and incubated at room temperature for at least 5 min. The absorbance at 595 nm was measured and proteins were quantified using BSA as the standard.

Results

The ATPase and ATP-dependent H^+pumping activities were both measured at different pH values and a maximal activity was observed at pH 6.7. The H^+ATPase of purified plasma membrane is specific for ATP. Its activity is weak when it was assayed in presence of ADP, GTP, pyrophosphate (PP_i) or phosphoenolpyruvate (PEP) as substrates (data not shown).

The Km_{app} (apparent Michaelis-Menten constant) of plasmalemma ATP-dependent H^+ pumping activity was determined for plants grown under SD (Fig.1) and from plants induced to flowering by transfer to 24 h of CL or by GA_3 treatment in SD as well. The H^+ pumping activity from induced plants was more efficient than that from vegetative (VEG) ones. However, the effect of GA_3 was lower in comparison with inductive light (Table 1). The ratios of the Km_{app} from vegetative plants to induced ones by 24 h of CL vary from 2 to 3. Sodium orthovanadate, a well-known poison able to specifically inhibit the plasma membrane H^+ATPase (DeMichelis and Spanswick 1986, Rasi-Caldogno *et al.* 1985, Pederson *et al.* 1987), reduces the H^+ accumulation rate into the purified plasma membrane vesicles of spinach leaves. Its effect was studied in the presence of increasing concentrations of ATP. The Lineweaver-Burk double reciprocal plots were used to determine the Km_{app} in absence and presence of 80 µM vanadate (Fig. 2). For vegetative spinach,

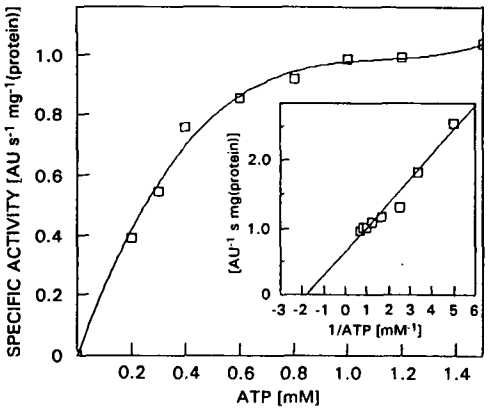


Fig. 1. Km_{app} determination for the ATP-dependent H^+ pumping activity from spinach leaves grown under short day (vegetative). Inset shows Lineweaver-Burk double reciprocal plot of values. Plasma membrane vesicles were incubated 10 min in the reaction medium (as described in Materials and methods) and the reaction was started by addition of indicated concentration of Mg ATP.

the Km_{app} decreased from 0.794 mM to 0.501 mM upon addition of vanadate but it increased from 0.252 mM to 0.463 mM for plants induced by 24 h of CL. Similar

inhibitions were observed in the presence of 120 μM vanadate. The two straight lines (absence and presence of vanadate) intersect in two different places when "vegetative" curves were compared to "induced" ones.

Table 1. Apparent Michaelis-Menten constant (Km_{app}) values of plasma membrane ATP dependent-H^+pumping activity from vegetative plants or plants induced by 24 h of continuous light or by GA_3 treatment as well. The values represent the mean of three independent experiments ± standard deviation.

Treatment	Km_{app} [mM ATP]
Control (SD vegetative plants)	0.697 ± 0.095
SD + 24 h CL	0.223 ± 0.023
SD + GA_3 (3 d)	0.412 ± 0.049

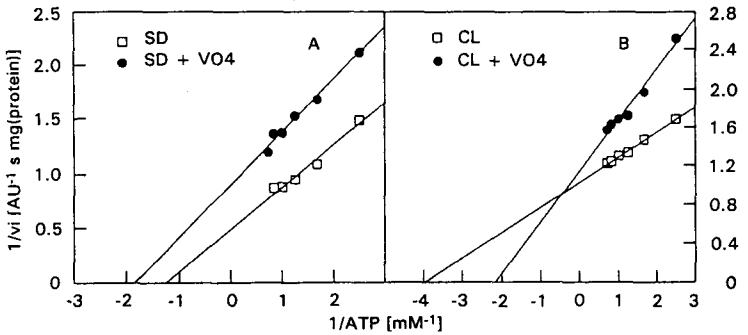


Fig. 2. Lineweaver-Burk double reciprocal plots for Km_{app} determination for the ATP-dependent H^+ pumping activity from spinach leaves grown under short days (*A*) or induced to flowering by 24 h of continuous light (*B*) either in absence (*open circles*) or presence of 80 μM vanadate (*closed circles*). Plasma membrane vesicles were incubated 10 min in the reaction medium, containing the vanadate or not (as described in Materials and methods). The reaction was started by addition of indicated concentration of Mg ATP.

Fatty acid methyl esters were prepared from plasma membrane of vegetative plants and induced ones by continuous light (24 h or 48 h) or by gibberellic acid treatment. The saturated to unsaturated and C18:1 to C18:2 phospholipid fatty acid ratios were calculated. The principal acyl component in all plasma membranes analysed was C18:2 (Table 2). After light induction, the level of the fatty acids C16:0 was not changed comparatively to that of vegetative plants. It weakly, but significantly, increased after GA_3 treatment. The level of the fatty acids C18:1 increased by about two times, and that of the fatty acids C18:2 and C18:3 decreased after induction by light. Accordingly, the C18:1/C18:2 phospholipid fatty acid ratio was increased after light induction of 24 h and remained stable till 48 h of CL. The same changes in the level of these fatty acids, but much weaker, were observed after treatment with GA_3, except for the fatty acid C18:3 where the level was not changed.

However, the saturated to unsaturated phospholipid fatty acid ratio remained unchanged after all treatments.

Table 2. Fatty acid composition of plasma membrane purified from vegetative spinach leaves and induced ones. The results were expressed as mol % and are representative of three experiments with three or four determinations. The ratios are the means of three experiments ± SD. Fatty acids were identified by co-chromatography with standards.

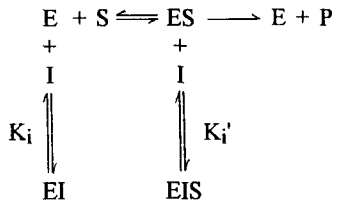
Fatty acids	Treatment VEG	24 h CL	48 h CL	GA_3
16:0	26.1 ± 0.36	26.1 ± 0.79	26.4 ± 0.81	27.2 ± 0.09
18:1	12.7 ± 0.25	24.0 ± 0.22	24.1 ± 0.64	13.6 ± 0.17
18:2	36.1 ± 0.21	29.3 ± 0.45	31.2 ± 0.99	34.0 ± 0.16
18:3	25.1 ± 0.19	20.5 ± 0.19	19.7 ± 0.88	25.1 ± 0.14
saturated/unsaturated	0.344 ± 0.023	0.347 ± 0.013	0.330 ± 0.026	0.364 ± 0.020
18:1/18:2	0.337 ± 0.012	0.799 ± 0.053	0.765 ± 0.020	0.427 ± 0.019

To examine the effect of the phospholipids on the ATP hydrolysis activity, we added to the incubation medium 0.05 % in methanol (m/v) of dioleoylphosphatidylcholine (PC C18:1) or phosphatidylethanolamine (PE C18:1). These two C18:1 phospholipids stimulated the ATPase activity by about 50 % when the polar group was choline and by about 20 % when the polar group was ethanolamine. To know whether the stimulation was specific for C18:1 acyl group, the dipalmitoylphosphatidylcholine (PC C16:0) was added and it appeared that it also weakly stimulated the ATP hydrolysis activity.

Discussion

In our previous paper (Bellamine *et al.* 1993) we reported a sensitivity change of plasma membrane H^+ATPase to IAA when plants were induced to flower by different methods. With additional studies presented here, we have obtained a plausible explanation for this change. *In vitro* studies of the efficiency of the H^+ATPase and its inhibition by vanadate for vegetative plants compared to induced ones suggested an intrinsic change of the H^+ATPase.

The Km_{app} of the ATP-driven H^+ transport into plasma membrane vesicles for vegetative plants was determined and is similar to that obtained for the same enzyme in other plants (DeMichelis and Spanswick 1986, Rasi-Caldogno *et al.* 1985, Pedchenko *et al.* 1990). The observed decrease in Km_{app} from vegetative plants and its increase from induced ones after inhibition by vanadate may be explained by the following non-competitive inhibition scheme. In such type of inhibition, the inhibitor binds to the enzyme before or after substrate fixation with the same probability ($K_i = K_i'$):



(E - enzyme, S - substrate, P - product, I - inhibitor, K_i and K_i' - inhibition constants)

In the "vegetative" case the inhibitor binds to the enzyme preferentially after substrate fixation ($K_i > K_i'$) and in "induced" case it binds to the enzyme preferentially before the substrate fixation ($K_i < K_i'$).

The decrease in the Km_{app} observed after induction suggests a conformational change of the H⁺ATPase within the plasma membrane, which could be the consequence of the lipid environment changes. This change in the enzyme conformation could be responsible for the diminution of the interaction between the enzyme and the auxin receptor and could explain the high IAA concentration needed to stimulate the H^+ pumping activity after plant induction (Bellamine *et al.* 1993).

The level of C18:2 fatty acids in spinach leaves plasma membrane exceeded the level of C18:3 fatty acids (Table 2), while in membranes of other plants, *e.g.* cucumber and maize, the level of C18:3 increased by greening (Murphy and Stumpf 1979, Hawke and Stumpf 1980). Fatty acid synthesis rate in spinach leaves may be stimulated by light and is strongly affected by the developmental stage of the leaves selected (Browse *et al.* 1981). The level of fatty acids and unsaturation degree may also be affected by temperature and by age as demonstrated for apple leaves (Ketchie and Kuiper 1979). The increase in the C18:1/C18:2 fatty acid ratio after induction by light or by GA_3 treatment, could influence the microdomain of the H^+pump or the fluidity of the plasma membrane leading to the change in H⁺ATPase activity. Such modifications could be described as an indirect effect of the lipids on enzyme activity. There are more and more evidence to believe that lipid components are important in the regulation of the plasma membrane H⁺ATPase activity.

The activity of isolated plant plasma membrane ATPases reconstitued into liposome vesicles depends on the type of phospholipid used (Cocucci and Marré 1984). This activity depends also on the type of phospholipid added to the purified plasma membrane as demonstrated by Kasamo and Nouchi in *Vigna radiata* L. (1987). The plasma membrane H⁺ATPase activity may be stimulated, and its Km_{app} decreased by lysophospholipids added exogenously to the purified plasma membrane or generated *in situ* by addition of phospholipase A_2 as demonstraded previously on corn and oat roots (Palmgren *et al.* 1988, Pedchenko *et al.* 1990). All these results suggest that lipids are implied in the regulation of the plasma membrane H⁺ATPase activity.

References

Auderset, G., Sandelius, A.S., Penel, C., Brightman, A., Greppin, H., Morré, D.J.: Isolation of plasma membrane and tonoplast fractions from spinach leaves by preparative free-flow electrophoresis and effect of photoinduction. - Physiol. Plant. **68**: 1-12, 1986.

Bellamine, J., Penel, C., Greppin, H.: Proton pump and IAA sensitivity changes in spinach leaves during the flowering induction. - Plant Physiol. Biochem. **31**: 197-203, 1993.

Bennet, A.B., Spanswik, R.M.: Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles: kinetic analysis of Cl-effects on a proton translocating ATPase. - J. Membrane Biol. **71**: 95-105, 1983.

Bernier, G.: The control of floral evocation and morphogenesis. - Annu. Rev. Plant Physiol. Plant mol. Biol. **39**: 175-219, 1988.

Browse, J., Roughan, P.G., Slack, C.R.: Light control of fatty acid synthesis and diurnal fluctuations of fatty acid composition in leaves. - Biochem J. **196**: 347-354, 1981.

Casal, J.J., Aphalo, P.J., Sanchez, R.A.: Phytochrome effects on leaf growth and chlorophyll content in *Petunia axalaris*. - Plant Sci. Environ. **10**: 509-514, 1987.

Chapman, D.J., Barber, J.: Polar lipids of chloroplast membranes. - In: Packer, L., Douce, R. (ed.): Methods in Enzymology. Vol. 148. Pp. 294-319. Academic Press, San Diego - New York - Berkeley - Boston - London - Sydney - Tokyo - Toronto 1987.

Cocucci, M., Marre, E.: Lysophosphatidylcholine-activated, vanadate inhibited, Mg^{2+}ATPase from radish microsomes. - Biochem. biophys. Acta **771**: 42-52, 1984.

Crespi, P., Crèvecoeur, M., Lefort, F., Greppin, H.: Changes in plasmalemma after gibberellic acid treatment. - Plant Sci. **62**: 63-71, 1989.

Crespi, P., Penel, C., Crèvecoeur, M., Greppin H.: Plasma membrane sterols and flowering induction. - Plant Sci. **89**: 153-160, 1993.

DeMichelis, M.I., Spanswick, R.M.: H^+ pumping driven by the vanadate-sensitive ATPase in membrane vesicles from corn roots. - Plant Physiol. **81**: 542-547, 1986.

Dieter, P., Marmé, D.: Partial purification of plant NAD kinase by calmodulin sepharose affinity chromatography. - Cell Calcium **1**: 279-286, 1980.

François, J.M., Bervillé, A., Rossignol, M.: Development and line dependent variations of *Petunia* plasma membrane H^+ATPase sensitivity to auxin. - Plant Sci. **87**: 19-27, 1992.

Greppin, H., Auderset, G., Bonzon, M., Penel, C.: Changement d'état membranaire et mécanisme de la floraison. - Saussurea **9**: 83-101, 1978.

Hawke, J.C., Stumpf, P.K.: Desaturation of oleic and linoleic acids by leaves of dark and light grown maize seedlings. - Plant Physiol. **65**: 1027-1030, 1980.

Karege, F., Penel, C., Greppin, H.: Detection de l'état végétatif et floral de la feuille de l'épinard: Emploi d'un indicateur biochimique. - Arch. Sci. Genève **35**: 331-340, 1982.

Kasamo, K., Nouchi, I.: The role of phospholipids in plasma membrane ATPase activity in *Vigna radiata* L. (mung bean) roots and hypocotyls. - Plant Physiol. **83**: 323-328, 1987.

Ketchie, D.O., Kuiper, P.J.C.: Fatty acid level in apple leaves of different age as affected by temperature. - Physiol. Plant. **46**: 93-96, 1979.

Kjellbom, P., Larsson, C.: Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light grown spinach and barley. - Physiol. Plant. **62**: 501-509, 1984.

Kristie, D.N., Jolliffe, P.A.: A rapid phytochrome-mediated growth response in etiolated *Sinapis alba* hypocotyls. - Can. J. Bot. **65**: 2017-2023, 1986.

Lefort, F., Thiellement, H., Greppin, H.: Polypeptide pattern modifications during floral induction in leaves of *Spinacia oleracea*. - Arch. Sci. Genève **44**: 227-234, 1991.

Métraux, J.P.: Gibberellins and plant cell elongation. - In: Davies, P.J. (ed.): Plant Hormones and Their Role in Plant Growth and Development. Pp. 296-317. Martinus Nijhoff Publishers, Dordrecht - Boston - Lancaster 1987.

Murphy, D.J., Stumpf, P.K.: Light-dependent induction of polyunsaturated fatty acid biosynthesis in greening cucumber cotyledons. - Plant Physiol. **63**: 328-335, 1979.

Palmgren, M.G., Sommarin, M., Ulvskov, P., Jorgensen, P.L.: Modulation of plasma membrane H^+ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A_2. - Physiol. Plant. **74**: 11-19, 1988.

Pedchenko, V.K., Nasirova, G.F., Palladina, T.A.: Lysophosphatidyl choline specifically stimulates plasma membrane H^+ATPase from corn roots. - FEBS Lett. **275**: 205-208, 1990.

Pederson, P.L., Carafoli, E.: Ion motive ATPases. I. Ubiquity, properties and significance to cell function. - Trends biochem. Sci. **12**: 146-150, 1987.

Ranjeva, R., Boudet, A.M.: Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. - Annu. Rev. Plant Physiol. **38**: 73-93, 1987.

Rasi-Caldogno, F., Pugliarello, F.M.C., DeMichelis, M.I.: Electrogenic transport of protons driven by the plasma-membrane ATPase in membrane vesicles from radish. - Plant Physiol. **77**: 200-205 1985.

Santoni, V., Vansuyt, G., Rossignol, M.: The changing sensitivity to auxin of the plasma-membrane H^+ATPase: Relationship between plant development and ATPase content of membranes. - Planta **185**: 227-232, 1991.

Thompson, W.F.: Physiological and molecular studies of light-regulated nuclear genes in higher plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. **42**: 423-466, 1991.