BIOLOGIA PLANTARUM 35 (4): 491-497, 1993

In vitro modification of spinach plasmalemma thickness

P. CRESPI, M. CRÈVECOEUR, C. PENEL and H. GREPPIN

Laboratory of Plant Biochemistry and Physiology, 3, Place de l'Université, 1211 Genève 4, Switzerland

Abstract

Floral induction in the long day plant spinach (*Spinacia oleracea*) has been shown to be accompanied by a thickening of plasmalemma. This change was observed at early evocation, in both shoot apices and leaves, as well as after inducing GA_3 treatment. To get further information on this thickening, plasma membranes from spinach leaves were isolated, in the present study, using aqueous two phase partitioning and the effect of various *in vitro* treatments on their thickness was investigated. The average plasmalemma thickness was unaffected by Na^+ and K^+ ions. It was increased upon the effect of either Ca^{2+} or gibberellic acid. A thickening of plasmalemma was also observed when plasma membranes from vegetative plants were incubated with a cytosolic preparation from photoinduced plants. The results were discussed in relation with the plasmalemma modifications previously reported in spinach.

Introduction

One hypothesis to explain photoinduction of flowering that resulted from investigations with the long day plant *Spinacea oleracea* is that of structural, biochemical and functional modifications in plasmalemma membranes (Greppin *et al.* 1987, Greppin *et al.* 1990). This one was then reinforced by more recent data indicating that this photoinduction was accompanied by a thickenning of leaf plasmalemma (Auderset *et al.* 1986). This change was reported to occur during the very first hours following the transfer of vegetative plants to continuous light as well as inducing GA_3 treatment in short days (Crespi *et al.* 1989). It was observed in both the leaves and the shoot apices (Penel *et al.* 1989, Crèvecoeur *et al.* 1992).

The only important biochemical modification detected until now in plasmalemma was in the sterols which exhibit qualitative and quantitative modifications (Penel *et al.* 1989), but their involvement in plasmalemma thickening still remain uncertain. To get further information on this change, we have investigated the effect of various *in vitro* treatments on its thickness.

Received 3 June 1992, *accepted* 21 September 1992.
Acknowledgment: This work was partly supported by a grant (no 31. 26510. 89) from the "Fonds National Suisse pour la Recherche Scientifique".

Materials and methods

Plant materials: Spinach plants (*Spinacia oleracea* L. cv. Nobel) were grown for 4 weeks under short day (photoperiod 8/16) in a growth chamber. The temperature was set at 23 °C and the relative humidity was maintained at 80 % during day and 60 % during night. Irradiance (400 μmol m^{-2} s^{-1}) was provided by fluorescent tubes (22 432-O *Sylvania*, USA).

Preparation of plasmalemma membranes: A microsomal pellet was obtained from the leaves as previously described (Penel *et al.* 1989) and used for isolation of pure plasma membrane by partition in a dextran-polyethylene glycol two phases system according to Rochester *et al.* 1987. Purified plasma membranes pellets were resuspended in 50 mM Hepes buffer (pH 7.5) containing 400 mM saccharose and 10 mM ascorbic acid. To examine potential contamination of our plasmalemma preparations by endomembranes, activities of cytochrome *c* reductase was used as endoplasmic reticulum marker (Hodges *et al.* 1974), latent inosine diphosphatase as Golgi apparatus marker (Quail 1980), cytochrome *c* oxidase as mitochondrial marker (Warton *et al.* 1967) and H^+ dependent pyrophosphatase as tonoplast marker (Wang *et al.* 1986). The activity of 1,3 ß glucan syntase II was used as plasma membrane marker and measured essentially as described by Kauss *et al.* 1985.

***In vitro* treatments of plasmalemma:** Purified plasma membranes (200 μg proteins) were incubated in Hepes buffer containing either $CaCl_2$, NaCl, KCl (10^{-5} M) or gibberellic acid (10^{-6} M) for 20 min at 20 °C.

Microsomal membrane pellets and supernatants from vegetative and from induced leaves as result of centrifugation at 30 000 × *g* (Auderset *et al.* 1986) were mixed. They were then recentrifuged at 30 000 × *g* during 30 min and the resulting pellet was submitted to aqueous two phase partitioning.

Electron microscopy: Plasma membranes were prepared for electron microscopy as previously reported (Crespi *et al.* 1989). Sections were stained with uranyl acetate and lead citrate. Electron micrographs were obtained with a Zeiss EM 10 at a magnification of 50 000 ×.

Measurements of membrane thickness: Measurements of plasmalemma thickness were made on negatives with a *BIOCOM* image analysis system (*Les Ullis*, France). A minimum of 300 measurements from 15 vesicles were made for each determination. The data were summed for each experimantal condition and their average analysed by the variance analysis and the Student's *t*-test.

Results

To establish the purity of the plasma membrane preparation obtained by aqueous two phase partitioning, we have assayed various biochemical markers (Table 1). Results

of these analysis indicate that the contamination of plasma membranes by other membranes was very low and mainly due to endoplasmic reticulum and Golgi apparatus. Purity of our plasma membrane can be estimated of 90 % and these were also completely free of chlorophyll (data not shown).

Table 1. Activities of the different enzymes markers in phases obtained by phase partitioning. U_1 represents the upper phase after two separations by phase partitioning and was enriched by plasmalemma vesicles. U_2 represents the upper phase after only one separation. L_3(lower phase) represents the rest of cell endomembranes. 100 % correspond to the activities found in $U1+U_2+L_3$.

Enzyme activities	U_1	U_2	L_3
1,3 β glucan syntase II	676	641	252
[cpm mg^{-1} protein 10^{-3}]	(43 %)	(41 %)	(16 %)
cytochrome *c* reductase antimycin A resistant	15.2	26.2	58.8
[ΔA_{550nm} min^{-1} mg^{-1} protein 10^{-5}]	(15 %)	(26 %)	(59 %)
cytochrome *c* oxidase	ND	ND	22.2
[ΔA_{550nm} min^{-1} mg^{-1} protein 10^{-5}]			(100 %)
H^+ dependent pyrophosphatase	ND	ND	1.2
[arbitrary units]			(100 %)
latent inosine diphosphatase	81	253	438
[arbitrary units]	(10 %)	(33 %)	(57 %)

ND - not detected

In leaf cells from short day spinach plants purified plasmalemma resuspended in buffer alone was 8.4 nm thick (Table 2). Plasmalemma thickness was not modified upon treatment with either NaCl or KCl. It showed increase upon the effect of $CaCl_2$. A similar thickening was also induced by treatment of plasma membranes with gibberellic acid. Variance analysis and Student t-test both indicate that the values of average thickness of plasmalemma obtained after the different experiments and increases observed were statistically significant ($P \leq 0.001$)

Table 2. Effect of various ions treatments on plasmalemma thickness from leaf cells [nm ± standard error of the mean]. These data correspond to one representative experiment. Each experiment was repeated at least three times.

Treatment	Average thickness
Control	8.28 ±1.10
KCl [10^{-5}M]	8.12 ±0.94
NaCl [10^{-5}M]	8.40 ±0.83
$CaCl_2$ [10^{-5}M]	9.43 ±1.40*
GA_3 [10^{-6}M]	9.00 ±1.00*

* - values statistically different from the thickness of untreated plasma membranes ($P \leq 0.001$).

In leaves from 24 h photoinduced plants (24 h light period) the plasma membranes were found to be significantly thicker than the control leaves (8 h light, 16 h dark) as previously reported (Table 3). A similar thickening was observed when plasma membranes from short day plants were treated with cytosolic preparation from photoinduced plants. On the other hand, the plasma membrane thickening observed as result of photoinduction was very slightly decreased by cytosolic preparation from vegetative short day plants.

Table 3. Effect of a cytosolic preparation on plasmalemma thickness [nm ± standard deviation of the mean]. These data correspond to one representative experiment. Each experiment was repeated at least three times.

Pellet from	+	Cytosol from	Average thickness
S.D. plants	+	S.D. plants	8.97 ± 0.97
L.D. plants	+	L D. plants	10.06 ± 0.98
S.D. plants	+	L.D. plants	10.18 ± 0.95
L.D. plants	+	S.D. plants	9.37 ± 0.85

* - values statistically different ($P \leq 0.001$) from control (S.D. + L.D. plants cytosolic preparation). S.D. - short day plants; L.D. - long day plants

Discussion

Experiments of the present study show that plasmalemma thickness can be increased by various *in vitro* treatments of purified plasma membranes from spinach leaf cells. Such a change in plasmalemma has been previously reported to occur in spinach, in both the leaves and the shoot apices, during floral induction (Auderset *et al.* 1986, Penel *et al.* 1989, Crèvecoeur *et al.* 1992). In leaf cells this change was observed *in situ* as well *in vitro* and after various staining procedures. We are conscious that these plasmalemma thickness increases reflect differences in the deposition of fixation and stain molecules. However these differences, regularly correlated with induction process, indicate modifications inherent in the membranes. Such a thickening of plasmalemma has been also reported in maize root cells during germination (Crèvecoeur *et al.* 1982) and in oat root cells during pinocytosis (Wheeler *et al.* 1973). A reversible change in the thickness of thylakoid membranes has been reported in *Ulva* and *Porphyra* upon illumination of dark thalli (Murakami *et al.* 1970). We show here that plasmalemma thickness was unaffected by Na^+ and K^+ whereas it was significantly increased by Ca^{2+}. Such an effect of calcium ions has been reported by Morré *et al.* (1976). These authors showed that plasmalemma thickness from soybean hypocotyls treated with 0.5 M calcium chloride was 15 to 20 % thicker than controls. This effect was abolished by EDTA treatment of the membranes. This change in plasmalemma was suggested to be related to some components removed by EDTA and membrane aggregation, fusion (Morré *et al.* 1974, Morré *et al.* 1976) or trafficking (Morré *et al.* 1992).

Plasma membranes from spinach leaf treated with gibberellic acid were also seen to become thicker than controls. This plant hormone is known to provoke or to favor flowering induction in some long day species (Pharis *et al.* 1985). In spinach, an increase of plasmalemma thickness was previously reported in GA_3 treated plants, in both leaves and shoot apices (Crespi *et al.* 1989, Crespi *et al.* 1990). A modification of plasmalemma dimension upon the effect of another plant hormone, the indole-3-acetic acid (IAA) has been described in soybean hypocotyls (Morré *et al.* 1976). It was shown that plasma membranes treated with IAA were 10 to 15 % thinner than controls. It may be suggested that the thickening observed in spinach, upon the effect of GA_3 could be due to the rearangement of membrane components at the time of fixation of GA_3 to membrane receptor (Ladyzhenskaya *et al.* 1987) or to the effect of GA_3 on ionic distribution (Nelles 1977).

An explanation for membrane thickening observed in spinach at evocation may be proposed on the basis of results in Table 3. They lead us to suggest that this modification could result from the effect of one or various cytosolic components whose concentration would increase during floral induction. These components could be enzymes, chemical substances or plant signals. Chemical substances could be for instance phenolic compounds which are known to react with osmium tetroxide and have been shown to increase in spinach leaves during floral induction (Fontana 1983). But other compounds like gibberellins or calcium, that are seen to provoke an increase of plasmalemma thickness *in vitro*, may also vary during floral induction. It is known that metabolism of gibberellins may be controlled by light (Gilmour *et al.* 1985, Metzger *et al.* 1980) and calcium ions have been shown to be involved in photoperiodic floral photoinduction (Friedman *et al.* 1989). However the only indication of a change in calcium concentration at floral evocation was given by Havelange (1989). This author described an increase of calcium concentration in shoot apices in *Sinapis alba* induced by a single day.

In conclusion, the experiments of present study indicate that plasmalemma thickness modification is a complex problem. The fact that this change was observed *in vitro* upon the effect of calcium and hormones lead us to suggest a possible involvement of a modification of signal transducers at plasmalemma level. Plasma membrane could be considered as an integrator of external and/or internal events, acting by a matrix of organic and ionic messengers. Physiological implication could also be the fitting *via* membranes of plant growth and development with environmental resources. This *in vitro* model could permit a simple approach of the first events in leaves during induction.

References

Auderset, G., Sandelius, A.S., Penel, C., Brightman, S.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.

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

Crespi, P., Crèvecoeur, M., Lefort, F., Greppin, H.: Modification of apical meristem plasmalemma during floral induction of *Spinacia oleracea* (cv. Nobel). - Physiol. Plant. **79**(2, Part 2): A 19, 1990.

Crèvecoeur, M., Deltour, R., Bronchart, R.: Quantitative freeze-fracture study of plasmalemma and nuclear envelope of *Zea mays* root cells during early germination. - J. Ultrastruct. Res. **80:** 1-11, 1982.

Crèvecoeur, M., Crespi, P., Lefort, F., Greppin, H.: Sterols and plasmalemma modifications in spinach apex during transition to flowering. - J. Plant Physiol. **139:** 595-599, 1992.

Fontana, D.: Thèse No. 2098, Univ. Genève, Genève 1983.

Friedman, H., Goldschmidt, E.E., Halevy, A.H.: Involvement of calcium in the photoperiodic flower induction process of *Pharbitis nil*. - Plant Physiol. **89:** 530-534, 1989.

Gilmour, S.J., Zeevaart, J.A.D., Schwenen, L., Graeber, J.E.: The effect of photoperiod on gibberellin metabolism in cell free extract from spinach. - Plant Physiol. **557:** 92-95, 1985.

Greppin, H., Auderset, G., Bonzon, M., Degli Agosti, R., Penel, C.: Flowering and leaf shoot interactions. - In: Wagner, E., Greppin, H., Millet, B. (ed.): The Cell Surface in Signal Transduction. Pp. 157-167, Springer - Verlag, Berlin 1987.

Greppin, H., Bonzon, M., Crespi, P., Crèvecoeur, M., Degli Agosti, R., Penel, C.: Physiological macrofunctions and indicators of the flowering process. - In: Millet, B., Greppin, H.(ed.): Intra- and Intercellular Communications in Plants. P. 107. INRA, Paris 1990.

Havelange A.: Levels and ultrastructural localization of calcium in *Sinapis alba* during the floral transition. - Plant Cell Physiol. **30:** 351-358, 1989.

Hodges, T.K., Leonard, R.T.: Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. - In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 32. Pp. 392-406. Academic Press, New York - San Francisco - London 1974.

Kauss, H., Jeblick. W.: Activations by polyamines, polycations and ruthenium of the Ca^{2+}-dependent glucan synthase from soybean cell. - FEBS Lett. **185:** 226-230. 1985.

Ladyzhenskaya, E.P., Dardzhaniya, L.G., Korableva, N.P.: [Influence of gibberellic and abscisic acid on some properties of the plasmalemma of potato tuber cells.] - Fiziol. Rast. **34:** 569-576, 1987. [In Russ.]

Metzger, J.D., Zeevaart, J.M.: Effect of photoperiod on the levels of endogenous gibberellin in spinach as measured by combined gas chromatography selected ion current monitoring. - Plant Physiol. **66:** 844-846, 1980.

Morré, D.J., Bracker, C.E.: Ultrastructural alternation in plant plasma membrane induced by auxin and calcium ions. - Plant Physiol. **58:** 544-547, 1976.

Morré, D.J., Penel, C., Greppin, H.: Protoplasma, submitted, 1993.

Murakami, S., Packer, L.: Light-induced changes in the conformation and configuration of the thylakoid membrane of *Ulva* and *Porphyra* chloroplasts *in vivo*. - Plant Physiol. **45:** 289-200, 1970.

Nelles, A.: Effect of gibberelic acid (GA_3)on the relative ion permeability (P_{Na}:P_K) of corn coleoptile cells. - Biochem. Physiol. Pflanzen **171**: 349-351, 1977.

Penel, C., Crespi, P., Crèvecoeur, M., Morré, D.J., Greppin, H.: Changes in plasma membrane sterols during floral induction. - In: Krekule, J., Seidlová, F. (ed.): Signals in Plant Development. Pp. 107-118. SPB Academic Publishing, The Hague 1989.

Pharis, R.P., King, R.W.: Gibberellins and reproductive development in seeds plant. - Annu. Rev. Plant Physiol. **36:** 517-568, 1985.

Quail, P.H.: Intracellular localization of phytochrome. - In: Trends in Photobiology. P. 485. Plenum Press, New York 1980.

Rochester, C.P., Kjellbom, P., Larsson, C.: Lipid composition of plasma membranes from barley leaves and roots, spinach leaves and cauliflower inflorescences. - Physiol. Plant. **71:** 257-263, 1987.

Wang, Y., Leigh, R.A., Kaestner, K.H., Sze, H.: Electrogenic H^+-pumping pyrophosphatase in tonoplast vesicles of oat roots. - Plant Physiol. **81:** 497-502, 1986.

Wharton, D.C., Tzagaloff. A.: Cytochrome oxidase from beef heart mitochondria. - In: Colowick, S.P., Kaplan, N.O. (ed.): Methods in Enzymology. Vol. 10. Pp. 245-250. Academic Press, New York - London 1967.

Wheeler, H., Baker, B.L.: Ultrastructure of thick membranes in plant roots exposed to uranyl and calcium salts. - J. Ultrastruct. Res. **43**: 355-361, 1973.

Communicated by J. KREKULE