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Autor: Bellamine, Jalil / Arrizabalaga / Greppin, Hubert

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CHARACTERIZATION AND IDENTIFICATION OF PLASMA MEMBRANE BEFORE AND AFTER FLOWERING INDUCTION BY FOURIER TRANSFORM INFRARED SPECTROSCOPY USING HORIZONTAL ATTENUATED TOTAL REFLECTANCE

BY

Jalil BELLAMINE*, Philippe ARRIZABALAGA & Hubert GREPPIN***

ABSTRACT

Horizontal Attenuated Total Reflectance - Fourier Transform Infrared spectroscopy (HATR/FTIR) has been used to analyse spinach (*Spinacia oleracea*) leaves plasma membrane before and after flowering induction by 24 h of continuous light or by gibberellic acid treatments. Pearson's correlation coefficient has been used to analyse similarities between spectra. We found that vegetative and induced plant plasma membrane spectra were mainly different in the 1800 to 1500 cm^{-1} window. This shows a compositional modification of plasma membrane which could be associated with flowering induction.

Key-words: *Spinacia oleracea*, flowering induction, gibberellic acid, vegetative.

Abbreviations: GA_3, gibberellic acid; Hepes, ((N-2-hydroxy ethylpiperazine)-N'-2-ethane-sulfonic acid); IAA, indole-3-acetic acid; Lum, continuous light; Tris, Tris (hydroxy methyl)-aminomethane; VEG, vegetative.

RÉSUMÉ

La spectroscopie HATR/FTIR a été utilisée pour analyser la membrane plasmique des feuilles d'épinard *Spinacia oleracea* avant et après induction florale. Le coefficient de corrélation de Pearson a été employé dans l'analyse des similitudes entre spectres. La différence principale entre les spectres de membranes de plantes végétatives et induites se situe entre 1800 et 1500 cm^{-1}. Cela montre que la composition de la membrane plasmique subit une modification qui pourrait être associée à l'induction florale.

INTRODUCTION

Modifications of several plasma membrane properties in spinach leaves and apices (*Spinacia oleracea*, cv. Nobel) are conspicuous soon after the transition from vegetative to floral state (GREPPIN *et al.*, 1987; CRESPI *et al.*, 1989; CREVECŒUR *et al.*, 1991; GREPPIN *et al.*, 1991; BELLAMINE *et al.*, 1993). These changes particularly concern: plasma membrane thickness, sterol composition and indole-3-acetic acid (IAA) and vanadate sensitivities of plasma membrane H^+ATPase.

* Laboratoire de Biochimie et Physiologie végétales, Place de l'Université 3, CH-1211 Genève 4.

** Ecotox, Avenue Sainte-Clotilde 23, CH-1211 Genève.

Nevertheless, the plasma membrane observation by electronic microscopy *in situ* or *in vitro* after purification, fixation and staining, gives several difficulties about interpretation of the obtained results.

The Fourier-transform infrared (FTIR) spectra of biological material are finger-print-like patterns which are reproducible and characteristic for different systems (URRY *et al.*, 1983; PURCEL & SUSI, 1984). This spectroscopic method has been used with success to classify and identify bacteria (HELM *et al.*, 1991).

The Horizontal Attenuated Total Reflectance (HATR) (WILKS & HIRSCHFELD, 1967; WILKS, 1968) gives transmission-like spectra for a very wide variety of samples, including products whose absorption is too intense for practical dispersive measurement (KHOO & ISHIDA, 1990). This method was utilised for nondestructive analysis in clinical studies of the internal organs (OZAKI & KANEUCHI, 1989).

The aim of the present study was to test whether this spectroscopic method (HATR/FTIR) can be used to identify and to characterize the isolated plasma membrane before and after flowering induction of spinach leaves.

MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea*, cv. Nobel) plants were grown in a phytotron for 4 weeks under non-inductive short days (SD) of 8 hours (08 am to 04 pm) and 16 hours dark (04 pm to 08 am). The temperature was set at 20 ± 1°C and the relative humidity at 80 ± 5% during light period and 60 ± 5% during dark period.

Floral induction

Floral induction was initiated in two ways:

a) Transfer to continuous light: Plants grown in SD for 4 weeks were exposed to 24 hours of continuous light (400 µmol $m^{-2}s^{-1}$) as described by AUDERSET *et al.* (1986). The light was provided by white fluorescent tubes (40 w, 244332 Sylvania USA).

b) Gibberellic acid treatments: Plants in short day were treated by 1 mM gibberellic acid during 3 days as described by CRESPI *et al.* (1989) and PENEL *et al.* (1988).

Preparation of microsomale vesicles

Leaves (16 g) were harvested and homogenised with a mixer at 4°C in a medium (4 ml per g of fresh weight) containing Hepes 50 mM, sucrose 400 mM, KCl 100 mM, $MgCl_2$ 1 mM and ascorbic acid 10 mM, adjusted to pH 7.5 with NaOH. 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. Plasmalemma

was purified by phase partitioning in an aqueous polymer two-phase system (6.2% dextran; 6.2% polyethylen glycoll) as described by Kjellbom and Larsson (1984). The pellet (crude microsomes) was resuspended in phosphate buffer (KH_2PO_4: Na_2HPO_4 5 mM pH 7.8) to give 16 g of the two phase system. This was mixed and the upper phase was washed with a fresh lower phase. The resultant upper phase (U_2) was diluted in Hepes-Tris 50 mM pH 6.7 and plasma membrane vesicles were recovered by centrifugation at 30,000 x g for 30 min and resuspended in the same buffer. The plasma membrane vesicles were analysed immediately.

Free flow electrophoresis

The plasma membrane purified by phase partitioning was analysed by free flow electrophoresis. The U_2 fraction was diluted in chamber buffer and centrifuged at 30,000 x g for 30 min. The pellet was resuspended in that buffer (500 µl).

The electrophoresis chamber buffer contained 0.03 M sucrose, 0.015 M triethanolamine, 0.004 M potassium acetate, 0.01 M glucose, 0.24 M glycine and adjusted to pH 7.5 with acetic acid. The electrode buffer contained 0.045 M triethanolamine, 0.012 M potassium acetate, 0.72 M glycine and adjusted to pH 7.5 with acetic acid. The electrophoresis equipment was Elphor VAR 21 (Bender and Hobein, Munich, FRG). Conditions for electrophoresis separations were as follows: constant current of 151 mA (about 1970 v), buffer flow 1.7 ml fraction^{-1} h^{-1}, sample injection 1 ml h^{-1} and constant temperature of 9°C. The distribution of membranes after separation was monitored from the absorbance at 280 nm.

The membrane vesicles were collected from separated fractions by centrifugation at 39,100 x g (18,000 rpm, Beckman, J A 20 rotor) for 30 min and resuspended in Hepes-Tris 50 mM pH 6.7 (100 µl).

ATPase activity

Membrane vesicles (50 µl) were incubated in 500 µl (final volume) of 50 mM KCl, 100 mM KNO_3, 1mM sodium molybdate, 5 mM sodium azide, 1.5 mM Mg ATP, and Hepes-Tris 50 mM pH 6.7 in presence or absence of 100 µM sodium orthovanadate. The reaction was started by addition of membrane vesicles and incubated at 36°C for 30 min. After this time, it was cooled in ice and centrifuged (12,000 rpm, Sorvall microfuge). Inorganic phosphate was determined by adding 200 µl of the resultant supernatant to 500 µl of 0.24% (w/v) ammoniummolybdate (in 0.5 M H_2SO_4), 0.96% (w/v) ascorbic acid and 0.8% trichloroacetic acid. After incubation at 36°C for 10 min the absorbance at 660 nm was measured (DIETER & MARMÉ, 1980).

Protein determination

Membrane aliquot (10 µl) was resuspended in 790 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.

Recording of the spectra

Plasma membrane vesicles (50 µg proteins) were deposited on crystal plate (ZnSe, refractive index 2.4 and 45° end faces) and dried under moderate vacuum between 65 and 60 KPa to obtain a film suitable for HATR/FR-IR measurements. All spectra were recorded between 4000 cm^{-1} and 650 cm^{-1} (wavenumbers) on FT-IR spectrophotometer (Perkin Elmer 1720-X) equiped with DTGS detector. Spectral resolution was 4 cm^{-1} and data point resolution was 1 point per cm^{-1} wavenumber. Second derivative of the original spectra were calculated using IRDM (Infrared Data Manager) software from Perkin Elmer.

Spectral windows

Spectral windows were selected considering their vibrators nature.

(1) The window between 3000-2800 cm^{-1} dominated by $-CH_3$, $-CH_2$ and -CH stretching vibrations present in the fatty acids, phospholipids, carbohydrates and sterols components of the membrane,

(2) The window between 1800 and 1500 cm^{-1} dominated by C=O stretching vibration of the carboxylic group present in fatty acid, and that of amide I and amide II present in proteins. This window is also dominated by C=C stretching vibration present in hydrocarbure chain of fatty acid and in aromatic compounds,

(3) The window between 1500 and 1400 cm^{-1} dominated by $-CH_3$ and $-CH_2$ bending vibrations,

(4) The window between 1200 and 900 cm^{-1} dominated by C-O stretching vibration of the functional group present in carbohydrates.

Analysis of spectral similarities

Second derivative spectra were digitized and the corresponding matrices were exported to Excel 3.0 software. We used Pearson's product moment correlation coefficient as a measure of similarity between spinach plasma membrane IR Spectra.

Since spectra were subdivided into several spectral windows, the similarity was calculated between two samples for each spectral window.

RESULTS

To analyse the purity of the plasma membrane fraction purified by phase partitioning, that fraction was subjected to a free flow electrophoresis. The distribution of the vanadate sensitivity of the ATPase (plasma membrane marker) from each fraction showed a single peak which superposed to that of the absorbance at 280 nm (Fig. 1). This indicates that our fraction contained high purified plasma membrane.

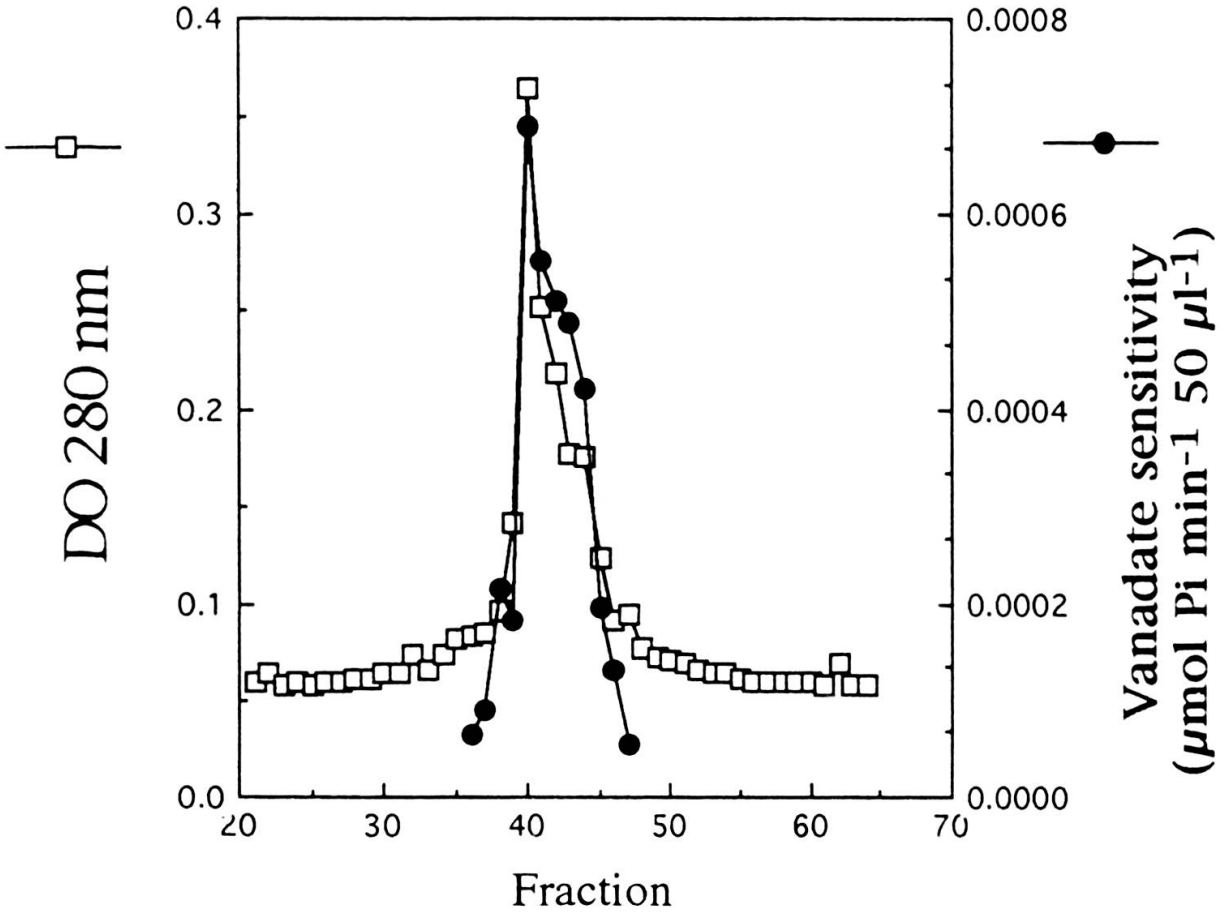

FIG. 1.

Free flow electrophoresis of the plasma membrane fraction purified by phase partitioning as described in Materials and Methods, and vanadate sensitivity of the ATPase from fractions separated by free flow electrophoresis. The vanadate sensitivity is determined as the difference between the ATPase activity in absence and in presence of 100 μM of vanadate. The ATPase activity was determined as described in Materials and Methods and expressed as μmole P_i min^{-1} 50 μl^{-1} protein. ATPase activity for fraction 41 was in absence of vanadate: 3.2 10^{-3} μmole min^{-1} 50 μl^{-1}, and in presence of 100 μM of vanadate: 2.52 10^{-3} μmole min^{-1} 50 μl^{-1}.

Plasma membrane vesicles were prepared from leaves in vegetative state, induced by 24 h of continuous light or treated by GA3 in short days. The analysis by HATR/FTIR spectroscopy of purified plasma membrane led to characteristic spectra (Fig. 2). Since absorption spectra obtained comprise vibrators of different plasma membrane components (proteins, lipids, polysaccharides), we expected a very complex spectrum due to the number of bands, peak positions, half width, and relative peak intensities. These factors led to overlapping bands, whose resolution can be enhanced by calculating, from original spectra, a second derivative using IRDM. It was showed by NAUMANN *et al.* (1988) that the second derivative of spectrum acts as a filter which helps to minimize experimental problems: slightly differing optical densities, base line shift and variable small amounts of water still present in plasma membrane films

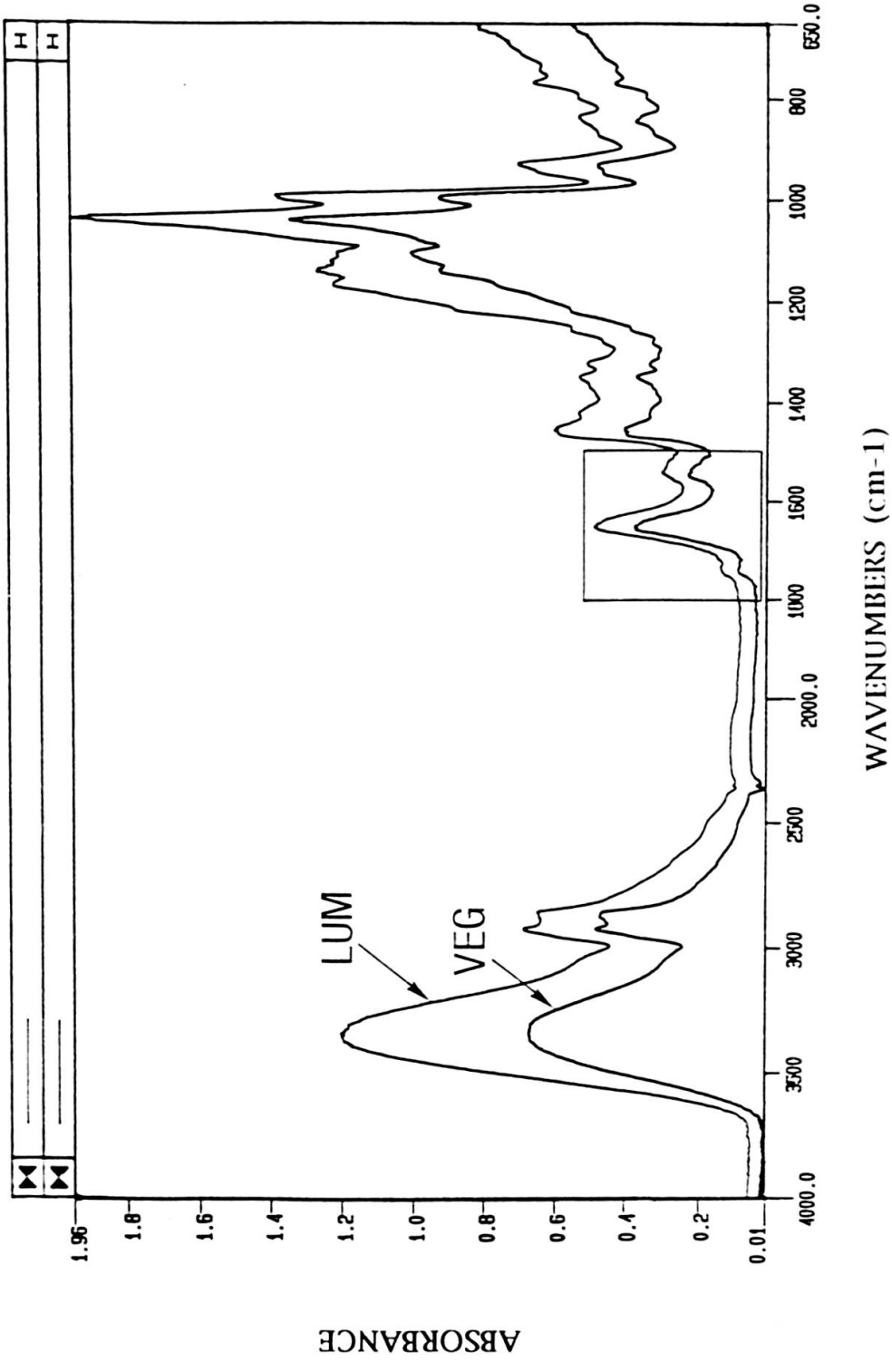

FIG. 2.

HATR/FTIR spectra of plasma membrane purified by partitioning phase from vegetative (VEG) and 24 h of continuous light induced spinach plants (LUM). The section within the frame shows selected spectra wich present the main dissimilarities between the two types of plants population. Spectra were recorded from plasma membrane vesicles (50 µg) as described in Materials and Methods.

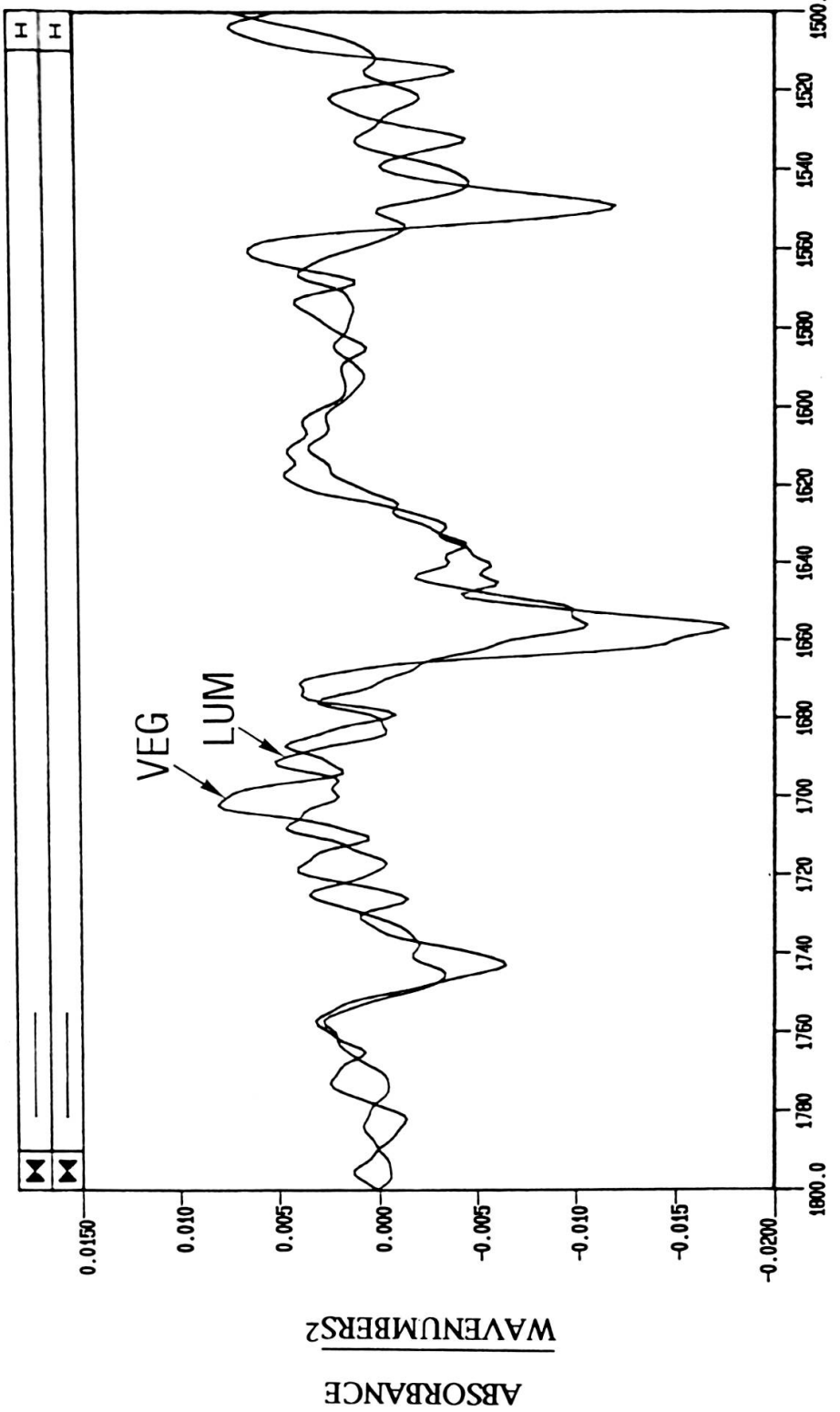

FIG. 3.

Second derivative spectra calculated from original spectra (spectral range: 1800-1500 cm^{-1}) of vegetative (VEG) and 24 h of continuous light (LUM) induced spinach plasma membrane. Original spectra were recorded from plasma membrane vesicles (50 μg) as described in Materials and Methods.

measured. We present in figure 3 an overplot of two second derivative spectra from vegetative and 24 h continuous light induced spinach plasma membrane preparations for the 1800-1500 cm^{-1} window.

The second derivative spectra were digitized and measured for similarities using Pearson's product moment correlation coefficient. When we purified *independently* the plasma membrane from *two sets* of vegetative plants, we got a high correlation coefficient for any window selected. This indicates that the degree of the purity is almost reproducible from one extraction to another, and the difference observed especially in 1800-1500 cm^{-1} window was not due to different level of impurities in plasma membrane preparation when plants were induced by light or by gibberellic acid (in short days) comparatively to vegetative plants (Table 1). Treatments by gibberellic acid was used here to verify whether the difference observed in 1800-1500 cm^{-1} was due to light effect after 24 h of continuous light treatment or actually to floral induction. High correlation coefficient was also obtained when two samples of the *same preparation* have been compared and indicated that the measurements by FTIR apparatus were reproducible (Table 1). For all windows selected we obtained a good Pearson's correlation coefficients (higher than 0.9). However, dissimilarities were observed for 1800 to 1500 and for 900 to 659 wavenumber ranges, when vegetative plants were compared to induced ones by 24 h of continuous light or by GA3 treatments (Table 1). Figure 4 A shows a good correlation, for the 1800-1500 cm^{-1} window, between two samples of the same vegetative plasma membrane preparation. However, when vegetative and induced by 24 h of continuous light plasma membrane preparations were compared, for the 1800-1500 cm^{-1} window, lower correlation was obtained (Fig. 4 B). Table 1 summarizes the Pearson's correlation coefficients for different windows selected of spectra recorded from vegetative and induced plants.

DISCUSSION

The flowering mechanisms and especially the leaves induction are, at this time not clearly known. So the discovery of some early flowering markers is of a great importance. For this reason, the HATR/FTIR of the purified plasma membrane should be useful to identify the floral state of plants as we have evidenced. The HATR/FTIR technique may lead to identification and characterization of the inductive state of spinach leaves plasma membrane. It is a simple, elegant and quick method (recording spectra within a minute after plasma membrane purification and drying) which should be applicable to nearly all types of plants.

The FTIR spectroscopy is a good tool to determine the presence of characteristic chemical vibrators. The absorbance spectra recorded from two samples seem to be identical for the reasons indicated in Results. The difference is more clear when second derivatives and then correlation coefficients are calculated. The second derivative spectra recorded from vegetative and induced plants were mainly different in the range

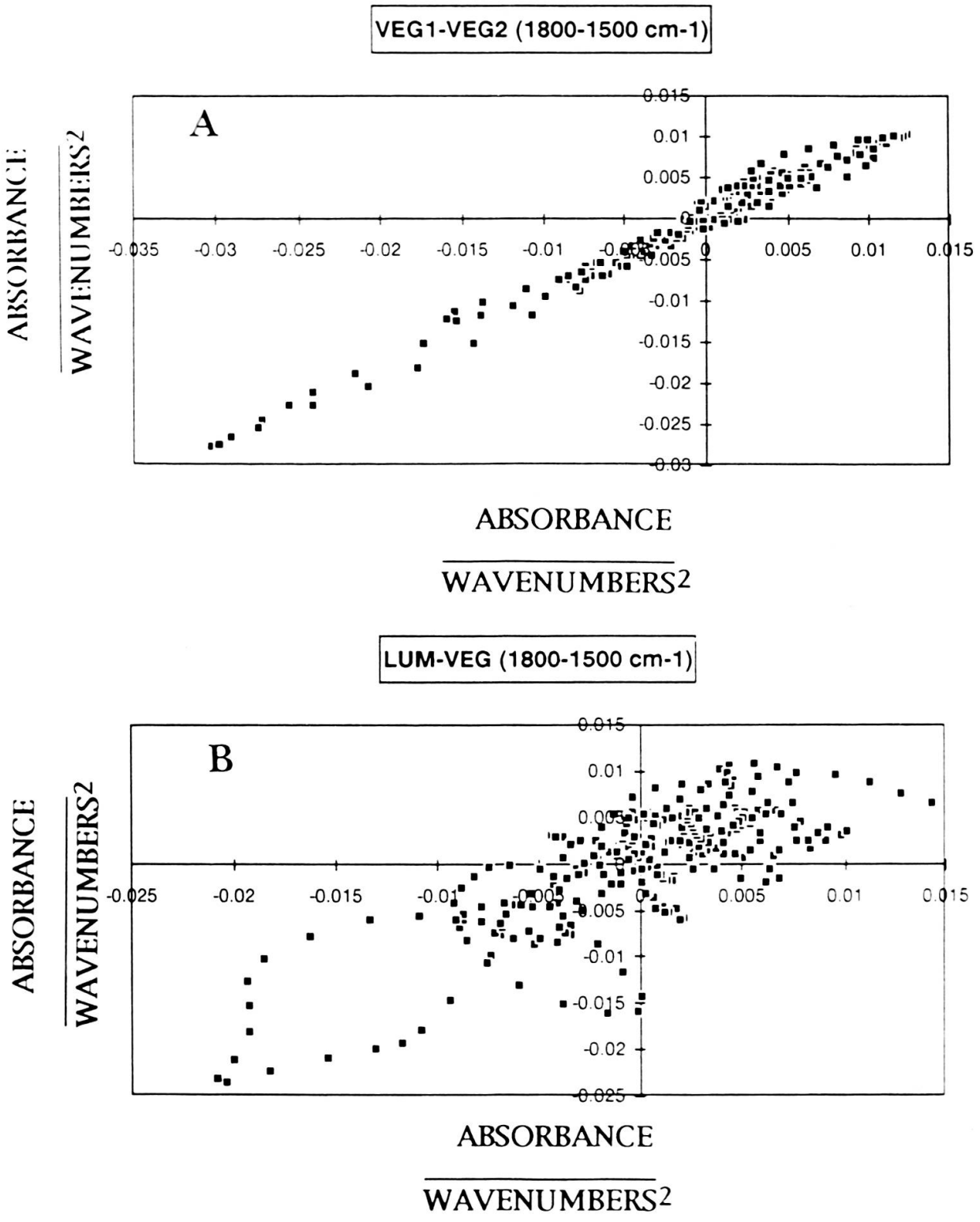

FIG. 4.

Correlation curves of second derivative spectra calculated from original spectra (spectral range: 1800-1500 cm^{-1}): A) The intensities of the second derivative spectra were compared between two samples of the same vegetative spinach plasma membrane preparation. B) The intensities of the second derivative spectra were compared between vegetative and 24 h of continuous light induced spinach plasma membrane preparations.

TABLE 1.

Pearson's correlation coefficients calculated from absorption spectra second derivatives, for different selected windows, recorded from vegetative and induced plants plasma membrane (50 µg proteins). All measurments have been done immediatly after plasma membrane purification. VEG, Lum and GA_3 were analysed in the same day. VEG_1 and VEG_2 are two spectra recorded from *the same vegetative* plasma membrane preparation. $VEGL_1$ and $VEGL_2$ are two spectra recorded from *independent* plasma membrane preparations from *two sets* of vegetative plants.

	3000-2800	1800-1500	1500-1400	1500-1200	1200-900	900-659	3747-659
AG3/VEG	0.8089	0.8087	0.9826	0.9251	0.9493	0.8900	0.9235
LUM/VEG	0.9045	0.6131	0.9597	0.8934	0.9627	0.8777	0.9319
LUM/AG3	0.9021	0.8839	0.9901	0.9898	0.9835	0.9666	0.9732
VEG1/VEG2	0.9269	0.9726	0.9987	0.9955	0.9909	0.9711	0.9751
VEGL1/VEGL2	0.9693	0.9117	0.9926	0.9945	0.9962	0.9952	0.9911

of 1800 to 1500 cm^{-1}. This window contains a characteristic band of C=O stretching vibration present in fatty acid (carboxylic group), and in proteins (amide I and amide II), and that of C=C stretching vibration present in fatty acid (hydrocarbure chain) or in aromatic compounds.

However, a plasma membrane spectrum is complex, and the characteristic band of a vibrator may be shifted by the presence of other neighbouring functions. Accordingly, we cannot certify the type of the chemical function present in the membrane which could be modified after flowering induction.

Until now, we did not find, soon after induction, any significant changes in protein pattern of the spinach leaves plasma membrane (LEFORT *et al.*, 1991). The fluidity may be changed by lipid content change. Two major factors which may alter the membrane fluidity are the plasma membrane sterols and the length and unsaturation degree of hydrocarbon chain of free fatty acid and acyl group of phospholipids (CHAPPMAN, 1975; COOKE & BUDEN, 1990). Results obtained by CRESPI *et al.* (1989) and PENEL *et al.* (1988) on spinach leaves plasma membrane demonstrated an increase of sterols after induction to flowering, following a transitory decrease during the first hours of flowering induction. The changes of membrane fluidity by changes in fatty acid unsaturation has not been demonstrated for spinach leaves plasma membrane. This will be a subject for next studies.

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