Electric and structural studies of hormone interaction with chloroplast envelope membranes isolated from vegetative and generative rape

Maria Filek, Maria Zembala, Anna Dudek, Peter Laggner, Manfred Kriechbaum

Institute of Plant Physiology, Polish Academy of Sciences, Podluzna 3, 30-239 Krakow, Poland
Institute of Catalysis and Surface Chemistry Polish Academy of Sciences, Niezapominajek 8, 30-239 Krakow, Poland
Institute of Biophysics and X-ray Structure Research, Austrian Academy of Sciences, Schmiedellstrasse 6, 8042 Graz, Austria

Received 7 December 2005; accepted 30 May 2006

KEYWORDS
Chloroplasts; Envelope membranes; Hormones; X-ray scattering; Zeta potential

Summary
The electric and structural properties of envelope membranes of chloroplasts obtained from vegetative and generative plants of rape and the effect of hormone (IAA, GA3 and zearalenone) treatment were determined by zeta potential and small-angle X-ray scattering (SAXS) methods. Chloroplasts were isolated from leaves cut off from the vegetative (before cooling) and generative apical parts of plants. The lipid composition of chloroplast envelope membranes were analyzed by chromatographic techniques. Envelopes from generative plants contained higher levels of digalactosyldiacylglycerol (DGDG) and smaller amounts of phospholipids (PLs) in comparison to those obtained from vegetative ones. Moreover, envelopes of generative plants were characterized by higher fractions of unsaturated fatty acids.

The zeta potential changes caused by hormone treatment were higher for chloroplasts isolated from vegetative plants in comparison to chloroplasts isolated from generative ones. An especially strong effect was observed for chloroplasts treated with IAA.

The thickness of bilayers of untreated chloroplasts from vegetative plants were larger by 0.4 nm when comparing to the thickness of layers obtained from generative ones. The effect of hormones (GA3 and zearalenone) was detected only for vegetative chloroplasts.

0176-1617/$ - see front matter © 2006 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2006.05.015
Both applied methods indicated differences in the properties of untreated and hormone-treated chloroplasts obtained from vegetative and generative plants. © 2006 Elsevier GmbH. All rights reserved.

Introduction

The transformation of plants from vegetative to generative stage during development is associated with several physiological events. The light and temperature requirements necessary for the flowering induction of winter plants indicate that chloroplasts and their membranes can play an essential role in generative development (Possingham, 1980; Miyamura et al., 1987; Masle, 2000; Samala et al., 1998).

Chloroplast membranes can be divided into two functional parts: envelope and thylakoid membranes. The first are formed of two achlorophyllous membranes, which separate the cytosol from the plastid compartments. The thylakoid membranes contain chlorophyll and take a key role in photosynthesis. Chloroplast envelopes and thylakoids are composed of proteins and lipids with galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG) (Moreau et al., 1998) as the dominant components. Therefore, the composition of chloroplast membranes is different from the composition of other membranes containing phospholipids (PLs) as a major lipid fraction. Both MGDG and DGDG are uncharged. In a different way from DGDG and from the majority of membrane lipids which form bilayers (Simidjiev et al., 1998; Lee, 2000) MGDG build-up non-bilayer structures. A negative charge of chloroplast membranes is related to the presence of sulfo-quinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). In addition, zwitterionic phosphatidyl choline (PC) is located in the envelope membranes (Moreau et al., 1998).

The unusual chemical composition of chloroplast lipids is responsible for their unusual packing and the formation of specific domains which can be important for interaction between lipids and membrane proteins (Zaitsev et al., 1999) as well as with other molecules adsorbed from the water phase (Shao et al., 1999; Tazzi et al., 1999). It has been suggested that cell surface galactolipids serve as recognition sites for counterpart lectins and cell adhesion receptors (Hakomori and Igarashi, 1995). High levels of polyunsaturated galactolipids in chloroplast membranes have been correlated with the maintenance of membrane fluidity and with the prevention of chilling injuries (Welburn, 1997; Mannock and McElhaney, 2004). Thus, modifications of the chloroplast membrane composition related to the maintenance of optimal membrane functions seem to be important also during the generative development of winter plants (which needs periodic chilling for flowering induction).

The reorganization of membrane lipids occurring during generative development can affect the formation of specific domains important to hormone interactions. Hormones, especially GA3 and IAA, play a significant role during generative development. Endogenous GA3 is essential in the photoperiodic control of grass flowering (Junttila et al., 1997). Moreover, transport of IAA from apex to roots is necessary for bud formation (Lomax et al., 1995). As it was presented in the literature (Kulaeva et al., 2000; Hole and Dodge, 1972; Misra and Biswal, 1980; Chaudhry and Hussain, 2001), hormones participate in the control of chlorophyll synthesis and its degradation during chloroplast aging (in vivo and in vitro).

The aim of the paper was to determine the electric and structural properties of chloroplast envelope membranes isolated from vegetative and generative winter rape and their changes caused by interaction with hormones of anionic type: GA3, IAA and non-ionic zearalenone (Meng et al., 1996). The studies are concentrated on the properties of envelope membranes of intact chloroplasts. Chloroplasts were isolated from vegetative and generative winter rape leaves which have a different morphological shape in these developmental stages. Comparison of electric and structural changes of chloroplasts obtained from generative and vegetative plants seems to be important in explaining the role of these organelles in the mechanism of flowering induction.

Material and methods

Plant material

Seedlings of Brassica napus L. var. oleifera cv. Górczański (winter) and cv. Młochowski (spring) were grown for 80 days in a controlled environment under a 16-h photoperiod (irradiance of 250 μmol m⁻² s⁻¹) and at day/night temperatures of 20/17 °C. Then, the plants were cooled to 5/2 °C...
(vernalization) for 63 days under the same conditions of day length and irradiance. Following the cold treatment, the plants were grown at 20/17 °C until the appearance of generative leaves. Apical segments that were 15 mm long including the youngest leaf of 10–20 mm long were cut off from the vegetative (before cooling) and generative plants and placed on the MS medium (Murashige and Skoog, 1962), which contained 0.1 mg dm⁻³ benzylaminopurine (BAP) and 3% sucrose. Under these conditions the plants were cultured during 1 week at day/night temperatures of 20/17 °C with a 16-h photoperiod (irradiance of 250 μmol m⁻² s⁻¹). Plants prepared this way were used for chloroplast isolation.

Chloroplast isolation

Intact chloroplasts were isolated from fresh leaf tissues by the method Block et al. (1983) in chloroplast isolation buffer (CIB), containing 50 mM Tris–HCl, 5 mM EDTA, 0.33 M sorbitol, pH 7.5 and separated from broken chloroplasts by centrifugation in a 40%/80% Percoll gradient. The chloroplasts were resuspended in CIB and, until further use, kept on ice in the dark. The measurements were performed within a time period not longer than 0.5 h after preparation. As is found in the literature (Polanska´ et al., 2004) and confirmed by our own observations, during this time the interval chloroplasts stayed intact in the sense of keeping unchanged shape (checked under microscope) and constant conductivity of the suspension.

Zeta potential measurements

The zeta potential values (ζ) were determined from electrophoretic mobility data measured with Zeta-PLUS apparatus (Brookhaven, USA). The palladium electrodes were used for an electric field application. Electric conductance was determined simultaneously for each measurement. The zeta potential measurements of chloroplasts were performed in media of defined ionic composition and ionic strength at 20 °C. The stock supporting electrolyte contained: 1 mM KCl, 0.3 mM NaCl and 1 mM MES–KOH buffer (pH 5.6) plus 0.6 mM mannitol. Indole-3-acetic acid (IAA), gibberellic acid (GA₃) or zearalenone (6-(10-hydroxy-6-okso-trans-1-undecenyl)-benzoic acid lactone) were introduced to the supporting electrolyte at the constant weight concentration equal to 25 mg dm⁻³, which corresponds to 143, 72 and 78.5 μM for IAA, GA₃ and zearalenone, respectively.

Small-angle X-ray scattering (SAXS) experiments

The intact chloroplast structure was determined from SAXS measurements carried out on a SWAX-camera (HECUS X-ray Systems, Graz, Austria) as described previously (Laggner and Mio, 1992; Laggner, 1994; Filek et al., 2005). Ni-filtered Cu–Kα-radiation (λ = 1.54 Å) originating from a rotating Cu-anode X-ray generator, (Rigaku, Japan) operating at 50 kV and 60 mA was used. SAXS angle calibration was done with silver stearate. Chloroplasts were used in a concentration of 100 μg chloroplast lipids/ml in CIB and the measurements were performed with samples placed in a 1-mm quartz capillary at 20 °C with an exposure time of 1800 s. The SAXS curves were analyzed after background subtraction and normalization in terms of distance distribution functions p(r) derived by indirect Fourier transformation (Laggner, 1988).

Lipid and fatty acids analysis

Chloroplast envelopes were separated from thylakoid membranes according to Poicelot’s (1977) method. Envelope membranes were homogenized in a mixture of chloroform: isopropanol (1:1, v/v) and lipids were re-extracted with chloroform (Bligh and Dyer, 1959). Glycolipids (MGDG and DGDG) and PL fractions were isolated using adsorptive and distributive column chromatography on silica acid under low nitrogen pressure. The purity of polar fractions was checked by thin-layer chromatography (Block et al., 1983). Individual lipid classes (MGDG, DGDG and PL) were transmethylated with BF₃ (14% in methanol). The obtained methyl esters were then quantified by gas chromatography (Hewlett Packard, USA) with a capillary column (30 m x 0.25 mm) at 170 °C. The identification of fatty acids was performed using appropriate standards. The quantification of fatty acid content was carried out using 17:0 acid as the internal standard.

Results

The composition of chloroplast envelope lipids indicated that generative development of rape plants is accompanied by an increase in the level of both galactolipids MGDG and DGDG while there is a decrease in the PL content (Table 1). Linolenic acid (18:3) was the major fatty acid of all lipids in both developmental stages. However, the unsaturation degree of DGDG and of PL, calculated as a ratio of
18:3/18:2 in a generative stage was higher than the ratio obtained in a vegetative one. By contrast, the unsaturation degree of MGDG was constant in both developmental stages with small differences noticed in 16:0 and 18:1 acids only. Traces of 16:1 fatty acid were detected in the DGDG fraction.

To choose appropriate conditions for the characterization of the electric properties of chloroplast envelope membranes, the zeta potential ($\zeta$) of intact chloroplasts was measured as a function of suspension conductance (Fig. 1). The suspension conductance was changed by dilution of the chloroplast isolation buffer (CIB) with water. The points are averages from 30 measurements $\pm$ SE.

**Figure 1.** Zeta potential ($\zeta$) of intact chloroplasts isolated from rape leaves measured as a function of medium conductance ($1/R$). The medium conductance was changed by dilution of the chloroplast isolation buffer (CIB) with water. The points are averages from 30 measurements $\pm$ SE.

18:3/18:2 in a generative stage was higher than the ratio obtained in a vegetative one. By contrast, the unsaturation degree of MGDG was constant in both developmental stages with small differences noticed in 16:0 and 18:1 acids only. Traces of 16:1 fatty acid were detected in the DGDG fraction.

To choose appropriate conditions for the characterization of the electric properties of chloroplast envelope membranes, the zeta potential ($\zeta$) of intact chloroplasts was measured as a function of suspension conductance (Fig. 1). The suspension conductance was changed by dilution of CIB with water. To determine zeta potential changes caused by hormone interaction, the CIB concentration of conductance of about 600 $\mu$S was chosen (which corresponds to 20 times dilution of the stock buffer). Under these conditions, $\zeta$ potential values for chloroplasts isolated from vegetative and generative plants were equal to $-20.2 \pm 1.2$ and $17.8 \pm 0.4$ mV, respectively (Fig. 2) and they were constant for about 5 h. These $\zeta$ potential values for reference chloroplasts were large enough to notice changes caused by hormones.

The absolute values of the $\zeta$ potential of chloroplasts isolated from vegetative and generative plants after applying all investigated hormones decreased (Fig. 2). The largest change was detected for IAA (despite the fact that its molar

### Table 1. Distribution of fatty acids among the polar lipids of chloroplast envelopes isolated from vegetative and generative winter rape

<table>
<thead>
<tr>
<th>Object</th>
<th>Lipid fractions (mol% of polar lipids)</th>
<th>Fatty acids (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td>Vegetative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGDG</td>
<td>43.2 ± 0.5</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>DGDG</td>
<td>16.7 ± 0.9</td>
<td>19.4 ± 0.3</td>
</tr>
<tr>
<td>PL</td>
<td>39.9 ± 0.8</td>
<td>34.2 ± 0.4</td>
</tr>
<tr>
<td>Generative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGDG</td>
<td>45.5 ± 0.6</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>DGDG</td>
<td>18.4 ± 0.9</td>
<td>11.9 ± 0.2</td>
</tr>
<tr>
<td>PL</td>
<td>35.9 ± 0.9</td>
<td>29.8 ± 0.3</td>
</tr>
</tbody>
</table>

Note: The data are averages of three independent replications $\pm$ SE.

MGDG, monogalactosyldiacylglycerols; DGDG, digalactosyldiacylglycerols; PL, phospholipids.
concentration was approximately two times lower than for the other hormones studied) and the smallest one for zearalenone. The effect of IAA was especially visible in the case of chloroplasts obtained from vegetative plants for which the $\zeta$ potential changed by about 15 mV, whereas in the case of generative ones, the change in the $\zeta$ potential amounted to only about 7 mV. For chloroplasts from vegetative and generative plants treated by GA$_3$, the same values of $\zeta$ potential were measured. However, when comparing to reference non-treated organelles the relative effect of this hormone was higher for vegetative membranes. The relative effect of zearalenone on chloroplasts of both types was similar.

The SAXS measurements for the chloroplasts studied resulted in continuous scattering curves characteristic for uncorrelated (with no regular long-range order) bilayer lipid membranes with a visible maximum reflecting a certain bilayer thickness (Fig. 3). The noticeable shift in the angular position of the maximum was observed when comparing results obtained for both investigated chloroplasts. Structural parameters in terms of the lamellar thickness of the lipid/water system were derived from the continuous Fourier transformation of the SAXS curves (Table 2).

The difference between bilayer thicknesses obtained for chloroplasts of vegetative and generative origin was about 0.4 nm. The treatment of chloroplasts of generative plants with all hormones investigated did not affect the bilayer thickness. However, in the case of chloroplasts of vegetative origin, a small decrease in bilayer thicknesses was detected after treatment with GA$_3$ and zearalenone whereas IAA did not cause any change in the value of this parameter (Table 2).

**Table 2.** Head-to-head group thickness of the lipid bilayer in average for all types chloroplast membranes after hormone (IAA, GA$_3$, zearalenone, 0 – without hormones) treatment, calculated from SAXS data for envelope chloroplasts obtained from vegetative and generative winter rape

<table>
<thead>
<tr>
<th>Chloroplasts</th>
<th>Membrane thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Vegetative</td>
<td>3.93 ± 0.10</td>
</tr>
<tr>
<td>Generative</td>
<td>3.52 ± 0.10</td>
</tr>
</tbody>
</table>

**Discussion**

The lipid and fatty acid composition of chloroplast envelope membranes of vegetative and generative rape is typical of plant chloroplasts and it is well represented by the data of Moreau et al. (1998). However, observed differences in lipid and fatty acid proportions (i.e., a decrease in the PL fraction and an increase in the fatty acid unsaturation degree) between generative and vegetative organelles can be connected with the generative development of a plant.

The negative charge of the investigated chloroplasts is connected with the presence of PLs in the envelope membranes. The various structural and functional features of the membranes can be correlated with their surface potential (Kraayenhof et al., 1996). The charged interface facilitates the formation of a hydration layer of oriented water molecules that may stabilize the lipid head-group region (Kraayenhof et al., 1996). In our earlier studies on wheat membranes, it was shown that negatively charged plasmalemma in wheat cells can adsorb negatively and positively charged hormones
as well as non-ionic zearalenone (Filek et al., 2002). This effect was discussed in terms of the interaction between the hormones investigated and the PL and protein domains.

The results presented here show that the treatment of rape chloroplast envelope membranes with the hormones studied led to a noticeable decrease in absolute values of zeta potentials. The larger effect of negatively charged IAA and GA3 in comparison to non-ionic zearalenone can be related to the interaction of hormones with zwitter-ionic PC, which represents a major fraction of the PLs present in a chloroplast envelope. This can also explain the weaker effect of all hormones on the zeta potential of chloroplasts of generative plants (whose membranes contain less PC in comparison to vegetative plants). The hormones studied can also interact with proteins present in membranes. However, taking into account the smaller content of proteins in envelope membranes (in comparison to plasmalemma as studied before (Filek et al., 2002)) as well as a comparable level of proteins in both types of chloroplasts (data not indicated), one can relate the changes in zeta potential as being caused predominantly by the interaction of hormones with lipids. Nevertheless, some contribution to these changes can be related to differences in protein hormone receptors characteristic for chloroplast envelopes of vegetative and generative origin.

The changes in zeta potential of hormone-treated membranes can be interpreted in terms of hormone adsorption. The higher changes in zeta potential obtained for envelopes of vegetative plants (especially large after treatment with IAA) in comparison to those of generative ones may be associated with a larger amount of adsorbed hormones. The treatment of chloroplasts of vegetative and generative origin by zearalenone did not produce noticeable changes in zeta potentials, indicating that adsorption of this hormone, if any, does not affect the electric state of the membranes.

Modification of envelope composition can be accompanied with structural changes in the chloroplast membranes of vegetative and generative plants. In spite of the many observations made on model membranes containing defined lipids occurring separately or in mixtures, little is known about the structural organization and properties of natural membranes. The bilayer thickness of model membranes results in the occurrence of low hydrated "pores" (Moreau et al., 1998).

The experiments presented indicate that the changes in the composition of chloroplast envelope membrane lipids during generative development lead to changes in electric and structural properties. These changes can also affect the adsorption of ionic and non-ionic hormones. The bilayer thickness of membranes of generative objects was not affected by the hormones studied whereas this parameter for vegetative membranes decreased after interaction with GA3 and zearalenone. The electric properties of vegetative chloroplasts were affected to a higher degree by treatment with hormones (especially IAA and GA3) in comparison to generative objects. The results presented show that zeta potentials more than structural parameters are sensitive to membrane surface modifications caused by hormone treatment. This last observation is related to the fact that electrokinetic parameters are determined by the outmost surface of the object whereas SAXS signal contains information about all membranes present in the system.

Acknowledgement

This work was partially supported by the MEiN grant No. 1 T09A 122 30.

References

Block AM, Dorne AJ, Joyard J, Douce R. Preparation and characterization of membrane fractions enriched in


