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EFFET DE LA TEMPERATURE ET DE LA LUMIERE SUR LA COMPOSITION EN LIPIDES DE LA MEMBRANE THYLACOIDALE PENDANT LA FORMATION DU CHLOROPLASTE DE COTYLEDONS DE COURGE - *UNE NOUVELLE HYPOTHESE*

THESE

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Effet de la température et de la lumière sur la composition en lipides de la membrane thylacoïdale pendant la formation du chloroplaste de cotylédons de courge - Une nouvelle hypothèse

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Publications

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Xu, Y. N., Siegenthaler, P. A. (1997) Low temperature treatments induce an increase in the relative content of both linolenic and Δ^3-*trans* hexadecenoic acids in thylakoid membrane phosphatidylglycerol of squash cotyledons. *Plant & Cell Physiol.* 38: 611-618.

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Xu, Y. N., Siegenthaler, P. A. (1996) Low temperatures induce an increase in both 18:3 and 16:1(3*t*) fatty acids in thylakoid membrane phosphatidylglycerol of developing squash cotyledons. *Plant Physiology and Biochemistry*, Special issue, 1996, 10th FESPP CONGRESS. S09-18.

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Low Temperature Treatments Induce an Increase in the Relative Content of Both Linolenic and Δ^3-*trans*-Hexadecenoic Acids in Thylakoid Membrane Phosphatidylglycerol of Squash Cotyledons

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The effect of low temperatures on the fatty acid composition of phosphatidylglycerol (PG) in thylakoid membranes, in particular on the ratios of nmol% 16:1(3*t*) (mg fresh weight)$^{-1}$ of cotyledons and nmol 16:1(3*t*) (mg chlorophyll)$^{-1}$ were measured during squash seedling growth. Plants were germinated and grown for one day at 30°C, then were either kept at 30°C (control plants) or transferred to low temperatures (18, 14 or 10°C). When plants were transferred from 30°C to low temperatures, the increase in fresh weight was gradually limited. The lower the temperature, the smaller was the fresh weight. In contrast, the relative content of 16:1(3*t*) and 18:3, as well as the ratios of nmol 16:1(3*t*) (mg chlorophyll)$^{-1}$ and mol% 16:1(3*t*) (mg cotyledon fresh weight)$^{-1}$ increased indicating that the increase of fresh weight and chlorophyll was more sensitive to low temperature than PG desaturation in thylakoid membranes. Furthermore, low temperatures induced an increase in 16:1(3*t*) and 18:3 (the final products of PG synthesis) at the expense of 16:0 and 18:1 (the initial products of PG synthesis). However, within a range of temperature from 10 to 18°C, the extent of these changes (nmol% of 18:3 or 16:1(3*t*) per day) was gradually limited by lower temperatures. We therefore propose that low temperatures inhibit both fatty acid synthesis and desaturation activities. However, at low temperatures the fatty acid synthesis is likely to be more strongly inhibited than the desaturation activities, thus explaining the observed increase in the relative content of PG-18:3 and PG-16:1(3*t*). Results are discussed in terms of the mechanism which could be involved in the metabolism of PG in squash cotyledons.

Key words: Chilling temperature — *Cucurbita moschata* Durch — Development — Linolenic acid — Phosphatidylglycerol — Δ^3-*trans*-hexadecenoic acid.

Low temperatures induce many changes in plant green tissues of chilling-sensitive plants. Among these, growth restriction (Lyons 1973, Brüggemann et al. 1992, Ulrich and Bournérias 1992), leaf chlorosis (Hugly et al. 1990, McWilliam and Naylor 1967), and an increase in membrane lipid unsaturation (Kodama et al. 1995) have been observed in a variety of plants. It is considered that the former two parameters are reliable symptoms of chilling injury (Lyons 1973, Hugly et al. 1990), while lipid unsaturation is one of the factor maintaining the membrane fluidity at low temperature for the survival of plants under chilling conditions (Kodama et al. 1995, Vigh et al. 1993).

At a critical temperature, plant growth stops. This is the so-called zero of vegetation, e.g. from 6 to 10°C for maize, from 10 to 13°C for rice and near 0°C for the winter cereals (Ulrich and Bournérias 1992). Chlorosis is a common symptom of chilling injury in chilling-sensitive species such as cucumber (Hasselt 1972), sorghum (Slack et al. 1974) and maize (McWilliam and Naylor 1967, Schapendonk et al. 1989). It was reported that chill-induced chlorosis in maize seedlings is partly the result of two metabolic blocks in the porphyrin pathway leading to chlorophyll synthesis (Hodgins and Huystee 1986). The chill-induced growth restriction and chlorosis are usually used to determine the chilling sensitivity of plants (Schapendonk et al. 1989, Hugly et al. 1990).

Changes in the lipid composition in response to low temperature have been observed to occur in a variety of chilling-sensitive and -resistant plants. In general, there is an increase in 18:3 when plants are grown at low temperature. If the desaturation of lipid induced by low temperature is the factor which maintains the membrane fluidity at low temperature, the desaturation of phosphatidylglycerol should be very important. First, of all thylakoid membrane lipids, only PG contains high levels of disaturated molecular species (Murata 1983). Second, the relative content of disaturated molecular species of PG is significantly higher in chilling-sensitive plants than in chilling-resistant ones, with only a few exceptions (Murata 1983, Roughan 1985). Several attempts have been made to determine whether the unsaturation of fatty acids contributes to the tolerance ability of plants at low temperature (Somerville 1995, Murata and Wada 1995). However, the results are controversial. Murata et al. (1992) demonstrated by genetic manipulations that changes in fatty acid unsaturation of PG can alter plant chilling sensitivity. Furthermore, in a

Abbreviations: DGDG, digalactosyldiacylglycerol; m:n, fatty acid containing m carbons and n cis double bonds; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; 14:0, myristic acid; 16:0, palmitic acid; 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

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mutant of *Arabidopsis* (*fab1*), leaf PG contains 43% of high-melting point molecular species, a percentage higher than in many chilling-sensitive plants. However, the mutant is completely unaffected, when compared with wild-type controls, by a range of low-temperature treatments that rapidly led to the death of other chilling-sensitive plants (Wu and Browse 1995).

The mechanisms leading to temperature-induced changes in the lipid composition of thylakoid membranes are not yet fully elucidated. We have previously shown that thylakoid membranes of squash cotyledons grown at 20°C (compared with those from plants grown at 30°C), a non-injurious temperature, not only contain higher level of 18:3, but also 16:1(3*t*) in PG. However, when squash plants having mature cotyledons were transferred from 30°C to 20°C, no significant changes are detected during 6 days (Xu and Siegenthaler 1996b). We therefore proposed that an increase in the level of the final desaturation products of PG at low temperature is likely to be the result of changes in the relative activities of the fatty acid synthesis and desaturase. To test this hypothesis, we studied the effect of low temperatures on the fatty acid composition of PG in thylakoid membranes, in particular on the relative content of 16:1(3*t*) based on the fresh weight of cotyledons and chlorophyll during growth of squash seedlings.

Our results indicate that the increase of cotyledon fresh weight and chlorophyll content are more sensitive to chilling temperature than the activity involved in PG desaturation during squash seedling growth. The observed increase of the relative level of PG-18-3 and -16:1(3*t*) induced by low temperatures appears to be the result of a relatively higher inhibition of fatty acid synthesis than of the desaturation reactions.

Materials and Methods

Plant materials and growth conditions—Squash plants (*Cucurbita moschata* Durch. cv. Shirakikuza) were grown from seeds in soil (Mio Plant Natura, Migros, Switzerland). The seeds were germinated in darkness at 30°C and the seedlings grown in controlled environment growth chambers equipped with both fluorescent and incandescent lights (Sanyo, Gallenkamp, U.K., Cabinet Model PG 660) under a 12 h photoperiod (photon flux density (PFD) : 100 μmol m^{-2} s^{-1}) at 30°C for one day. At the end of the first light period, control plants remained under the same growth conditions, whereas other seedlings were transferred to various temperatures (18°C/60% RH, 14°C/65% RH and 10°C/70% RH) with the same light regime. Chloroplasts were immediately isolated from cotyledons collected after the desired daily light period.

Isolation of thylakoid membranes—Cotyledons (about 20 g corresponding to 40 to 120 cotyledons depending on the period of harvest) were ground and chloroplasts, then thylakoids isolated according to the method described by Xu and Siegenthaler (1996b). The chlorophyll concentration was determined according to Bruinsma (1961).

Lipid extraction and purification—Lipids were extracted from thylakoid membranes according to Siegenthaler et al. (1989). Lipid classes were separated by thin layer chromatography (TLC) on silica gel plates (pre-coated silica gel plates, Merck 5626) in two dimensions. The first developing solvent was acetone/toluene/H_2O (91 : 30 : 8, by volume) and the second was chloroform/methanol/25% NH_3/H_2O (65 : 35 : 3 : 2, by volume). The plates were dried shortly in air and lightly sprayed with 0.01% primuline and viewed under UV light.

Determination of fatty acids—The individual thylakoid membrane lipids separated by TLC were transesterified with 5% H_2SO_4 in MeOH for 1 h at 85°C. The fatty acid methyl esters were separated on a Hewlett-Packard 5890 gas chromatography supplied with an hydrogen flame ionization detector and a capillary column FFAP (30 m; i.d. 0.53 mm). The column was isothermally run at 190°C and the detector was held at 230°C. Arachidic acid (from Sigma) was used as an internal standard.

Results

Effect of growth temperature on cotyledon fresh weight—To examine the effect of low temperatures on plant growth, the fresh weight of developing cotyledons is a good and simple criterion as illustrated in Fig. 1. Cotyledons reached their maximum fresh weight (about 430 mg per cotyledon) when they were grown at 30°C for only three days after the transfer (control plants). The cotyledon growth was greatly restricted by lower temperatures. When plants were transferred from 30°C to 18°C (a non-chilling temperature) the fresh weight of their cotyledons reached after 6 days of transfer the same level as those of control plants. When plants were transferred to 14°C (a temperature which is close to that causing chilling injury), the increase of cotyledon fresh weight was after 6 days about half of that of control plants. Meanwhile, no chilling sym-

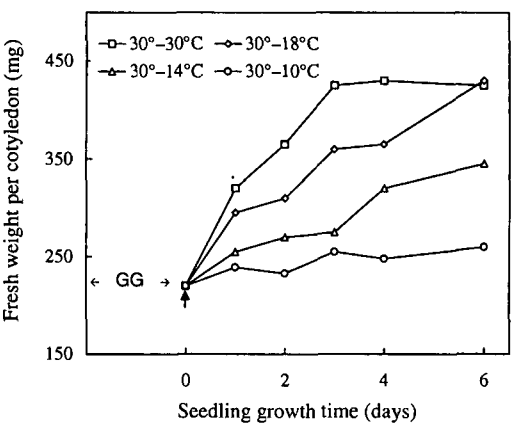

Fig. 1 The effect of low temperatures on the fresh weight of developing squash cotyledons. GG corresponds to 5 d of seed germination in darkness at 30°C, followed by one day of seedling growth at the same temperature with a 12 h photoperiod and a PFD of 100 μmol m^{-2} s^{-1}. At the end of this one day light period, seedlings were transferred from 30°C to 18, 14, or 10°C with the same light regime. The vertical arrow indicates the time at which seedlings were transferred to lower temperatures.

ptoms were observed. In contrast, when plants were transferred to 10°C (a chilling temperature), the cotyledon growth was almost stopped and the chilling symptoms (e.g. damage to the root with appearance of necrotic lesions) appeared after 6 days. The death of the plants began at the 6th day and all plants died after 8 days. Plants were also transferred from 30°C to 5°C but they survived only for 3 days.

Effect of growth temperature shift on the relative molar content of glycerolipids and fatty acid composition of glycolipids in thylakoid membranes—Preliminary results (not shown) revealed that the relative molar content of each of the four glycerolipid classes did not depend on the growth temperature and light energy as well as on the developmental stage of squash cotyledons. The relative levels of MGDG, DGDG, SQDG and PG were 53, 30, 6 and 11 mol%, respectively. In contrast, the fatty acid composition of glycerolipids underwent changes which were particularly marked in PG. The fatty acid composition of glycolipids (MGDG, DGDG and SQDG) were determined as a function of growth time (i.e. at 0, 1, 2, 3, 4 and 6 days) under four temperature shift conditions. For the sake of simplicity, only the results obtained at 0, 1, 2 and 4 days are presented (Table 1). Under all conditions, the content of 18:3 increased mainly at the expense of 18:2 and to a lesser extent of 18:1 and 18:0 in the three lipid classes. These changes occurred especially during the first two days of growth after the shift temperature. In contrast, no significant change was observed at the level of 16:0 during growth in the three glycolipids when the temperature remained constant (30°/30°C). However, when it was lowered, 16:0 content diminished progressively as a function of growth time in galactolipids but not in SQDG. The data confirm that growth temperature shift did not affect markedly the fatty acid composition of glycolipids and that the fatty acid composition of PG will be the interesting one to be considered.

Effect of growth temperature on the relative composition of fatty acids in thylakoid membrane PG—Low temperature treatments induce usually an increase in 18:3 of membrane lipids (Graham and Patterson 1982). We have previously found that in squash, this change occurs only in the developing cotyledons and furthermore that low temperature (20°C compared to 30°C) induces an increase in the content of 16:1(3*t*) in PG during growth (Xu and Siegenthaler 1996b). Figure 2 shows the effect of different low temperatures on the 18:3 content of PG in developing cotyledons of squash. When plants were isothermally grown at 30°C, PG contained about 8 mol% of 18:3, a level which remained constant during growth. When plants were transferred from 30°C to lower temperatures, an increase in 18:3 was detected after two d. After six d, the 18:3 content reached about 12 mol%, which corresponded to an increase of about 50% compared to that of control plants. Fig. 2 also shows that between the 2nd and 4th day, the increase of 18:3 levels was gradually limited by low temperatures. The effect of low temperatures on the 16:1(3*t*) content in thylakoid PG of squash cotyledons is illustrated in Fig. 3. The 16:1(3*t*) level increased during cotyledon growth under all conditions. When plants were grown isothermally at 30°C, the relative content of 16:1(3*t*) increased from about 5 to 15 mol% during the first 3 d, then remained constant. Lower temperatures enhanced the 16:1(3*t*) content but the lower the temperature, the lesser was the enhancement.

The relationship between the relative content of 18:3 and the sum of the other C_{18}-fatty acids in thylakoid PG from cotyledon squash plants grown at various temperatures is shown in Fig. 4. For all transfer temperatures tested, the correlation between the content of 18:3 and the

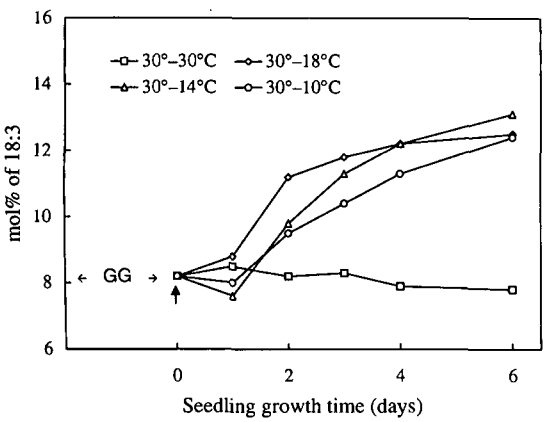

Fig. 2 The effect of low temperatures on the relative linolenic acid (18:3) content in thylakoid phosphatidylglycerol from developing squash cotyledons. Growth conditions and symbols are described in the legend of Fig. 1.

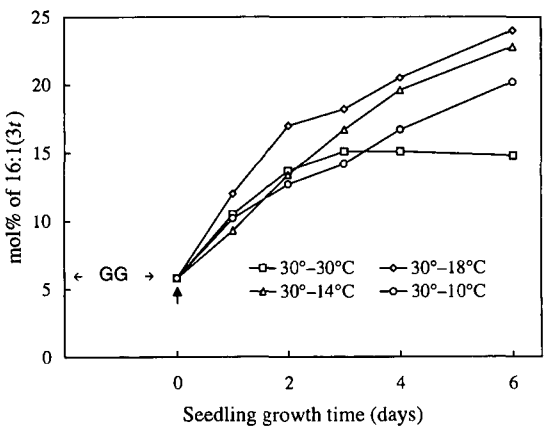

Fig. 3 The effect of low temperatures on the relative Δ^3-*trans*-hexadecenoic acid [16:1(3*t*)] content in thylakoid phosphatidylglycerol from developing squash cotyledons. Growth conditions and symbols are described in the legend of Fig. 1.

Table 1 Effect of temperature shift of squash plants on the fatty acid composition of thylakoid membrane glycerolipids (MGDG, DGDG, and SQDG)

Lipides	Temperature shift	Growth days	Fatty acid composition (mol%)				
			16:0	18:0	18:1	18:2	18:3
MGDG	30°-30°C	0	1.5	0.0	1.1	6.4	90.9
		1	1.6	0.0	0.5	2.9	95.0
		2	1.7	0.0	0.6	2.8	94.9
		4	2.0	0.0	0.5	3.0	94.4
	30°-18°C	1	1.4	0.0	0.8	3.9	93.8
		2	1.2	0.0	0.3	2.1	96.4
		4	1.0	0.0	0.2	2.2	96.6
	30°-14°C	1	1.7	0.0	1.1	5.4	91.9
		2	0.9	0.0	0.2	1.9	97.0
		4	0.8	0.0	0.1	1.5	97.5
	30°-10°C	1	1.7	0.0	1.1	6.0	91.2
		2	1.4	0.0	0.6	2.7	95.4
		4	1.0	0.0	0.2	1.4	97.4
DGDG	30°-30°C	0	9.9	1.6	0.9	5.9	81.7
		1	9.4	0.9	0.7	3.6	85.5
		2	9.3	0.7	0.7	3.5	85.9
		4	9.4	0.5	0.2	3.2	86.7
	30°-18°C	1	9.6	1.2	0.9	5.2	83.0
		2	8.1	0.9	0.6	3.3	87.1
		4	6.7	0.3	0.4	1.5	91.1
	30°-14°C	1	9.3	1.5	1.0	4.3	83.9
		2	8.0	0.8	0.7	4.0	86.5
		4	7.0	0.5	0.4	2.4	89.7
	30°-10°C	1	8.4	1.2	0.9	4.2	85.2
		2	7.8	0.7	0.7	3.4	87.4
		4	7.2	0.4	0.4	2.2	89.8
SQDG	30°-30°C	0	26.6	6.4	3.2	1.3	50.6
		1	26.9	5.7	2.9	8.5	56.0
		2	27.6	4.7	2.9	7.5	57.3
		4	29.4	3.9	1.9	6.7	58.0
	30°-18°C	1	27.1	8.8	3.4	10.3	50.3
		2	25.3	6.0	2.8	6.9	59.0
		4	26.0	4.8	1.4	5.6	62.2
	30°-14°C	1	27.0	7.0	2.9	12.1	50.9
		2	25.7	8.1	3.1	8.1	55.1
		4	25.0	4.8	1.1	5.2	63.9
	30°-10°C	1	25.6	6.9	3.4	12.0	52.0
		2	25.5	5.5	2.5	9.3	57.2
		4	25.9	5.7	2.0	5.7	60.7

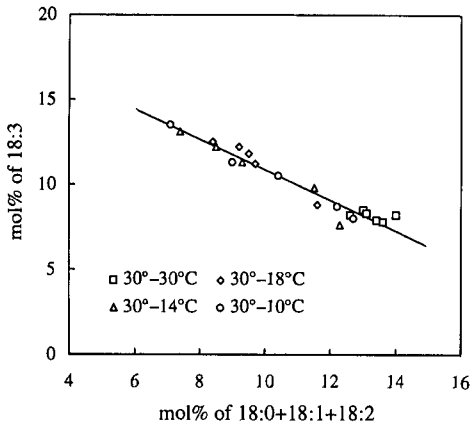

Fig. 4 Relationship between the relative content of 18:3 and the sum of 18:2, 18:1 and 18:0 in thylakoid phosphatidylglycerol from developing squash cotyledons. Plant growth conditions are described in the legend of Fig. 1. The equation of the straight line was $y=-1.02x+20.93$ ($r=0.98$).

sum of 18:2, 18:1 and 18:0 was linear and decreasing ($y=-1.02x+20.93$). Under all temperature conditions, an increase in 18:3 content occurred mainly at the expense of 18:1 and to a lesser degree of 18:2 and 18:0 (results not shown). A similar relationship was calculated for the C_{16}-fatty acid series (Fig. 5). Again, for all growth temperatures studied, the correlation between the content of 16:1(3*t*) and 16:0 was linear and decreasing ($y=-1.21x+87.05$). The excellent correlations between 18:3 and the sum of 18:2, 18:1 and 18:0 relative content ($r=0.98$) as well as between 16:1(3*t*) and 16:0 relative content ($r=0.99$) indicate that low temperatures did not affect the sum of the fatty acids within each of the C_{18}- and C_{16}-series.

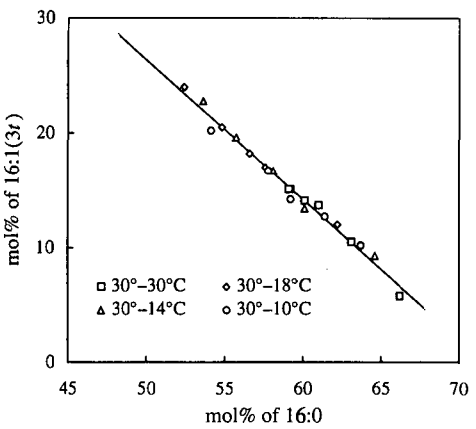

Fig. 5 Relationship between the relative content of 16:0 and 16:1(3*t*) in thylakoid phosphatidylglycerol from developing squash cotyledons. Plant growth conditions are described in the legend of Fig. 1. The equation of the straight line was $y=-1.21x+87.05$ ($r=0.99$).

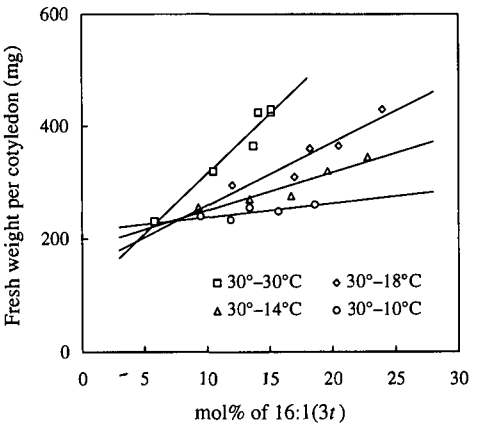

Fig. 6 Effect of low temperatures on the relationship between the fresh weight of developing cotyledons and the relative molar content of 16:1(3*t*) in phosphatidylglycerol of thylakoid membranes. Plant growth conditions are described in the legend of Fig. 1. The equation of the straight lines was $y=22.34x+87.50$ at 30°C; $y=11.26x+145.54$ at 18°C; $y=6.80x+181.77$ at 14°C; and $y=1.97x+203$ at 10°C.

Relationship between growth temperature and the slope expressing the fresh weight versus the content of 16:1(3t) in PG—During the growth of squash seedlings, the curves expressing the fresh weight of cotyledons (Fig. 1) and the relative content of 16:1(3*t*) [Fig. 3] displayed a similar pattern at each temperature. Therefore, a linear correlation between these two parameters should exist. The results of Fig. 6 show that each squash plant grown at a given temperature was characterized by its own ratio between fresh weight of cotyledons and the relative content of 16:1(3*t*) in thylakoid membrane PG. The equations of

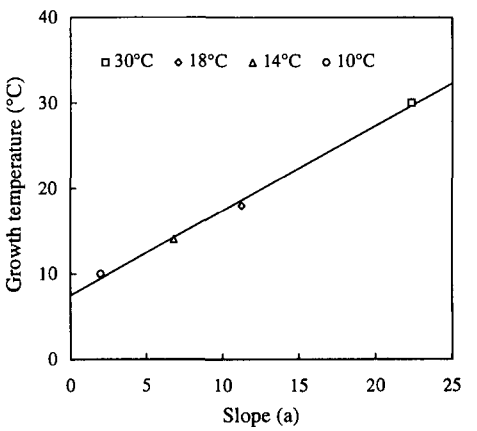

Fig. 7 Relationship between the growth temperature after the transfer of seedlings (30° → 18°, 14° or 10°C) and the slope (a) of the straight lines obtained in Fig. 6, i.e. $a=22.34$ for a transfer from 30°C → 30°C, $a=11.26$ (30° → 18°C), $a=6.80$ (30° → 14°C) and $a=1.97$ (30° → 10°C). The equation of the straight line is $y=0.99x-7.51$ ($r=0.99$).

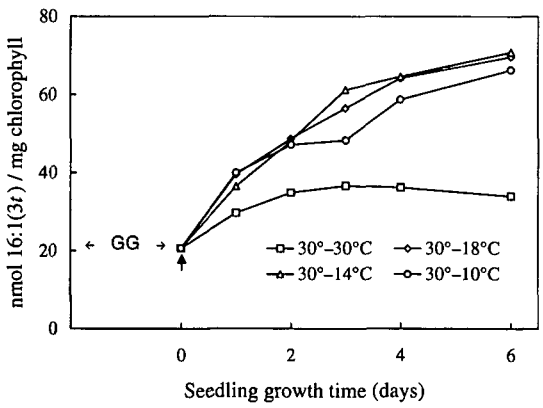

Fig. 8 Effect of low temperatures on the ratio of 16:1(3*t*) content in thylakoid phosphatidylglycerol and chlorophyll as a function of growth time in developping squash cotyledons. Growth conditions are described in the legend of Fig. 1. The vertical arrow indicates the time at which seedlings were transfered at lower temperatures.

the straight lines were $y=22.3x+87.5$ (at 30°C), $y=11.3x+145.5$ (at 18°C), $y=6.8x+181.8$ (at 14°C), $y=2.0x+203$ (at 10°C). The relationship between the growth temperature after the transfer of seedlings and the slope of the straight lines (see Fig. 6) is illustrated in Fig. 7. It can be seen that the lower the temperature, the smaller was the slope. Therefore, the growth temperature after transfer and the slope [fresh weight versus the content of 16:1(3*t*)] displayed a perfect linear correlation $y=0.99x-7.51$ ($r=0.99$). The extrapolation of the straight line to the slope zero corresponded to a growth temperature of 7.5°C.

Effect of growth temperature on the 16:1(3t) content to chlorophyll ratio as a function of seedling growth—Chlorosis is a common sympton of chilling injury in several chilling-sensitive plant species, namely maize (Schapendouk et al. 1989). Therefore, chlorophyll content is frequently used to assess chilling damage (Schapendonk et al. 1989, Hugly et al. 1990). Under the conditions used in this study it was of interest to compare the relative increase of 16:1(3*t*) during seedling growth on the basis of chlorophyll. Fig. 8 shows that in control plants (30°C) the nmol 16:1(3*t*)/mg chlorophyll ratio increased slightly during the first two days of growth (from about 20 to 35) then remained constant. At lower temperatures (18°, 14° and 10°C), the ratio increased progressively to reach after 6 d of growth a value which was 3.5 times higher than the initial one.

Discussion

Growth restriction and leaf chlorosis are the common symptoms of chilling injury in chilling-sensitive plants (Lyons 1973, Schapendonk et al. 1989, Hugly et al. 1990). Concerning the lipid metabolism, the fatty acid composition of PG, compared to the other lipid classes of thylakoids, is the most affected by temperature, not only at injurious (Table 1; Fig. 2, 3) but also at non-injurious temperatures (Xu and Siegenthaler 1996b).

To investigate the role of PG in chilling-damage, we have studied the changes of fatty acid composition induced by low temperatures and compared them with the extent of growth and chlorophyll bleaching. Our results show that when plants were grown at 30°C (control plants), the fresh weight of cotyledons (Fig. 1) as well as the relative content of 16:1(3*t*) expressed in mol% (Fig. 3) or nmol per mg chlorophyll (Fig. 8) increased during the first two days of growth. All these parameters reached their maximal level after two days, whilst the relative 18:3 content (mol%) did not change during growth (Fig. 2). In contrast, when plants were transferred to lower temperatures the increase of cotyledon fresh weight was gradually diminished whereas the relative contents of 18:3 (mol%) and of 16:1(3*t*) [mol% and nmol per chlorophyll] increased. Interestingly, when plants were grown at 10°C, the growth of cotyledons almost stopped, although 18:3 and 16:1(3*t*) increased dramatically. This indicates that even at chilling temperature, the PG desaturases in thylakoids of squash cotyledons were still active. Altogether, these results suggest that in thylakoid membranes chlorophyll accumulation and growth (expressed by fresh weight increase) are more sensitive to low temperatures than the desaturation of PG leading to 18:3 and 16:1(3*t*) molecular species (Xu and Siegenthaler 1996a). Indeed, it was reported that chill-induced chlorosis in maize seedlings is partly the result of two metabolic blocks in the porphyrin pathway leading to chlorophyll synthesis and that the temperature range of the impaired chlorophyll synthesis coincides with that of chlorosis, i.e. from 17° to 10°C (Hodgins and Huystee 1986).

Changes in the lipid composition induced by low temperature have been observed in a variety of plant and cyanobacteria membranes. These changes are generally considered to be one of the main factors conferring low temperature tolerance by keeping the adequate membrane fluidity (Vigh et al. 1993, Murata and Wada 1995, Kodama et al. 1995). In accordance with this concept, we found that low temperatures induced always an increase in 18:3, mainly at the expense of 18:1 and to a lesser degree of 18:0 and 18:2 in the thylakoid PG of squash cotyledons. However, low temperatures induced also an increase in 16:1(3*t*) (Fig. 3). This fatty acid is known to display physical properties (e.g. high melting point, configuration, etc.) which are similar to those characterizing 16:0 (Bishop and Kenrick 1987). Moreover, the relative increase in the content of PG-16:1(3*t*) induced by low temperature was greater than that of PG-18:3. For example, when squash plants were transferred from 30° to 14°C for 6 d (Fig. 2, 3), the thylakoid PG of cotyledons contained 8.0 mol% more 16:1(3*t*) and 5.3 mol% more 18:3 than the corresponding thylakoid PG

from plants kept at 30°C for the same time. This difference suggests that 16:1(3*t*) may contribute to the formation of not only 18:3/16:1(3*t*) PG molecular species but also of other species such as 16:0/16:1(3*t*), as found recently in our laboratory (Xu and Siegenthaler, unpublished data). Thus, the conversion of 16:0 to 16:1(3*t*) induced by low temperature in thylakoid membranes of squash cotyledons does not result in an increase of membrane fluidity. At this stage, it is therefore risky to claim that low-temperature induced PG desaturation always leads to an increase of the membrane fluidity.

The above results could be explained by a mechanism involving the synthesis of the different molecular species of PG in the chloroplast. The fatty acid synthesis in plant cells takes place exclusively within the plastid (Ohlrogge and Browse 1995). The final products of the synthesis of fatty acids are 16:0 and 18:1. After these fatty acids have been incorporated into a PG molecule, 18:1 at the *sn*-1 position of the glycerol is desaturated into 18:2 and 18:3 (Ohlrogge and Browse 1995), whilst 16:0 at the *sn*-2 position is desaturated into 16:1(3*t*) (Ohnishi and Thompson 1991). Thus, 18:1- and 16:0-containing PG are the initial products of PG synthesis whilst 18:3- and 16:1(3*t*)-containing PG can be considered as the final desaturation products of PG. Both types of PG are constituents of thylakoid membranes. Our results show that squash plants grown at 30°C, contained low levels of 16:1(3*t*) and 18:3 and high levels of 16:0 and 18:1 in their thylakoid PG. When plants were transferred from 30°C to lower temperatures (18°, 14° and 10°C), both 18:3 and 16:1(3*t*) contents increased indicating that low temperatures induce an increase in the final products of PG at the expense of the initial ones. This increase is unlikely to be due to a higher absolute activity of the desaturases because the formation rate of 18:3 and 16:1(3*t*) was gradually limited by lowering the temperature within the 10° to 18°C range (Fig. 2, 3). Alternatively, these changes might reflect that the rates of fatty acid synthesis and PG desaturation are differentially affected by low temperatures, i.e. the fatty acid synthesis appears to be more strongly impaired by low temperature than the desaturation reactions.

Another interesting feature of this investigation is the finding that each squash plant grown at a given temperature was characterized by its own ratio of cotyledon fresh weight and relative content of 16:1(3*t*) in thylakoid membranes (Fig. 6). For instance, slopes of the straight line characterizing this relationship (or ratio) was increased as a function of growth temperature. Thus, a low temperature affects more significantly the fresh weight (which reflects the global physiological activity of the plant) than the conversion of 16:0 to 16:1(3*t*) (which is the expression of a membrane-bound lipid desaturase). Interestingly, the extrapolation of the straight line to zero (slope=0) displayed a temperature equal to 7.5°C (Fig. 7). At this temperature, the growth of squash cotyledons was completely abolished. In conclusion, the sensitivity of squash cotyledons towards temperature can be characterized by the equation of the above straight line (i.e. growth temperature versus slope): $y=0.99x+7.51$. This equation takes into consideration two parameters: the global physiological activity of the plant (i.e. the fresh weight) and a specific membrane bound enzyme (i.e. a lipid desaturase). One can expect that each plant is characterized by a different equation. We are currently investigating this hypothesis.

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Effect of Non-Chilling Temperature and Light Intensity during Growth of Squash Cotyledons on the Composition of Thylakoid Membrane Lipids and Fatty Acids

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The lipid composition, in particular the content of fatty acids in phosphatidylglycerol (PG), is believed to be crucial for the understanding of the chilling injury mechanism occurring in the thylakoid membrane (TM) of higher plants. In this investigation, we have studied the effect of growth conditions (e.g. temperature and light) on the composition of glycerolipid classes and their respective fatty acids during the maturation period of squash cotyledons. We have found that the changes in the lipid fatty acid composition of TM which are induced by different temperature and light growth conditions occurred only during the development of cotyledons but not when these latter had reached their maturity. The major changes were an increase of 18:3 and a decrease of 16:0 in galacto- and sulfolipids, and an increase of 16:1(3*t*) and 18:3 with a concomitant decrease of 16:0 and 18:1 in PG, when the temperature was low (20°C compared to 30°C). We conclude that low temperature conditions of growth induced an increase of the end acyl products [18:3 and/or 16:1(3*t*)] in PG and galactolipids. The possible mechanism is discussed in terms of the relative temperature dependence of fatty acid synthesis and desaturation processes. The light intensity of growth affected only the fatty acid composition of PG, i.e. when it was high (350 compare to 100 μmol m^{-2} s^{-1}), an increase of 16:1(3*t*) at the expense of 16:0 was observed. Whatever the growth conditions, the level of 16:1(3*t*) increased during the maturation of cotyledons and was characterized by an increasing linear correlation (r=0.99) with the fresh weight. In contrast, a decreasing linear relationship (r=−0.99) was found between the fresh weight and 16:0. Thus, we propose that a constant level of 16:1(3*t*) is a good criterion for defining the chloroplast maturity and that it is highly advisable to study the effect of temperature and light on the lipid composition when the cotyledons have reached their maturity. Under these conditions, these effects can be considered regardless of developmental factors.

Key words: Δ^3-*trans*-hexadecenoic acid — *Cucurbita moschata* Durch — Development — Galactolipids — Maturity — Phosphatidylglycerol.

Low temperature is one of the major environmental factors which limits the growth, the geographical distribution and productivity of plants on the earth. Most plants of temperate climate have the ability to survive at chilling temperature (e.g. from 5 to 15°C) and are called chilling-resistant plants. In contrast, most tropical and subtropical plants are unable to survive under such temperatures and are considered as chilling-sensitive plants (Lyons 1973, Levitt 1980, Murata and Nishida 1990). However, the resistance of chilling-sensitive plants can be increased by a pretreatment at low, but not injurious temperature (Anderson et al. 1994, Kodama et al. 1995). Several mechanisms have been proposed to explain the chilling sensitivity of plants. One of the first hypothesis was that bulk membrane lipid phase transitions at a critical temperature result in the formation of gel phase lipids (Lyons 1973). Consequently, the gel phase leads to increased permeability or leakiness of cellular and organelle membranes, loss of compartmentation and physiological functions, then to cell death. More recently, Murata (1983) established a correlation between the content of desaturated molecular species of phosphatidylglycerol [16:0/16:0, 16:0/16:1(3*t*), 18:0/16:0 and 18:0/16:1(3*t*)] and the chilling sensitivity of plants, i.e. chilling-sensitive plants contain much higher proportions of desaturated PG than resistant plants. These "high-melting point" PG molecular species are supposed to form gel phase domains in the thylakoid membranes at chilling temperatures. Furthermore, an addition of small amounts (as little as 1 to 2%) of disaturated phospholipids to mixtures of leaf membrane polar lipids significantly increases the temperature at which phase transition occurred in the mixture (Raison and Wright 1983). Experiments with transgenic plants have confirmed the causal link between the amount of desaturated PG and low-temperature-induced injury (Murata et al. 1992).

However, several results are difficult to interpret in terms of the "high-melting point" PG molecular species hypothesis: (1) The correlation between the amount of desaturated PG and chilling sensitivity is not unambiguous be-

Abbreviations: DGDG, digalactosyldiacylglycerol; m:n, fatty acid containing m carbons and n cis double bonds; MGDG, monogalactosyldiacylglycerol; MOPS, 4-morpholinopropanesulfonic acid; PFD, photon flux density; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography; 14:0, myristic acid; 16:0, palmitic acid; 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

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cause of the lack of a quantitative measure for chilling sensitivity (Somerville 1995); (2) Not all chilling-sensitive plants contain high proportion of desaturated PG, such as solanaceous and other 16:3-plants and C_4 grasses (Roughan 1985); (3) When the level of desaturated PG was determined in several plant species (Murata et al. 1982, Roughan 1985, Kenrick and Bishop 1986) no special precautions were taken to grow plants under similar light and temperature conditions. However, it is known that these two combined growth parameters can influence greatly the content of the fatty acids and lipid molecular species (Dubacq and Trémolières 1983 and references therein; Xu et al. 1992). Moreover, in earlier studies, the developmental stage of the plant tested is rarely known or defined; (4) According to Murata's hypothesis, low-temperature acclimated plants which are more chilling-resistant should contain less high-melting point PG molecular species than the control plants. This is apparently not the case for the plant species *Nerium oleander* L. (Orr and Raison 1987). (5) In a mutant of *Arabidopsis* (*fab1*), leaf PG contains 43% of high-melting point molecular species, a higher percentage that is found in many chilling-sensitive plants. However, the mutant was completely unaffected, when compared with wild-type controls, by a range of low-temperature treatments that rapidly led to the death of other chilling-sensitive plants (Wu and Browse 1995).

As a further step in understanding the relationship between chilling sensitivity and the level of membrane polar lipids, we have studied the composition of acyl lipids and fatty acids in thylakoids from squash cotyledons. In this investigation, we demonstrate that in the growth temperature/glycerolipid composition relationship, it is very important to define exactly the growth conditions of plants as well as the development stage of the cotyledons. In addition, the presence of the end acyl products (16:1(3*t*) and/or 18:3) in galactolipids and PG of squash plants grown at low temperature will be discussed in terms of the temperature dependence of the enzymes involved in fatty acid synthesis and desaturation.

Materials and Methods

Plant materials and growth conditions—Plants of squash (*Cucurbita moschata* Durch. cv Shirakikuza), a chilling-sensitive plant (Murata et al. 1982), were grown from seeds in soil (Mio plant Natura, Migros, Switzerland). The seeds were germinated in darkness at 30°C and the seedlings grown in controlled environment growth chambers (Sanyo Gallenkamp, U.K., Cabinet Model PG 660) under a 12 h photoperiod and various temperatures (20 or 30°C) and light (100 or 350 μmol m^{-2} s^{-1}) conditions as indicated in Figure 1. Chloroplasts were immediately isolated from cotyledons collected after the desired daily light period.

Isolation of thylakoid membranes—All operations were performed at 4°C. The cotyledons (20 g, 40 to 120 cotyledons) were ground shortly (5 s) using a Waring Blendor in 160 ml of a grinding medium containing 330 mM sorbitol, 30 mM MOPS-KOH (pH 7.8), 2 mM EDTA-Na_2 and 0.15% BSA. The mixture was filtered through six layers of cheesecloth, and the filtrate was subsequently centrifuged at 1,910 × *g* for 1 min. The supernatant was discarded by aspiration whilst the intact chloroplast pellet was resuspended with a smooth brush in a lysis medium containing 10 mM Tricine-KOH (pH 7.8) first in 4 ml, then diluted with 30 ml of the same solution. After 1 min, isotony was restored by adding 4 ml of 3 M sorbitol, followed by mixing and centrifuging at 4,300 × *g* for 2 min. After discarding the supernatant by aspiration, thylakoids were resuspended in a washing medium containing 300 mM sorbitol and 10 mM Tricine-KOH (pH 7.8) and further centrifuged at 4,300 × *g* for 2 min. The thylakoid pellet was resuspended in the same washing medium and adjusted to about 2 mg chlorophyll per ml. The chlorophyll concentration was determined according to Bruinsma (1961).

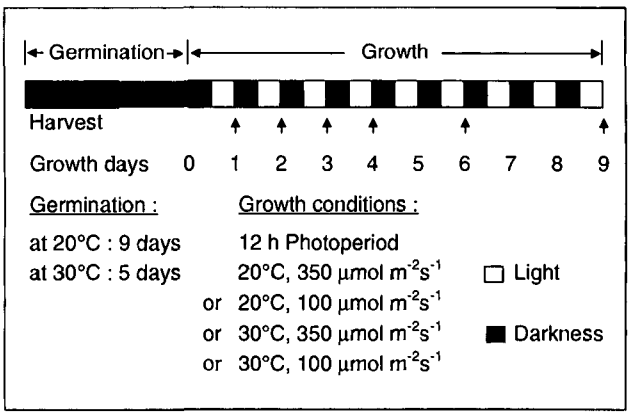

Fig. 1 Germination and growth conditions of squash plants. Cotyledons were harvested after 1, 2, 3, 4, 6 and 9 days of growth and thylakoids were isolated immediately for lipid analyses as described in Material and Methods.

Lipid extraction and purification—Lipids were extracted from thylakoid membranes according to Siegenthaler et al. (1989). The lipid classes were separated from each other by TLC on silica gel plates (pre-coated silica gel plates, Merck 5626). The developing solvents were: acetone/toluene/H_2O (91 : 30 : 8, by volume) for separating PG and chloroform/methanol/25% NH_3/H_2O (65 : 35 : 3 : 2, by volume) for separating MGDG, DGDG and SQDG. The plates were dried shortly in air and lightly sprayed with 0.01% primuline and viewed under UV light. Zones of interest (MGDG, DGDG, SQDG and PG) were scraped into glass tubes and the quantification of each lipid class was carried out by determining its fatty acids content by gas chromatography (Xu and Siegenthaler 1996).

Determination of fatty acids—The individual thylakoid membrane lipids separated by TLC were transesterified with 5% H_2SO_4 in MeOH for 1 h at 85°C. The fatty acid methyl esters were separated on a Hewlett-Packard 5890 gas chromatography supplied with an hydrogen flame ionization detector and a capillary column FFAP (30 m; i.d. 0.53 mm). The column was isothermally run at 190°C and the detector was held at 230°C. Arachidic acid (Sigma) was used as an internal standard.

Results

Effect of cotyledon development upon the fatty acid composition of glycerolipid classes—Table 1 shows the changes of fatty acid composition in MGDG, DGDG and SQDG during the cotyledon maturation when seedlings were grown, after germination, at isothermal temperature (20 or 30°C) and at two photon flux densities (100 or 350 μmol m^{-2} s^{-1}). During growth and under all temperature and light conditions, the content of 18:3 increased mainly

Table 1 The effect of the cotyledon development on the fatty acid composition of thylakoid membrane glycerolipids (MGDG, DGDG and SQDG)

Lipids	Growth condition		Growth days	Fatty acid composition (mol%)				
	Temp. °C	PFD, μmol m^{-2} s^{-1}		16:0	18:0	18:1	18:2	18:3
MGDG	30	350	1	1.5	0.0	1.3	5.7	91.5
			2	1.6	0.0	0.7	2.6	95.1
			3	1.6	0.0	0.7	2.7	95.0
	30	100	1	1.5	0.0	1.1	6.4	90.9
			2	1.6	0.0	0.5	2.9	95.0
			3	1.7	0.0	0.6	2.8	94.9
	20	350	1	1.1	0.0	0.8	7.1	90.9
			2	1.1	0.0	0.3	2.6	96.0
			3	1.0	0.0	0.2	1.8	97.0
	20	100	1	1.1	0.0	0.9	6.9	91.0
			2	1.0	0.0	0.4	2.7	95.9
			3	1.1	0.0	0.3	2.1	96.5
DGDG	30	350	1	9.6	1.4	1.0	5.2	82.9
			2	9.5	0.7	0.7	3.1	86.0
			3	9.5	0.5	0.6	2.8	86.5
	30	100	1	9.9	1.6	0.9	5.9	81.7
			2	9.4	0.9	0.7	3.6	85.5
			3	9.3	0.7	0.7	3.5	85.9
	20	350	1	5.0	1.0	0.6	6.0	87.5
			2	6.3	0.4	0.3	3.1	89.9
			3	6.2	0.4	0.2	1.9	91.3
	20	100	1	7.2	1.0	0.6	5.8	85.4
			2	6.4	0.5	0.4	3.5	89.2
			3	6.2	0.4	0.3	2.6	90.4
SQDG	30	350	1	26.7	5.8	3.6	11.8	52.0
			2	26.2	4.8	2.3	7.2	59.5
			3	27.2	4.6	2.9	6.5	58.8
	30	100	1	26.6	6.4	3.2	13.1	50.6
			2	26.9	5.7	2.9	8.5	56.0
			3	27.6	4.7	2.9	7.5	57.3
	20	350	1	25.3	6.0	2.8	12.1	53.7
			2	24.3	5.8	2.2	7.2	60.5
			3	24.9	5.3	1.8	5.0	62.9
	20	100	1	25.2	6.0	2.9	11.9	54.0
			2	23.4	5.9	2.5	7.7	60.5
			3	23.5	5.4	2.0	6.1	62.9

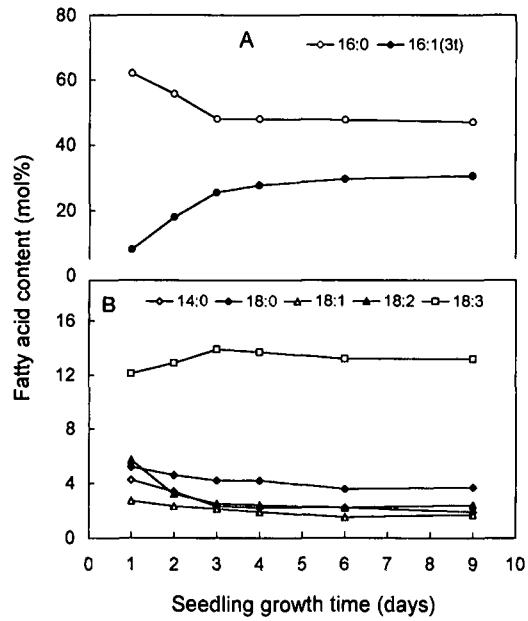

Fig. 2 Changes in fatty acid composition of phosphatidylglycerol in thylakoid membranes during cotyledon development. Plants were germinated and grown at 20°C. The photoperiod was 12 h and the PFD 350 μmol m^{-2} s^{-1}. A: changes in the content of 16:0 and 16:1(3*t*); B: changes in the content of 14:0, 18:0, 18:1, 18:2 and 18:3.

at the expense of 18:2 and to a lesser degree of 18:1 and 18:0 in the three lipid classes. These changes occurred especially during the first two days of growth. In contrast, no significant change was observed at the level of 16:0 during the first three days of growth.

Phosphatidylglycerol displayed the most complex pattern in fatty acid composition. Seven fatty acids were indeed found in thylakoid membranes of squash, i.e. 14:0, 16:0, 16:1(3*t*), 18:0, 18:1, 18:2 and 18:3. Fig. 2 shows, as an example, the changes in the composition of fatty acids occurring during the first 9 days of growth at 20°C and 350 μmol photons m^{-2} s^{-1}. The comparison of the results of Fig. 2B and Table 1 reveals that the behaviour of 18:3 and 18:2 in PG was very close to that found in the other lipid classes, i.e. an increase in 18:3 content from 12.1 to 13.9± 0.4 mol% at the expense of 18:2 (from 5.7 to 2.9±0.2) during the first three days of growth. However, when seedlings were grown at a higher temperature (30°C), the content of 18:1 increased from 3 to 6.6±0.4 mol% whilst the level of 18:3 remained constant at about 8.4±0.3 mol% (results not shown). The most significant change occurred at the level of the C_{16} series as shown in Fig. 2A. During the first 3 days of growth, there was a significant decrease in 16:0 with a concomitant increase in 16:1(3*t*). After 3 days, the molar ratio 16:0/16:1(3*t*) remained constant.

Relative content of glycerolipid classes during seedling growth—Preliminary experiments revealed that whatever growth conditions were (see Fig. 1), the content of MGDG, DGDG, SQDG and PG remained constant. Cotyledons were collected 6 times during growth (as shown in Fig. 1) and the content of lipid classes was measured in each cotyledon thylakoid samples. The mean values (±standard deviation) which are reported in Table 2 show that, within the limits of environmental conditions used, the relative molar content of each lipid class did not depend on the growth temperature and light energy as well as on the maturity degree of the cotyledons.

Effect of growth temperature and light on the fatty acid composition—We have observed previously (Table 2 and Fig. 2) that beyond 4 days of seedling growth, whatever the growth conditions, the content of each lipid class and of each fatty acid (within one lipid class and one growth conditions) remained constant. This means that the development of cotyledons did not affect the composition of thylakoid glycerolipids and fatty acids beyond 3 days of growth. This situation appears to be ideal for studying the effect of growth temperature and/or light on these biochemical parameters, regardless of developmental factors.

Cotyledons were harvested after 4, 6 and 9 days of growth, i.e., when they had reached their full maturity. The content of thylakoid glycerolipids and their respective fatty acids was measured. Data are presented as mean

Table 2 The glycerolipid composition of thylakoid membranes isolated from squash cotyledons which were grown under different temperature and light conditions

Growth condition		Lipid composition (mol%)			
Temp. °C	PFD, μmol m^{-2} s^{-1}	MGDG	DGDG	SQDG	PG
30	350	53.9±0.9	29.5±1.4	5.7±0.2	10.9±0.6
30	100	54.4±0.7	29.1±1.1	5.7±0.4	10.8±0.3
20	350	51.8±1.1	31.7±0.7	5.8±0.2	10.8±0.7
20	100	53.1±0.7	30.2±0.6	6.0±0.2	10.8±0.6

Cotyledons were collected after 1, 2, 3, 4, 6 and 9 days of growth. Data are expressed as mean values±SD (n=6). See text for detailed experimental conditions.

values (±standard deviation) in Table 3. Low growth temperature (20°C), compared to high temperature (30°C), induced an increase of 18:3 and a decrease of 16:0 level. In contrast, low (100 μmol $m^{-2} s^{-1}$), compared to high (350 μmol $m^{-2} s^{-1}$) light, had no effect on the content of 18:3 and 16:0 as well as on the content of 18:0, 18:1 and 18:2. The above observations were similar in all three glycolipids (MGDG, DGDG and SQDG).

Table 3 shows also that thylakoid PG of squash cotyledons contained high proportions of 16:0 and 16:1(3*t*) fatty acids. The content of these individual fatty acids was influenced by both the light and temperature (Table 3). At constant growth temperature (30 or 20°C), the thylakoid PG of plants grown at high light (350 μmol $m^{-2} s^{-1}$) contained higher level of 16:1(3*t*) (about 8 mol%) and a concomitant lower level of 16:0, compared to those of plants grown at low light (100 μmol $m^{-2} s^{-1}$). The 14:0 fatty acid had the same behaviour as that of 16:0, though to a lesser extent. Light intensity had no significant effect on the content of the C_{18}-fatty acid series. At constant growth light (350 or 100 μmol $m^{-2} s^{-1}$), the thylakoid PG of plants grown at high temperature (30°C) contained lower level (about 4 mol%) of 16:1(3*t*) and a concomitant higher level of 16:0, compared to that of plant grown at a low temperature (20°C). The major changes occurring in the C_{18}-fatty acid series was found at the level of 18:1 and 18:3. Lowering the temperature induced an increase in 18:3 content (about 5 mol%) at the expense of 18:1. Table 3 shows also that when cotyledons had reached their maturity (after 5 days of growth) a temperature transition from 30 to 20°C at low light (100 μmol $m^{-2} s^{-1}$) had no effect on the fatty acid composition of PG.

Though the content of 16:0 and 16:1(3*t*) in PG depended on the temperature and light of growth (Table 3), the sum of these two fatty acids (about 74 mol%) was independent of these two growth parameters. Indeed, Fig. 3 shows that under all conditions tested in this study, the correlation between the content of 16:0 and 16:1(3*t*) was linear and decreasing ($y = -1.29x + 89.31$; $r = 0.99$).

Effect of growth temperature on the cotyledon fresh weight—The above results showed that the content of 16:0 and 16:1(3*t*) depended not only on growth temperature and light conditions (Table 3) but also on the maturity state of the cotyledons (Fig. 2). Therefore, it was necessary to express the level of the two above fatty acids as a function of a parameter characterizing the growth of cotyledons, e.g. the fresh weight. Fig. 4 shows the changes of cotyledon fresh weight at two growth temperatures. Though the in-

Table 3 The effect of growth temperature and light on the fatty acid composition of thylakoid membrane glycerolipids (MGDG, DGDG, SQDG and PG) isolated from squash cotyledons

Lipids	Growth condition		Fatty acid composition (mol%)						
	Temp. °C	PFD, μmol $m^{-2} s^{-1}$	14:0	16:0	16:1(3*t*)	18:0	18:1	18:2	18:3
MGDG	30	350	—	2.1±0.3	—	—	tr	2.8±0.3	94.7±0.3
	30	100	—	2.1±0.5	—	—	tr	3.1±0.5	94.2±0.9
	20	350	—	0.9±0.2	—	—	tr	1.8±0.1	97.1±0.3
	20	100	—	1.2±0.1	—	—	tr	2.2±0.4	96.4±0.4
DGDG	30	350	—	9.8±0.2	—	tr	tr	2.6±0.2	86.7±0.3
	30	100	—	9.6±0.3	—	tr	tr	3.4±0.3	86.0±0.8
	20	350	—	5.7±0.5	—	tr	tr	1.6±0.3	92.1±1.0
	20	100	—	6.3±0.1	—	tr	tr	2.2±0.3	90.9±0.6
SQDG	30	350	—	30.2±1.7	—	4.5±0.4	2.0±0.1	5.5±0.2	57.9±2.2
	30	100	—	29.6±1.8	—	4.8±0.4	2.2±0.4	6.8±0.4	56.5±1.6
	20	350	—	26.4±1.2	—	5.1±0.6	1.9±0.3	4.4±0.6	62.2±0.7
	20	100	—	24.9±1.2	—	4.9±0.7	2.3±0.4	5.2±1.0	62.6±0.4
PG	30	350	1.8±0.1	49.3±0.3	25.3±0.3	5.0±0.3	7.1±0.2	2.7±0.1	8.8±0.2
	30	100	2.8±0.2	56.9±0.6	17.3±0.6	5.0±0.2	6.6±0.4	3.0±0.1	8.4±0.3
	20	350	2.1±0.2	47.5±0.5	29.3±1.5	3.8±0.3	1.6±0.2	2.3±0.1	13.4±0.3
	20	100	3.8±0.5	51.2±2.1	21.4±2.4	4.1±0.2	2.7±0.2	2.9±0.1	13.8±0.4
	30/20[a]	100	3.3±0.1	54.4±0.2	18.7±0.4	5.7±0.3	5.4±0.8	3.3±0.1	8.9±0.7

Data were obtained from cotyledons collected after 4, 6 and 9 days of growth and are expressed as mean values±SD (n=3). See text for detailed experimental conditions.

[a] After germination at 30°C in darkness plants were grown at 30°C and at a photon flux density (PFD) of 100 μmol $m^{-2} s^{-1}$ for 5 days, then plants were incubated at 20°C, at a PFD of 100 μmol $m^{-2} s^{-1}$. Cotyledons were harvested after 1, 2, 4 and 6 days of growth following the transition time. Data are expressed as mean values±SD (n=4). tr, trace amounts (<0.5%).

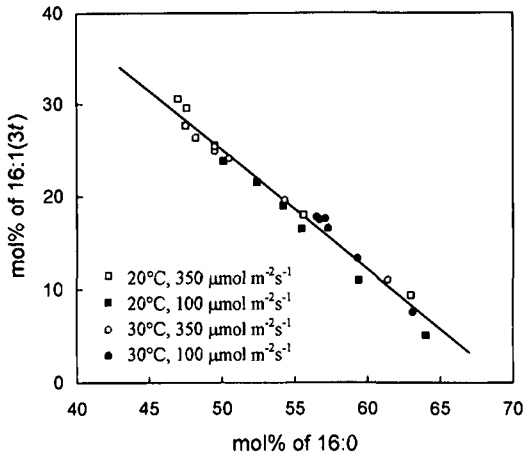

Fig. 3 Relationship between the relative content of 16:0 and 16:1(3*t*) in phosphatidylglycerol of thylakoid membranes from squash cotyledons. Plants were grown and cotyledons harvested under the conditions shown in Fig. 1. The equation of the straight line was $y=-1.29x+89.31$ ($r=0.99$).

itial fresh weights were different at 30°C (21 g/100 cotyledons) and 20°C (17 g/100 cotyledons), their increase rates were very similar up to 3 days of growth. Beyond this first period, both fresh weights increased slowly, the rate at 20°C being slightly greater than at 30°C. After 6 days, the fresh weights were the same and remained constant.

Relationship between cotyledon fresh weight and the content of 16:1(3t) and 16:0—The comparison of Fig. 2 and 4 reveals that the growth curve and the corresponding changes in 16:1(3*t*) displayed about the same pattern. Fig. 5 shows the correlation existing between the cotyledon fresh

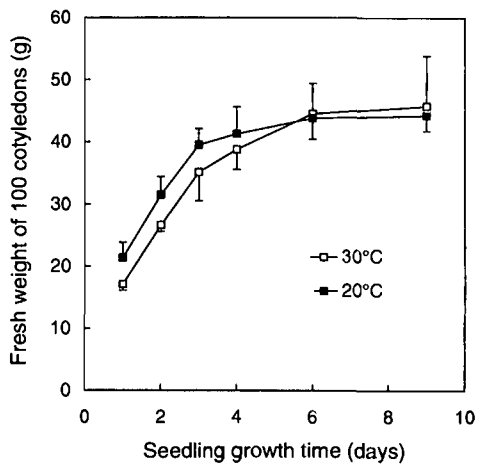

Fig. 4 Effect of two growth temperatures on the cotyledon fresh weight during the cotyledon development. Plants were germinated and grown at 30 or 20°C. The photoperiod was 12 h and the PFD 100 μmol m^{-2} s^{-1}. Visible greening appeared after 2 h of light and progressed rapidly. Data are reported as the mean values ± SD ($n=3$).

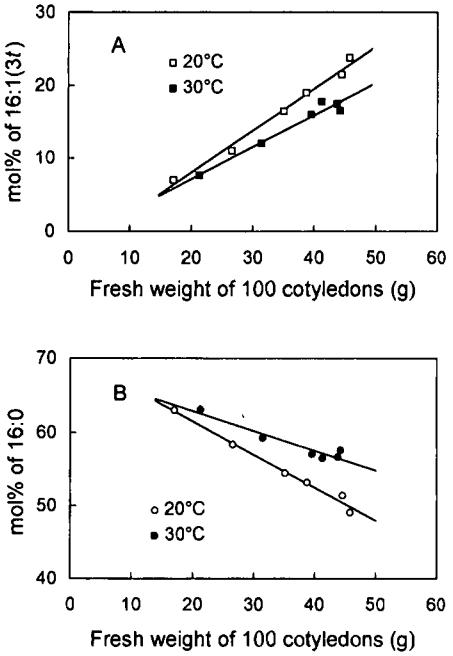

Fig. 5 Effect of temperature on the relationship between cotyledon fresh weight and the relative molar content of 16:1(3*t*) (A) and of 16:0 (B) in phosphatidylglycerol of thylakoid membranes. Plants were grown and cotyledons harvested under the conditions shown in Fig. 1. Light intensity was 100 μmol m^{-2} s^{-1}. In A, the equation of the straight lines was $y=0.58x-3.52$ at 20°C and $y=0.44x-1.72$ at 30°C. In B, the equations were $y=-0.45x+70.65$ at 20°C and $y=-0.27x+68.36$ at 30°C.

weight and either 16:1(3*t*) or 16:0 level at two growth temperatures but at the same low PFD (100 μmol m^{-2} s^{-1}). An increasing linear correlation ($r=0.99$ at 20°C and 0.98 at 30°C) was found between the fresh weight and 16:1(3*t*) as shown in Fig. 5A. The slope of the straight line was higher for low temperature samples ($a=0.58$) than for the high ones ($a=0.44$). In contrast, a decreasing linear relationship ($r=-0.99$ at 20°C and -0.95 at 30°C) was found between the fresh weight and 16:0 (Fig. 5B). The slope at low temperature ($a=-0.45$) was lower than that at high temperature ($a=-0.27$).

Discussion

The changes in fatty acid composition are associated with the cotyledon development—In the present investigation, we show clearly that the changes in the lipid fatty acid composition of thylakoid membranes which are induced by different temperature and light growth conditions take place only during the development of cotyledons but not when these latter have reached their maturity. Indeed, when cotyledons were mature, i.e., when the fresh weight remained constant (after 4 days of growth as shown in

Fig. 4), the composition of their lipid fatty acids was different and depended on the growth conditions (temperature and light) which preceeded cotyledon maturity. The main differences observed in PG were an increase of 18:3 and 16:1(3*t*) when the growth temperature was lower and an increase of 16:1(3*t*) accompanied by a decrease in 16:0 when the light intensity of growth was higher (see Table 3). However, after 5 days of growth at 30°C and 100 μmol m^{-2} s^{-1} of light, a transfer of plants to 20°C conditions resulted in no significant changes in the 18:3 content of PG and only slight changes at the level of 16:0 and 16:1(3*t*) (Table 3, compare lines 2 and 5 of PG). Thus, these results indicate that growth temperature- and light-induced changes in fatty acid composition of PG can occur only in developing cotyledons but not in mature ones.

The chloroplast maturity can be estimated by the 16:1(3t) content—During plant growth, the relative molar proportion of the 4 main lipid classes encountered in thylakoids (MGDG, DGDG, SQDG and PG) did not undergo any significant changes. These results are in agreement with previous studies (e.g. Bulder et al. 1991). Similarly, varying the growth conditions (e.g. various temperatures and light intensities) did not affect the relative molar content of these lipid classes (cf. Table 2). However, during cotyledon development, certain glycerolipid fatty acids underwent significant changes. The prominent ones were an increase in 16:1(3*t*) and a concomitant decrease in 16:0 content in PG (Fig. 2). The 18:3 level in all lipid classes increased also, though to a lesser extent. It is noteworthy that the increase of 16:1(3*t*) and the fresh weight of cotyledons displayed the same pattern.

Thus, as far as the growth of squash cotyledons in relationship with its thylakoid lipid and fatty acid content is concerned, two phases can be distinguished. The first one, corresponding to the development of cotyledons, is characterized by an increase of both the fresh weight and 16:1(3*t*) level (see Fig. 2, 4). The second phase, corresponding to the maturity of cotyledons, displayed no change in fresh weight, constant levels of 16:1(3*t*) and of the 16:1(3*t*)/16:0 molar ratio. Since the 16:1(3*t*) fatty acid is present only in chloroplast membranes, we propose that the increase of 16:1(3*t*) is associated with the chloroplast development whereas a constant level of this fatty acid indicates that the chloroplast has reached its maturity. This proposal is substantiated by the increasing linear correlation found between the fresh weight of cotyledons and the 16:1(3*t*) content (see Fig. 5A). It is of interest that a decreasing linear correlation existed between the fresh weight and 16:0. Assuming that a constant level of 16:1(3*t*) is a good criterion for defining the chloroplast maturity, one can estimate the degree of maturity of chloroplasts (probably also of thylakoids) during its development. For instance, when plants were grown under 20°C and 350 μmol m^{-2} s^{-1} conditions, the thylakoid PG contained 8, 18 and 26 mol% of 16:1(3*t*) after one, two and three days of growth, which corresponded to 27, 60 and 87% of chloroplast maturity.

The fact that the growth temperature and light influence the lipid fatty acid composition only during the development phase but not when the cotyledons (or chloroplasts) have reached their maturity, underline the importance of defining the development stage of cotyledons (or leaf) which are submitted to temperature and light stress. This is probably why conflicting data about the effect of growth temperature on the lipid fatty acid composition are found in the literature (de la Roche 1979, Chapman et al. 1983, Vigh et al. 1985, Orr and Raison 1987, Krupa et al. 1987, Huner et al. 1989).

High PFD induces an increase in 16:1(3t) fatty acid—The effect of two light conditions (350 and 100 μmol m^{-2} s^{-1}) during the development of cotyledons resulted in no differences of the relative content of galactolipid and SQDG classes and of their fatty acids. In contrast, at high PFD, the content of 16:1(3*t*)PG was higher and the 16:0PG lower than those at low PFD (Table 3). Earlier reports established a close correlation between the level of 16:1(3*t*)PG and the extent of granal stacking during the greening of etiolated leaves. This correlation extends to the accumulation of chlorophyll *b* and LHCII, especially of its oligomeric form, in developing leaves (Lemoine et al. 1982, Dubacq and Tréolières 1983). Moreover, Hobe et al. (1995), have found that the trimer formation of LHCII is stimulated by adding PG to the reconstitution incubation mixture containing LHCII monomers. Altogether, these data suggest that high PFD enhances the formation of 16:1(3*t*)PG molecular species in order to build up functional LHCII trimers.

Low temperatures induce an increase in both 18:3 and 16:1(3t) fatty acids—It is well known that low-temperature injury can be decreased by a pretreatment of plants at low, non-injurious temperatures. This low temperature acclimation is a general phenomenon which has been described in several plants such as maize (Anderson et al. 1994), wheat (de la Roche 1979) and potato (Chen and Li 1980) and results in an increase of the unsaturation degree of membrane acyl lipids, and consequently in a higher membrane fluidity (Graham and Patterson 1982 and ref. therein). These two combined phenomena are thought to insure the survival of plants at chilling temperatures whilst a high content of saturated fatty acids in membrane lipids (corresponding to a decrease of the membrane fluidity) may be responsible for chilling injury (Murata et al. 1982). Thus, low temperature conditions appear to result in both an increase of polyunsaturated fatty acids (e.g. 18:3) and a decrease of saturated fatty acids. In agreement with the above concept, our data show that the growth of squash plants at a low temperature compared to a higher one, induced an increase of 18:3 and a decrease of 16:0 levels in the three glycolipid classes (see Table 3). However, these lipid classes contain

such a high level of unsaturated fatty acids that it is highly improbable that they form a solid phase at chilling temperature (Murata and Yamaya 1984). Moreover, even at 30°C growth temperature, the fatty acid composition of MGDG, DGDG and SQDG in thylakoids of squash (a chilling-sensitive plant) is very similar (see Table 3) to that of chilling-resistant plants such as *Arabidopsis* (Kunst et al. 1989), pea (Chapman et al. 1983) and spinach (Murata and Yamaya 1984). Thus, despite the fact that growth at 20°C induced a significant increase of 18:3 in MGDG, DGDG and SQDG in comparison with growth at 30°C, we do not think that these changes contribute to the modulation of chilling sensitivity.

In contrast to the glycolipid classes, phosphatidylglycerol seems to play a crucial role in chilling sensitivity of plants (Murata et al. 1982, Murata and Yamaya 1984). In squash cotyledons, the PG of thylakoid membranes contain 80% of saturated fatty acids [16:0, 18:0 and 16:1(3*t*)]. The fatty acid 16:1(3*t*) has a phase transition temperature very similar to that of 16:0 (Bishop and Kenrick 1987). Since the glycerol *sn*-2 position of PG is occupied only by 16:0 or 16:1(3*t*), while the *sn*-1 position is occupied by either 16:0 or C_{18} fatty acids, PG can form desaturated molecular species like 16:0/16:0, 16:0/16:1(3*t*), 18:0/16:0 and 18:0/16:1(3*t*) (Xu and Siegenthaler 1996). These species are thought to be responsible for chilling injury (Murata et al. 1982). However, our results show that a lower temperature of growth (compare results at 20 and 30°C in Table 3) resulted in an increase of the 18:3 level in PG at the expense of 18:1 as well as a concomitant slight decrease of 18:0, but in no change of the sum of 16:0 and 16:1(3*t*) fatty acids. Though this sum remained constant, it is noteworthy that lower growth temperature induced also a decrease in 16:0 and a concomitant increase in 16:1(3*t*). As discussed above, these changes have no major effects on the fluidity of the membrane.

All lipids derived from the prokaryotic pathway contain only 18:1 and 16:0 fatty acids when they are synthesized in the chloroplast envelope (Browse and Somerville 1991). In PG, the 16:0 present at the *sn*-2 position is converted to 16:1(3*t*) whilst the 18:1 at the *sn*-1 position is desaturated stepwise, first to 18:2, then to 18:3 in the chloroplast. In galactolipids, the desaturation occurs either by the prokaryotic pathway in the chloroplast or via the eukaryotic pathway via the endoplasmic reticulum as in squash plants. In PG, the end products of the desaturation process are 18:3 at the *sn*-1 position and 16:1(3*t*) at the *sn*-2 position whereas in galactolipids the end products are 18:3 at both the *sn*-1 and *sn*-2 positions. In conclusion, our results show that low temperature conditions of growth induce an increase of the end acyl products in both PG and galactolipids.

Possible mechanisms—In order to explain our results, we have to consider that the effects of temperature on the composition of fatty acids should depend on the relative rates of the enzyme activities involved in fatty acid synthesis and desaturation. Indeed, in safflower seed, Browse and Slack (1983) have found that as the temperature is lowered, the rates of both fatty acid synthesis and desaturation diminish. However, it is noteworthy that the rate of desaturation relative to fatty acid synthesis is increased. Furthermore, in plasma membrane of leek cells, Moreau et al. (1994) have observed that the synthesis of unsaturated C_{18}-fatty acids is significantly less affected by low temperature than that of C_{16}-fatty acids. The proposed mechanism is further substantiated by the data of Orr and Raison (1987) who studied the changes in the proportion of the molecular species of PG and SQDG of *Nerium oleander* L. thylakoids when the plants are grown at two non-injurious temperatures. As an example, they found that when the growth temperature of this plant is decreased from (45°C, day/35°C, night) to (20°C, day/15°C, night), the major PG molecular species containing 16:1(3*t*) increase, in particular the PG 18:1/16:1(3*t*) content increases from 25 to 51 mol%. Finally, another parameter which has to be considered is that temperature also alters the lipid assembly process and the export of the lipids from their site of synthesis (the inner envelope membrane) to their final destination (the thylakoid membrane) as found recently (Rawyler et al. 1995).

In conclusion, based on our results, we propose that the mechanism involved in the formation of highly unsaturated chloroplast lipids at low temperature is likely to be due to faster fatty acid desaturation compared to fatty acid synthesis reactions. Moreover, the mechanisms involved in the changes of the fatty composition of lipids induced by temperature and light are distinct ones.

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Phosphatidylglycerol Molecular Species of Photosynthetic Membranes Analyzed by High-Performance Liquid Chromatography: Theoretical Considerations

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ABSTRACT: A reversed-phase high-performance liquid chromatography technique was developed to separate, identify, and quantify individual phosphatidylglycerol (PG) molecular species in thylakoid membranes isolated from higher plant leaves. PG was first separated by thin-layer chromatography; then the dinitrobenzoyl derivatives of diacylglycerols produced after phospholipase C hydrolysis of PG were separated by a C_{18} reversed-phase column and detected at 254 nm. A linear response of the detector was observed in the range of 0.025 to 12 nmol of PG molecular species. It was established that there was an excellent correlation (r = 0.996) between the carbon and double-bond number in the aliphatic residues and the relative retention time of dinitrobenzoyl derivatives. A new equivalent carbon number value (ECN*) which takes into consideration the number of *cis*-(n_c) and *trans*-(n_t) double bonds per molecular species was defined as ECN* = CN − $2n_c$ − n_t, where CN is the number of carbon atoms in the aliphatic residues. The logarithm of the retention time increased linearily as a function of ECN* value. However, in this type of correlation, it may happen that two molecular species of PG having distinct relative retention times had the same ECN* value. In this case, the two molecular species can be identified by the linear correlation (r = 1) existing between the reciprocal of the relative retention time and the number of double bonds ($0 \leq n \leq 3$) in the separate 18:n/Δ3-*trans*-hexadecenoic acid [16:1(3*t*)] and 18:n/16:0 molecular species series. The advantages of this method are good separation, short elution time, quantitative precision, and predictable retention times of PG molecular species from chloroplast membranes. The method has been used routinely to identify the ten PG molecular species of thylakoid membranes in squash, potato, lettuce, and spinach leaf: 18:3/16:1(3*t*), 18:3/16:0, 18:2/16:1(3*t*), 18:2/16:0, 18:1/16:1(3*t*), 18:1/16:0, 18:0/16:1(3*t*), 18:0/16:0, 16:0/16:1(3*t*), and 16:0/16:0.

Lipids 31, 223–229 (1996).

Phosphatidylglycerol (PG) represents about 10 mol% of the total acyl lipids in thylakoid membranes of higher plants (1). In spite of its small proportion, this lipid is characterized by several unique and important features. First, PG is thought to be the only anionic phospholipid in higher plant thylakoid membranes (2). It is asymmetrically distributed across the membrane with a significant enrichment (69 mol%) in the outer monolayer (3). It also contains a special fatty acid, the Δ^3-*trans*-hexadecenoic acid [16:1(3*t*)], which is always linked to the glycerol molecule at the *sn*-2 position (4,5): There is evidence that 16:1(3*t*)-containing PG is involved in the molecular organization of the LHCII receptor complex (6). We have recently found that the phospholipid population which is located in the inner monolayer sustains most of the uncoupled noncyclic electron flow activity (3,7). Finally, because of its high-melting point fatty acids [16:0, 18:0, and 16:1(3*t*)] in plants sensitive to chilling, PG can form a gel phase in the membrane at cold temperatures and, consequently, induce chilling damage (8,9).

Thus far, most of the information about PG concerns its fatty acid composition. However, molecular species may be even more important than total fatty acid composition in the structure/function relationship of this lipid. Subtle differences in molecular species composition significantly may affect the physical properties and physiological activities of a membrane (10).

In order to separate, identify, and quantify the molecular species of PG, several methods have been proposed in the literature, e.g., thin-layer chromatography (TLC) on $AgNO_3$ in silica gel (5,11), capillary column gas chromatography using volatile derivatives and coupled gas chromatography/mass spectrometry (12). However, the argentation method cannot be used to resolve certain PG molecular species such as PG 16:0/16:0, 16:0/16:1(3*t*), 18:0/16:0, and 18:0/16:1(3t) (5). Kito *et al.* (13) have developed a sensitive method for the quantitative analysis of phospholipid molecular species by high-performance liquid chromatography (HPLC). Diacylglycerols (DAG) were prepared from phospholipids by phospholipase C (PLC) treatment and converted to the corresponding dinitrobenzoyl (DNB) derivatives which were detected at 254 nm. This method has been successfully used to analyze the molecular species of phosphatidylcholine and phosphatidylethanolamine extracted from animal cells (14).

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Abbreviations: DAG, diacylglycerol; DNB, dinitrobenzoyl; ECN, equivalent carbon number; HPLC, high-performance liquid chromatography; PG, phosphatidylglycerol; PLC, phospholipase C; RRT, relative retention time; TLC, thin-layer chromatography; 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid.

Using the method described by Kito's group (13,14), we report here on the quantitative separation of PG molecular species in thylakoid membranes isolated from plant leaves. Based on the relationship between the number of carbon atoms as well as of double bonds and the relative retention time (RRT) of PG DNB derivatives, the corresponding molecular species of PG can be identified.

EXPERIMENTAL PROCEDURES

Chemicals. PLC, EC. 3.1.4.3 (grade 1, from *Bacillus cereus*) was from Boehringer Mannheim (Rotkreuz, Switzerland). Pyridine and 3,5-DNB chloride were provided by Fluka Chemie AG (Buchs, Switzerland), and diethyl ether extra pure, acetonitrile, and 2-propanol (gradient grade) were from Merck (Darmstadt, Germany). All other solvents were of reagent grade.

Plant material. Squash (*Cucurbita pepo* L. cv Quintal), potato (*Solanum tuberosum* L. cv Granola), and spinach (*Spinacia oleracea* L. cv Nobel) plants were grown in a growth chamber under the following conditions: 12 h of light (350 μmol • s^{-1} • m^{-2})/22°C/50% relative humidity and 12 h darkness/18°C/60% relative humidity. For squash plants, the two first leaves were harvested when the third leaf appeared (about two weeks after germination). For potato and spinach plants, mature leaves were harvested after about one month of growth. Lettuce (*Latuca sativa* L. cv Laitue Romaine de Morges) leaves were from the local market.

Preparation of thylakoid membranes. Deribbed leaves (about 30 g) were ground in a Waring Blender for 5 s in 160 mL of a grinding medium containing 330 mM sorbitol/30 mM 4-morpholinopropanesulfonic acid–KOH (pH 7.8)/2 mM. The mixture was filtered through six cheesecloth layers; then the filtrate was spun down at 1910 × *g* for 1 min. The supernatant was discarded by aspiration while the intact chloroplast pellet was resuspended in a lysis medium containing 10 mM *N*-Tris[hydroxymethyl]methylglycine–KOH (pH 7.8) first in 4 mL with a smooth brush, then in 30 mL. After 1 min, isomolarity was restored by adding 4 mL of 3 M sorbitol. The mixture was spun down at 4300 × *g* for 2 min. After discarding the supernatant by aspiration, thylakoids were resuspended in a washing medium containing 300 mM sorbitol/10 mM *N*-Tris[hydroxymethyl]methylglycine–KOH (pH 7.8) then sedimented for 2 min at 4300 × *g*. The thylakoid pellet was resuspended in the same washing medium and adjusted to 1 to 2 mg chlorophyll/mL. The chlorophyll concentration was determined according to Bruinsma (15).

Lipid extraction. To 1 mL of thylakoid suspension (1 to 2 mg chlorophyll/mL), 4 mL of chloroform/methanol (1:1, vol/vol) and 0.8 mL of 100 mM KCl were added. The mixture was centrifuged (1300 × *g* for 5 min). The lower phase was collected and dried under N_2. The pellet was resuspended in chloroform/methanol (8:2, vol/vol).

PG separation. The acyl lipids contained in the chloroform/methanol extract were separated by two-dimensional TLC on silica gel 60 plates (pre-coated silica gel plates, Merck 5626). The total lipid extract corresponding to 0.15 mg chlorophyll was spotted on the plate using an automatic sample applicator (Linomat IV; Camag, Switzerland). TLC plates were developed in acetone/toluene/water (91:30:8,by vol). After drying in an N_2 atmosphere, TLC were developed in the second dimension with a mobile phase consisting of chloroform/methanol/25% NH_3/water (65:35:2:3,by vol). Lipids were revealed with 0.01% primuline in acetone/H_2O (60:40, vol/vol) and the band corresponding to PG was scrapped and introduced into a glass tube. After the addition of 30 μg of distearoyl phosphatidylcholine as internal standard, the mixture was washed twice with chloroform/methanol (1:1, vol/vol) to eliminate silica gel then dried under N_2.

PLC treatment and preparation of DNB derivatives. The removal of the polar head groups of PG was achieved by treatment with the PLC and the preparation of the 3,5-DNB derivatives of PG was carried out according to the method of Kito *et al.* (13), however, with some modifications. The purified PG was resuspended by sonication in 1.25 mL of 10 mM Tris–HCl (pH 7.5) containing 30 mM H_3BO_3, 1 mM $ZnSO_4$, and 5 mM $CaCl_2$. After the addition of ten units of PLC (5 μL) and 2 mL of water-saturated diethyl ether, the tubes were shaken at 30°C for 60 min. The diethyl ether layer containing DAG was collected, whereas the remaining aqueous phase was washed twice with 2 mL of water-saturated diethyl ether. The diethyl ether fractions were then dried under N_2.

HPLC analysis of 3,5-DNB derivatives. HPLC was performed on a Kontron HPLC-system 400 with detection at 254 nm. The column used was the KS250/6/4/nucleosil 100-5C_{18} mounted with a guard column KS 11/6/4/nucleosil 120-7C_{18} from Macherey-Nagel (Düren, Germany). Elution was achieved isocratically with acetonitrile/2-propanol (80:20, vol/vol).

Fatty acid analysis. Each HPLC peak corresponding to 3,5-DNB PG derivative molecular species was collected and dried under N_2 flow. The fatty acid constituents of each PG derivative were transesterified with 5% H_2SO_4 in methanol at 85°C for 1 h under N_2 (1). The extraction of the methyl esters was achieved with 1.5 mL pentane after addition of 1 mL water. The extract was evaporated to dryness with N_2, taken up in 150 μL hexane and injected (5 μL) automatically into the gas chromatograph Hewlett-Packard 5890 (Palo Alto, CA) mounted with a capillary column Hewlett-Packard free fatty acid phase (30 m; i.d. 0.53 mm). The column was isothermically run at 190°C with helium as carrier (14.8 mL/min) while the detector (flame-ionization detector) was held at 230°C. Retention times of fatty acid methyl esters were compared with those of standard methyl esters of 18:0, 18:1, 18:2, 18:3, 16:0, and 16:1(3*t*), allowing the identification of the fatty acids contained in each HPLC peak. The quantification of fatty acids was carried out by adding a known amount (30 μg) of arachidic acid (20:0) in each sample, just before the transesterification step.

RESULTS AND DISCUSSION

Molecular species of thylakoid membrane PG in four plant species. Figure 1 shows the HPLC profile of 3,5-DNB deriv-

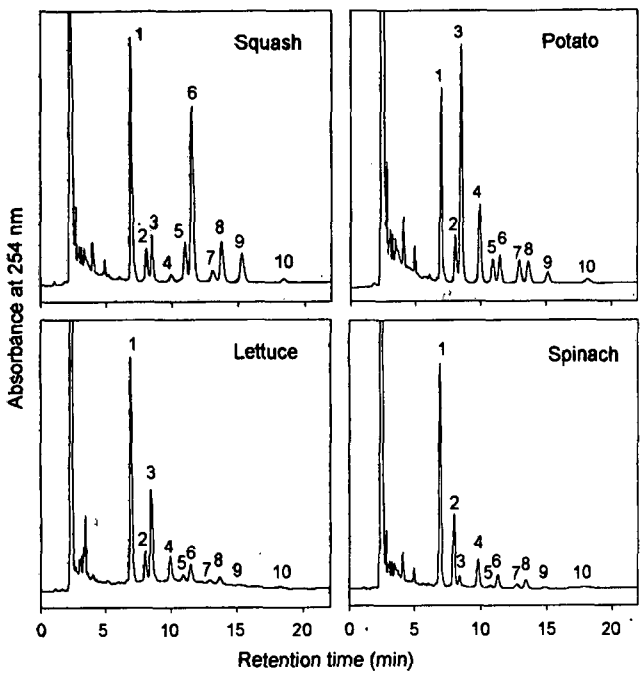

FIG. 1. High-performance liquid chromatography profile of 3,5-dinitrobenzoyl derivatives of phosphatidylglycerol isolated from thylakoid membranes of squash, potato, lettuce, and spinach. Peak numbers correspond to those in Tables 1 and 2. The retention time of the internal standard (3,5-dinitrobenzoyl derivative of 18:0/18:0 phosphatidylcholine) was 25.02 min (data not shown).

atives of DG isolated from thylakoid membranes of four plant species. All thylakoid membranes were characterized by ten HPLC peaks numbered from 1 to 10. It is noteworthy that, under our experimental conditions, all compounds were eluted from the HPLC column during 20 min only. The peaks appearing during the first 7 min were not due to lipid derivatives since an HPLC profile of a control sample (e.g., without added PG DNB derivatives) gave exactly the same profile as those illustrated in Figure 1 (results not shown). In addition, no methyl esters of fatty acids were detected by gas chromatography in the first fractions of the HPLC eluate. As an example, the composition in fatty acids of each HPLC peak from squash thylakoid PG (Fig. 1) is shown in Table 1. Each peak included two main fatty acids, except peak 8, which contained essentially 16:0. Within experimental errors, the amount of these two fatty acids was the same. However, each peak included small amounts of fatty acid contaminants due to manual collection of the eluted sample. In our hands, contaminants never exceeded 0.8 mol% of total PG (e.g., the contamination by 18:1 in peak 6). This indicates that each peak corresponded to one DNB–DAG molecular species. Table 1 also shows that each HPLC peak always contained either 16:0 or 16:1(3*t*) as one of the main fatty acids, the other one being a fatty acid in the C_{18} series, in agreement with the finding of Roughan (5). However, there are two exceptions to this general rule, i.e., peak 6 containing both 16:0 and 16:1(3*t*) and peak 8 containing essentially 16:0 (in double amount). Several authors have reported that in leaf PG of higher plants, the *sn*-1 position is occupied either by a fatty acid of the C_{18} series or by 16:0, whereas the *sn*-2 position always contains a fatty acid of the C_{16} series. This last finding has been verified by hydrolyzing the ester bond of PG at the position *sn*-2 with snake venom phospholipase A_2 according to Chapman and Barber (16). Moreover, when present, 16:1(3*t*) is always located at the *sn*-2 position (4,5,8,17). On the basis of these findings, it was possible to assign a PG molecular species to each of the HPLC peak, as illustrated in Table 1. It can be seen that about 50 mol% of PG in squash (a plant sensitive to chilling) thylakoid membranes contained highly saturated molecular species [e.g., 16:0/16:1(3*t*), 16:0/16:0, 18:0/16:1(3*t*), 18:0/16:0]. In contrast, the PG from potato, and even more from lettuce and spinach (chilling-resistant plants) thylakoid membranes, was characterized by much more unsaturated molecular species [e.g., 18:3/16:1(3*t*), 18:2/16:1(3*t*); see Fig. 1]. These results are qualitatively in agreement with earlier observations (12,18). However, Sekiya *et al.* (19) have reported that in leaves of various *Citrus* species, there are also additional mol-

TABLE 1

Determination by Gas Chromatography of the Fatty Acid Composition and Content of Each High-Performance Liquid Chromatography Peak of Phosphatidylglycerol (PG) Isolated from Squash Thylakoid Membranes

Peak[a] number	Fatty acid composition (mol%)						Molecular species *sn*-1/*sn*-2[b]	Mol% of total PG
	16:0	16:1(3*t*)	18:0	18:1	18:2	18:3		
1	4.1	47.3[c]		1.3	1.0	46.4	18:3/16:1(3*t*)	28.9
2	48.3	1.4		1.3	1.8	47.3	18:3/16:0	5.5
3	5.2	45.8		1.1	45.3	2.6	18:2/16:1(3*t*)	6.5
4	46.8	3.1		12.7	33.3	4.0	18:2/16:0	1.3
5	6.9	45.9		47.2			18:1/16:1(3*t*)	7.7
6	47.4	48.2	1.7	2.6			16:0/16:1(3*t*)	31.0
7	47.1	5.1		47.8			18:1/16:0	2.5
8	94.7	1.2	3.1	1.1			16:0/16:0	8.6
9	1.8	48.9	49.3				18:0/16:1(3*t*)	7.1
10	48.0	1.8	48.5	1.7			18:0/16:0	1.0

[a]Peak numbers correspond to those indicated in Figure 1.

[b]The *sn* position of fatty acids was not determined but assumed from other reports (see text); 16:1(3*t*), Δ^3-*trans*-hexadecenoic acid.

[c]The underlined numbers correspond to the main fatty acids in each peak.

shows that the amount of DNB–DAG injected in the HPLC was linearly correlated with the peak area. This was true for the ten PG molecular species encountered in the thylakoid membranes of squash. From these data and those reported in Table 1, one can calculate the range limits within which the peak areas were proportional to the amounts of DNB–DAG. The upper limit (see arrow in Fig. 5) was found for the DNB–DAG [16:0/16:1(3*t*)] which corresponded to about 12 nmol (i.e., 40 nmol of DNB–DAG injected × 31 mol% of total PG/100 = 12.4 nmol). The lower limit (see arrow in Fig. 5) was obtained for the DNB–DAG (18:0/16:0) which corresponded to 0.025 nmol (i.e., 2.5 nmol of DNB–DAG injected × 1 mol% of total PG/100 = 0.025 nmol). Though not shown in Figure 5, the detection limit of DNB–DAG (18:0/16:0) can be lowered to 6 pmol, an amount which is generally sufficient for characterizing the PG molecular species in higher plants. When higher sensitivities are needed, new techniques such as electrospray mass spectrometry (22) or HPLC/mass spectrometry using electrospray ionization (23) allow the quantification of phospholipid molecular species as low as approximately 0.5 pmol (23).

Conclusions. The fact exists that PG molecular species encountered in photosynthetic membranes from higher plants contain, in addition to *cis*-double-bond fatty acids, one *trans*-double bond in 16:1(3*t*), required a new theoretical approach on the relationship between the HPLC experimental data (e.g., retention time or its reciprocal) and the structural characteristics of each molecular species (carbon and double-bond number). A new ECN value $ECN^* = CN - 2n_c - n_t$, where CN corresponds to the number of carbon atoms in the aliphatic residues and n_c and n_t to the number of *cis*- and *trans*-double bond per molecular species. Moreover, the ten PG molecular species, except 16:0/16:0 and 16:0/16:1(3*t*), fit into the two series: 18:n/16:1(3*t*) and 18:n/16:0 ($0 \leq n \leq 3$) where the characteristics of the molecular species are determined by the number of double bonds of the C_{18}-fatty acid at the *sn*-1 position. The reciprocal of the retention time and the number of double bonds in the two C_{18} series are linearly correlated (r = 1).

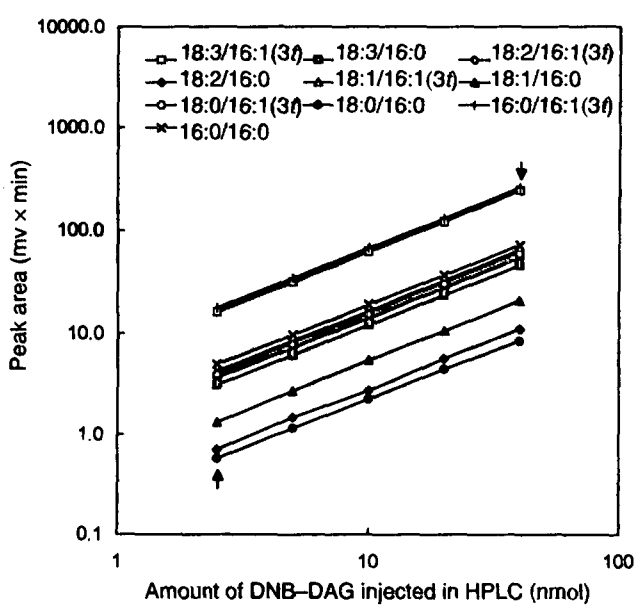

FIG. 5. Proportionality between the amounts of DNB–DAG injected in the HPLC column and the peak areas of each DNB–DAG molecular species. For further explanation, see the text. See Figures 3 and 4 for abbreviations.

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TEMPERATURE AND LIGHT CONDITIONS DURING THE GROWTH OF SQUASH SEEDLINGS INFLUENCE GREATLY THE RELATIVE CONTENT OF PHOSPHATIDYLGLYCEROL MOLECULAR SPECIES IN COTYLEDONS

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Photosynthetic membranes contain 10 molecular species of phosphatidylglycerol (PG) [1,2]. These species have been divided into two groups: the first one contains 16:0 at the *sn*-2 position (18:3/16:0, 18:2/16:0, 18:1/16:0, 18:0/16:0 and 16:0/16:0); the second one contains 16:1(3*t*) at the *sn*-2 position [18:3/16:1(3*t*), 18:2/16:1(3*t*), 18:1/16:1(3*t*), 18:0/16:1(3*t*) and 16:0/16:1(3*t*)]. The thylakoid transmembrane distribution of total PG and of its molecular species is asymmetric i.e. the outer monolayer is highly enriched (2/3) in PG [2].
PG is a unique phospholipid in the thylakoid membrane because half of its molecular species contains *trans*-Δ^3-hexadecenoic acid (16:1(3*t*)). In contrast to the other acyl lipid classes, PG contains high levels of saturated fatty acids (16:0, 18:0) and of the particular 16:1(3*t*). Moreover it has been suggested that PG is involved in the molecular organization of the light collection receptor complex (LHCII) [3] and in the mechanism of chilling sensitivity of plants [4]. Indeed, a distinct difference between chilling-sensitive and chilling-resistant plants is found in the composition of PG molecular species [1]. However, Murata et al. [4] pointed out that the relative content of 16:0 and 16:1(3*t*) varies greatly in both types of plants.
The aim of this study was to determine the relative content of PG molecular species in cotyledons from squash, a chilling-sensitive plant, which was grown under various temperature and light conditions.

Materials and Methods

Plants. After germination of squash plants (var. Quintal) in darkness at 20, 30, and 35°C, seedlings were grown at the same above temperature and in continuous light (20,000 lux).
Lipid extraction and separation. Immediately after the cotyledons were harvested, lipids were extracted with chloroform/methanol (1/1, v/v) [5]. Total lipids obtained were separated into individual lipid classes by two-dimensional TLC on a Silica gel 60 plate using aceton / toluene / acetic acid / H_2O (100/25/1/9, v/v) then chloroform / methanol / NH_3 25% / H_2O (65/35/3/2, v/v).
HPLC analysis of 3,5-dinitrobenzoyl derivatives. Purified PG were hydrolyzed by phospholipace C (*Bacillus cereus)* and the resulting 1,2-diacylglycerides were esterified with 3,5-dinitrobenzoylchloride. 3,5-dinitrobenzoyl derivatives of PG were separated by HPLC C18 column (5 mm, 4.6 x 250 mm) with acetonitrile / 2-propanol (80/20, v/v) [6].
HPLC peak analysis. Each peak was collected and fatty acids were transesterified with 5% H_2SO_4 in methanol for 1 h at 85°C and then analyzed with GC.

Results and Discussion

Fig. 1 shows that when squash seedlings were grown at a constant temperature (30°C), an exposure to continuous light (20,000) lux resulted in a progressive increase in all PG molecular species containing 16:1(3*t*), at the expense of the 16:0 ones. The PG molecular species undergoing the highest increase were 16:0/16:1(3t), 18:3/16:1(3*t*) and 18:0/16:1(3*t*), whereas those showing the greatest decrease were 16:0/16:0, 18:3/16:0 and 18:0/16:0. These changes occurred during the first 48 h in the light, then the content of the above molecular species remained constant. Decreasing the light intensity and photoperiod resulted in a slower rate and extent of these changes (results not shown). This suggests that light is the main factor which determines the content of fatty acid 16:1(3*t*). Therefore, it is important to control the light intensity and the photoperiod during plant growth when 16:1(3*t*) is analyzed.

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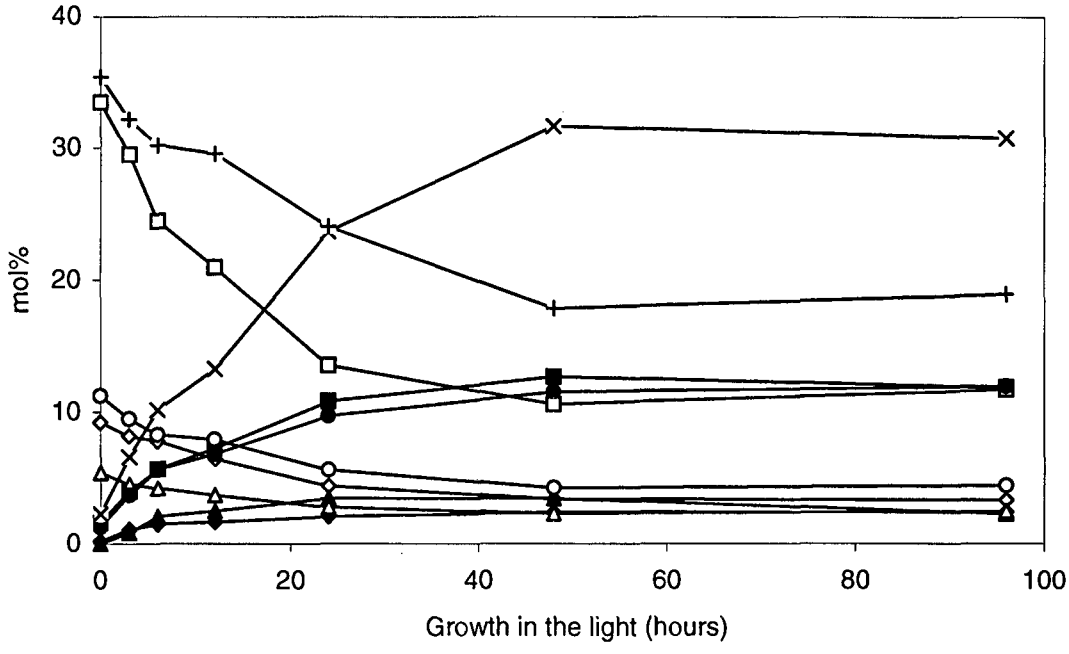

Fig. 1. Changes in the content of PG molecular species from squash cotyledons during the growth of seedlings. After germination in darkness, plant were grown in continuous light (20,000 lux). The temperature was 30°C during germination and growth.
■ 18:3/16:1(3*t*), □ 18:3/16:0, ♦ 18:2/16:1(3*t*), ◊ 18:2/16:0, ▲ 18:1/16:1(3*t*), Δ 18:1/16:0,
● 18:0/16:1(3*t*), ○ 18:0/16:0, X 16:0/16:1(3*t*), + 16:0/16:0.

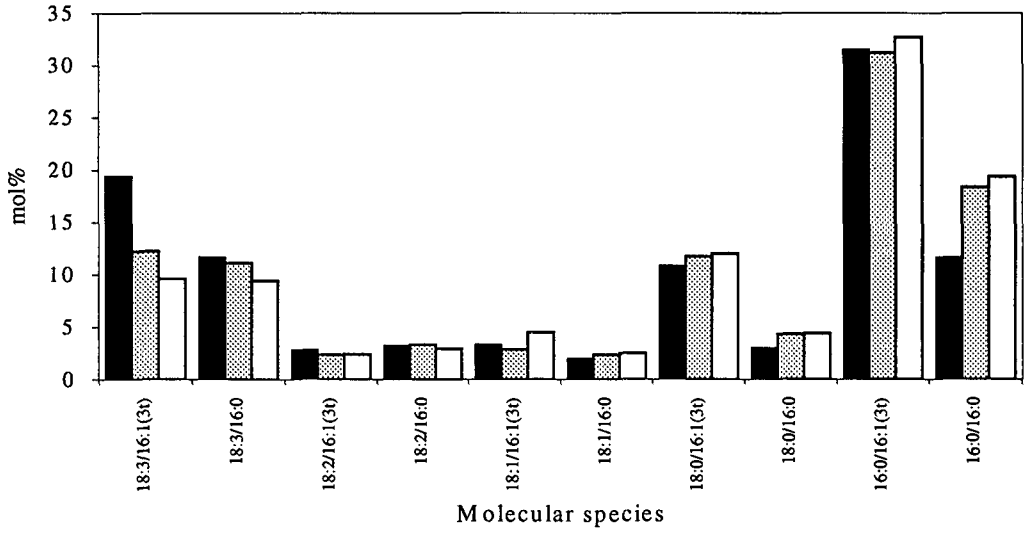

Fig. 2. Molecular species composition of PG from squash cotyledons grown at 20°C ■, 30°C ■ and 35°C □. Analysis of the PG molecular species was carried out after 48 h growth under continuous light (20,000 lux).

As can be seen in Fig.2, decreasing the growth temperature from 35°C to 20°C resulted in an increase in 18:3/16:1(3t) from about 10 to 19%, in 18:3/16:0 from 9 to 12 %, and a concomitant decrease in 16:0/16:0 from about 19 to 13% in squash cotyledon PG. Diminishing further the growth temperature down to 10°C resulted in a greater and faster increase in 18:3/16:1(3*t*) and decrease in 16:0/16:0 (data not shown). This suggests that the fatty acid unsaturation induced by low temperature was due to an increase in the molecular species associated with C18:3, especially 18:3/16:1(3*t*).

It has been reported that growth at low, cold-hardening temperature resulted in a specific 67% (thylakoids) to 74% (whole leaves) decrease in the 16:1(3*t*) acid level associated with winter rye PG [8]. However, our results show that in squash cotyledons, lowering the growth temperature induced an increase in 16:1(3*t*), mainly in PG 18:3/16:1(3*t*) molecular species (Fig. 2).

We conclude that both light intensity and photoperiod are the main factors affecting the content of the 16:1(3*t*)-containing molecular species of PG. Higher light intensity and longer photoperiod result in an increasing content of these molecular species. On the other hand, temperature affects the degree of unsaturation in PG molecular species. Decreasing the growth temperature resulted in an increase in molecular species containing 18:3 (18:3/16:1(3*t*) and 18:3/16:0) and a decrease in 16:0/16:0 in squash cotyledon PG. (Supported in part by the Swiss National Science Foundation)

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THOUGHTS CONCERNING A NEW PARADIGM OF THE PHOTOSYSTEM II REGION OF THE THYLAKOID MEMBRANE BASED ON LIPID STRUCTURE AND FUNCTION

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Each type of membrane is characterized by the amount and kind of its proteins and lipids. When considering the numerous models which have been published in the literature on the molecular organization of the thylakoid membrane (TM), it is surprising that almost nobody has attempted to represent the acyl lipids according to their topological distribution and associated functions. This failure is probably due to the fact that, contrarily to their counterpart protein moiety, lipids have no catalytic properties by themselves. The purpose of this communication is to propose a tentative paradigm shedding light on the molecular architecture of the TM in which not only proteins but also acyl lipids are considered. The concept of this model is based on recent studies [1-5].

Murata et al. [1] have studied three different photosystem II (PSII) preparations and compared their polypeptide and glycerolipid components as well as their photosynthetic activities. Several of their findings can bring interesting informations for the construction of a new paradigm of the molecular organization of acyl lipids in the TM : (1) The reaction center (RCII) which contains five proteins (the D1 and D2, the large and small subunits of cytochrome b_{559} and the 4.8 kDa hydrophobic protein) is tightly associated with 1 mol of MGDG (monogalactosyldiacylglycerol) and 0.1 mol PG (phosphatidylglycerol) per mol P_{680}. The presence of these two lipids is probably necessary for the photochemical charge separation; (2) the PSII core complex contains two additional antenna core proteins (43 and 47 kDa), the 33 kDa extrinsic protein and one hydrophobic protein. These proteins which bound firmly MGDG, DGDG (digalactosyldiacylglycerol) and PG (3-4 mol / mol P_{680} for each lipid) confer to this complex the capacity of performing O_2 evolution; (3) the molar ratio of glycerolipids to P_{680} reveals that PSII membranes (i.e. PSII core complex + LHCII + the 23 and 18 kDa extrinsic proteins) can loose about 97, 90 and 99% of their MGDG, DGDG and PG molecules, respectively, without affecting the ability to support charge separation and O_2 evolution. This is an indication that most lipids are in the bulk phase and only a few of them are involved in protein-lipid interactions and consequently may play particular structural and/or functional role; (4) when compared to

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the bulk phase lipids the relative content of saturated fatty acids (16:0; 18:0) increases and that of trienoic acids (18:3) decreases in the PSII core complex and even more in the RCII complex.

Trémolières et al. [2] have determined the lipid and fatty acid composition in various purified light-harvesting complexes II, i.e. in the major (Lhcb1 : 28 kDa; Lhcb2 : 27 kDa) and the minor LHCII (Lhcb3 : 25 kDa; Lhcb4 : CP 29; Lhcb5 : CP26; Lhcb6 : CP 24). Their data support the view that a great heterogeneity exists in the lipid class distribution and in the degree of unsaturation in this region of the TM: (1) the minor LHCII complexes retain high amounts of lipids in the decreasing order MGDG > DGDG >> PG. The estimated lipid/polypeptide molar ratio varies from 15 to 25 for MGDG, 3 to 10 for DGDG and about 1 for PG; (2) in contrast, in the major LHCII complexes this ratio drops to about 1 for all the above lipids; (3) the relatively high amount of MGDG and to some extent of DGDG molecules which are associated with the minor complexes suggests that these lipids have linking properties between the PSII core complex and the antennae (Lhcb 3-6). Concerning the PG molecule, it might be anchored in the apoprotein-chlorophyll domains of these minor complexes; (4) in the major LHCII complexes, the low lipid/polypeptide ratio excludes the view that lipids can play a cohesion role in the organization of these antennae but rather a specific role in the function of LHCII; (5) in LHCII complexes, the galactolipids are slightly unsaturated and PG much more saturated than in the corresponding bulk lipids.

Siegenthaler's group [3-5] has studied the bulk lipid phase of the TM : (1) when acyl lipids are expressed in mol % of total lipids, MGDG (35 mol %) + DGDG (4 mol %) in the outer monolayer and DGDG (21 mol %) + MGDG (19 mol %) in the inner monolayer are prominent - both galactolipids representing about 40 mol % of the total lipid in each monolayer; (2) the minor lipid classes SQDG (sulfoquinovosyldiacyl-glycerol), PG and PC (phosphatidylcholine) represent not more than 10% in each monolayer. (3) Altogether, these results strongly suggest that most galactolipids have a structural role in the bulk lipid phase whereas the minor lipids and a few saturated galactolipid molecules display a particular functional role in the TM.

In addition to these topological studies, we have shown [5] - using a lipid depletion technique, that the population of PG molecules (70 mol % of the lipid class) localized in the outer monolayer is likely to have a structural role in the bulk phase. In contrast, the inner PG population includes several yet undefined subpopulations, some of them, very discrete (3 mol %) being highly efficient in supporting electron flow and O_2 evolution activities. Thus, these subpopulations of PG have a particular role to play in the TM function.

Based on these results [1-5], we have built a new paradigm of the molecular organization of the PSII region of the TM (Fig. 1). This model inspired from that of Jansson [6] underlines the topological distribution of acyl lipids. All big circles, squares and triangles represent lipids which are firmly bound to proteins and thus are likely

to play a special structural and/or functional role. Small circles and stars represent MGDG and DGDG which are located in the bulk lipid phase of TM. All open symbols underline the fact that lipids closely bound to proteins, especially in the RCII, are saturated. Though we have tried to respect the stoichiometry between lipids and polypeptides, one has to keep in mind that the use of detergents for the isolation of PSII preparations may cause the loss of lipids and the perturbation in their topological distribution. Anyhow, this model shows clearly a great heterogeneity of lipid classes and of their molecular species.
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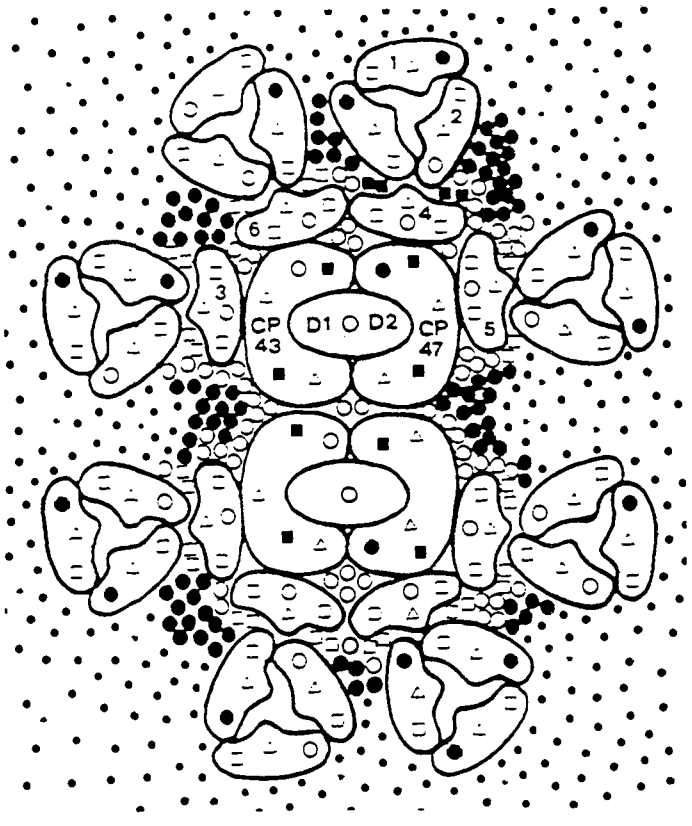

Fig. 1. A new paradigm of the PSII region of the thylakoid membrane (top view). ○●, saturated and unsaturated MGDG; □ ■, saturated and unsaturated DGDG; △ ▲, saturated and unsaturated PG; ● ✶, MGDG and DGDG of the bulk lipid phase of TM. The reaction center is represented only by the D1 and D2 proteins, the core complex by additional proteins CP43 and CP47, and the different LHCII complex by 1 and 2 (Lhcb1,2), 3,4,5 and 6 (Lhcb3-6), according to Jansson [6].

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MOLECULAR ORGANIZATION AND FUNCTIONS OF ACYL LIPIDS IN SPINACH CHLOROPLAST MEMBRANES

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SYNOPSIS

The mature spinach thylakoid membrane (TM) contains 10 PG molecular species. Only three of them displayed values > 1 mol %. The relative proportion of all these species, especially the 18:3/16:1(3t) one which accounted for about 83 mol % in TM, was almost equal in the two TM monolayers. However, probably due to interactions with specific proteins, inner PG molecular species mediated at least 80% of the electron flow activity of TM, whereas outer species preserved the integrity of the LHCII complex. In chloroplast envelope membranes, PG and PC, but not PI, were found to support, to different extents, the phosphorylation of the 67, 26 and 14 kDa proteins.

INTRODUCTION

It is well established that not only chlorophyll-protein complexes but also all acyl lipid classes are heterogeneously distributed across TM. This asymmetric arrangement is needed to support the vectorial properties needed for energy conservation. Concerning acyl lipids, we have shown that the outer monolayer is enriched in MGDG and PG, whereas DGDG is essentially confined in the inner monolayer (Rawyler and Siegenthaler, 1985; Giroud and Siegenthaler,1988; Siegenthaler et al., 1989). One of the most intriguing questions concerns the role of inner and outer lipids in TM functions. The enzymatic approach (use of specific lipolytic enzymes), followed by a BSA treatment to remove hydrolysis products from the membranes, allowed us to show that

ABBREVIATIONS : BSA, bovine serum albumin; CEM, chloroplast envelope membranes; DAG, diacylglycerol; DGDG (MGDG), di(mono)galactosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA_2, PLC, phospholipase A_2 or C; TM, thylakoid membrane.

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certain lipid classes, depending on their localization (outer and/or inner TM monolayer), support particular functions. For instance, the phospholipid and MGDG populations which sustain the uncoupled non-cyclic electron flow activity are mainly localized in the inner TM monolayer (Siegenthaler et al., 1989; Rawyler and Siegenthaler, 1989). Furthermore, a close correlation has been shown between the functional state of the CFo/CF1 complex and the packing pressure of MGDG in the outer monolayer, e.g. only when thylakoids actively photosynthesize ATP does the packing pressure of MGDG decrease (Rawyler and Siegenthaler, 1989). Another example concerns the numerous changes occurring in the binding and inhibitory properties of urea/triazine-type herbicides upon phospholipid and galactolipid depletion in the outer monolayer of TM (Siegenthaler and Mayor, 1992). In contrast to the above examples, investigation on the role of acyl lipids in the functions of CEM are just at their beginning. Recently, we have been interested in the phosphorylation of proteins in CEM and in the role phospholipids may play in this process (Siegenthaler and Bovet, 1992). In this contribution, we address the two following questions : (1) What is the transmembrane distribution of PG molecular species in TM and the consequence of their removal on the LHCII structure and PSII photosynthetic electron flow ? (2) Do phospholipids play a role in the protein phosphorylation process in CEM ?

METHODS

Conditions of incubation in the presence of PLA_2, the techniques for the depletion and quantification of PG are described elsewhere (Siegenthaler et al., 1989). Identification and quantification of PG molecular species by HPLC was carried out by the method of Kito et al. (1985). Fatty acid analysis by GC was performed according to Rawyler and Siegenthaler (1980). Protein phosphorylation and electrophoretic conditions are as described by Siegenthaler and Bovet (1992).

RESULTS AND DISCUSSION

TM PG molecular species : transmembrane distribution and functions

The enrichment of PG in TM outer monolayer has been reported in spinach (Siegenthaler et al., 1989), oat (Giroud and Siegenthaler, 1988), barley, lettuce and pea (Unitt and Harwood, 1985). These last authors found that in all three species tested, 16:1(3t), one of the main fatty acid of PG, was exclusively located in the outer monolayer. One consequence of this finding is that no PG molecular species

containing 16:1(3t) should be found in the inner monolayer. Results of Table 1 show that out of the 10 PG molecular species found in spinach TM, only three displayed values > 1 mol %. The relative proportion of these latter species, especially the 18:3/16:1(3t) one (which accounted for 83.4 mol % in TM), was almost equal in the two monolayers. Similar results were found in lettuce TM (not shown). These results indicate that the essential role of inner PG population(s) in sustaining electron flow activity (Siegenthaler et al., 1989) does not lay on particular PG molecular species but rather on specific interactions with certain inner proteins or protein domains. Interestingly, it was found that upon PG and PC depletion in the outer monolayer (PLA_2, 2°C, 1h), the two major polypeptides, characterizing the LHCII complex in SDS-gels, displayed smaller Mr (of about 2 kDa), at the expense of their respective parent polypeptide (not shown). This suggests that outer molecular species may preserve the integrity of the LHCII complex (maybe from an endogenous TM peptidase).

Role of phospholipids in protein phosphorylation in CEM

When CEM were incubated in vitro with [γ-^{32}P]ATP, the autoradiogram pattern of the SDS-gel (A') revealed the presence of several labelled polypeptides, three of them being major ones (67, 26 and 14 kDa). The PLC treatment prior to the addition of [γ-^{32}P]ATP

Table 1. Relative proportion of PG molecular species in outer and inner monolayers of spinach thylakoid membranes

PG Molecular species	mol % of Total PG[2]	Inner PG[3]	Outer PG[4]
18:3 / 16:1(3t)	83.4	79.0	85.0
18:3 / 16:0	8.2	9.2	7.8
16:0 / 16:1(3t)	4.7	6.0	4.2
Other[1]	3.7	5.8	3.0

[1] 18:2/16:1(3t); 14:0/16:1(3t); 18:2/16:0; 18:1/16:1(3t); 18:1/16:0; 16:0/16:0; 18:0/16:1(3t). [2] Outer/inner PG molar ratio : 73/27. [3] Intact TM were treated by PLA_2 at 2°C for 1 h, then the hydrolysis products (free fatty acids and lyso-PG) were removed by a treatment with BSA (Siegenthaler et al., 1989). [4] Outer PG molecular species were calculated by difference between total and inner values.

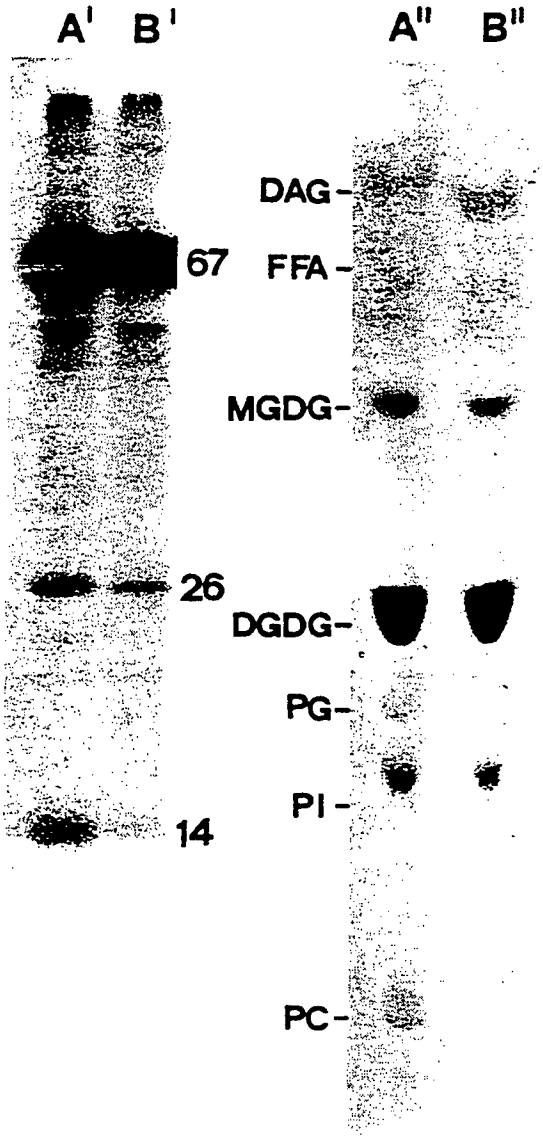

Figure 1. Role of PG and PC in protein phosphorylation in CEM : CEM were incubated for 20 min without (A',A") or with (B'B") PLC, then [γ-^{32}P]ATP was added. After 5 min, CEM proteins were separated by SDS-PAGE and stained by Coomassie blue (not shown), then the gels were autoradiographed (A',B'). Aliquots were taken for lipid separation by TLC (A",B"). Mr are indicated in kDa.

did not alter the Coomassie blue polypeptide pattern (not shown) but affected significantly, but to different extents [Fig.1,A'B'], the incorporation of ^{32}P into the three major proteins (61% inhibition : 67 kDa; 50% : 14 kDa; 29% : 26 kDa protein). Simultaneously, PLC caused a complete hydrolysis of PG and PC with a concomittant increase in diacylglycerol (DAG). The level of all other acyl lipids remained constant [Fig.1,A",B"]. These results indicate that the phosphorylation of the 67, 26 and 14 kDa proteins is partially dependent on PG and PC; however, this does not exclude that other acyl lipids may be involved in this process.

In conclusion, it appears that, depending on the activities or structural parameters considered, distinct classes or populations of acyl lipids are involved in TM and CEM functions.

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Low temperatures induce an increase in both 18:3 and 16:1(3*t*) fatty acids in thylakoid membrane phosphatidylglycerol of developing squash cotyledons

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INTRODUCTION

Low-temperature injury can be decreased by a pretreatment of plants at low, non-injurious temperatures. Meanwhile an increase in the unsaturation degree of acyl lipids and in membrane fluidity is observed (Graham and Patterson, 1982). Therefore, it is considered that fatty acid desaturation during chilling acclimation is one of the factors involved in conferring low temperature tolerance (Kodama *et al.*, 1995) and that a high content of saturated fatty acids in membrane lipids is likely to be responsible for chilling injury (Murata *et al.*, 1982). However, in the mutant *fab1* of *Arabidopsis*, high levels of saturated phosphatidylglycerol (PG) do not induce chilling sensitivity (Wu and Browse, 1995). In order to explain these apparent contradictions, we have studied the effect of low temperatures on the PG fatty acid and molecular species composition in thylakoids. Our results will be discussed in terms of the possible mechanism(s) involved in the formation of 16:1(3*t*) and 18:3 at low temperature.

METHODS

Plants of squash (*Cucurbita moschata* Durch. cv Shirakikuza) were grown in controlled environment growth chambers. Seeds were germinated in darkness at 30°C, then the seedlings were grown for one day at the same temperature with a 12 h photoperiod (PFD: 100 μmol $m^{-2}s^{-1}$). At the end of the first light period, some plants were transferred to 18°, 14° and 10°C under the same light regime. Thylakoid membranes were immediately isolated from cotyledons collected after the desired daily light period and lipids, fatty acids and PG molecular species were determined (Xu and Siegenthaler, 1996).

RESULTS

The major fatty acids of thylakoid membrane PG of squash cotyledons are 16:0, 16:1(3*t*), 18:0, 18:1, 18:2 and 18:3 (Xu and Siegenthaler, 1996). Transferring plants to low temperatures resulted in no change in the C_{16}/C_{18} molar ratio. However, within the C_{16} fatty acid series, the content of 16:1(3*t*) increased at the expense of 16:0. Similarly, the level of 18:3 increased at the expense of the other C_{18} fatty acids, especially 18:1. The extent of the increase in 16:1(3*t*) and 18:3 levels was lower when the temperature of the transfer was lowered. All lipids derived from the prokaryotic pathway contain only 18:1 and 16:0 fatty acids when they are synthesized in the chloroplast envelope. In PG, the 16:0 present at the *sn*-2 position is converted to 16:1(3*t*) whilst the 18:1 at the *sn*-1 position is desaturated stepwise, first to 18:2, then to 18:3 in the chloroplast (Browse and Somerville, 1991). Therefore, we propose that the formation of highly unsaturated chloroplast lipids at low temperature(s) is likely to be due to faster fatty acid desaturation compared to fatty acid synthesis reactions.

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EFFECT OF PHOSPHOLIPASE A_2 ON THE RATE AND EXTENT OF PHOSPHATIDYLGLYCEROL MOLECULAR SPECIES HYDROLYSIS IN SPINACH AND SQUASH THYLAKOIDS

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The thylacoid membrane contains 10 molecular species of phosphatidylglycerol. The relative amount of these species depends on the plant species and growth conditions. Using phospholipase A_2 at 0°C to digest preferentially phosphatidylglycerol in the outer monolayer of spinach thylacoid membranes, we have shown previously that this monolayer was enriched in phosphatidylglycerol (ca 70%). The purpose of this investigation was to determine the transmembrane distribution of each phosphatidylglycerol molecular species. To this aim, we have studid the hydrolysis kinetics of each species in the presence of phospholipase A_2 in spinach and squash thylacoid membranes containing different relative proportion of molecular species. The hydrolysis rate and extent of the molecular species depended on the unsaturation degree of the fatty acid at the *sn*-1 position as well as on the presence or absence of trans-3-hexadecenoic acid [16:1(3*t*)] at the *sn*-2 position. For instance, the hydrolysis rate of 18:3/16:1(3*t*) was greater than that 16:0/16:0.

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RELATIVE CONTENT OF PHOSPHATIDYLGLYCEROL MOLECULAR SPECIES IN THYLAKOIDS OF FOUR PLANTS SPECIES DURING GROWTH UNDER VARIOUS TEMPERATURES

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The aim of this study was to determine the relative content of PG molecular species of thylakoids from four plant species which were grown under various temperature conditions. In thylakoids of squash, potato, lettuce and spinach, two groups of five PG molecular species were identified:

(1) 16:0/16:0, 18:0/16:0, 18:1/16:0, 18:2/16:0, 18:3/16:0

(2) 16:0/16:1(3*t*), 18:0/16:1(3*t*), 18:1/16:1(3*t*), 18:2/16:(3*t*), 18:3/16:1(3*t*)

Their relative proportions were different in each plant species.

When squash plants were grown under three temperature regimes (20, 30 and 35°C) and under continuous light (20'000 lux), the PG molecular species of group 2 increased at the expense of those of the group 1. On the other hand, low temperatures favored the formation of 18:3/16:0 and/or 18:3/16:1(3t) molecular species at the expense of 18:0/16:0 and/or 16:0/16:0. These results will be discussed in terms of the influence of growth temperature on the degree of unsaturation in these different PG molecular species. (Supported by the SNSF 3100-33693.92).

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INFLUENCE OF LIGHT AND TEMPERATURE ON THE FATTY ACID COMPOSITION OF PHOSPHATIDYLGLYCEROL IN SQUASH COTYLEDONS

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Phosphatidylglycerol (PG) appears to play several roles in the thylakoid membrane, among which its involvement in the mechanism of chilling sensitivity of plants is most important. Here, we study the influence of light and temperature on the fatty acid composition of PG in cotyledons of squash, a chilling-sensitive plant. We show that during growth, the sums (mol%) [16:0 + 16:1(3*t*)] and (18:0 + 18:1 + 18:2 + 18:3) in PG remained constant. Whereas the proportion between the fatty acids of the C_{18} series did not change during growth, 16:1(3*t*) increased at the expense of 16:0. The rate of this change depended on the plant growth rate, on the day length and on light intensity. However, lowering the temperature increased the unsaturation of the C_{18} series fatty acids. We conclude that in a given plant variety, the constant level of the C_{16} and C_{18} series fatty acids in PG is controlled genetically. In contrast, the changes occurring in the C_{18} series fatty acids depend on the temperature and those in the C_{16} series fatty acids on the light. These results are discussed in terms of the pathways of fatty acid synthesis.

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