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Effect of Cold Acclimation on the Lipid Composition of the Inner and Outer Membrane of the Chloroplast Envelope Isolated from Rye Leaves[1]

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The lipid composition of the inner and outer membranes of the chloroplast envelope isolated from winter rye (*Secale cereale* L. cv Puma) leaves was characterized before and after cold acclimation. In nonacclimated leaves the inner membrane contained high proportions of monogalactosyldiacylglycerols (MGDG, 47.9 mol% of the total lipids) and digalactosyldiacylglycerols (DGDG, 31.1 mol%) and a low proportion of phosphatidylcholine (PC, 8.1 mol%). The outer membrane contained a similar proportion of DGDG (30.0 mol%); however, the proportion of MGDG was much lower (20.1 mol%) and the proportion of PC was much higher (31.5 mol%). After 4 weeks of cold acclimation, the proportions of these lipid classes were significantly altered in both of the inner and outer membranes. In the inner membrane the proportion of MGDG decreased (from 47.9 to 38.4 mol%) and the proportion of DGDG increased (from 31.1 to 39.3 mol%), with only a slight change in the proportion of PC (from 8.1 to 8.8 mol%). In the outer membrane MGDG decreased from 20.1 to 14.8 mol%, DGDG increased from 30.0 to 39.9 mol%, and PC decreased from 31.5 to 25.4 mol%. Thus, both before and after cold acclimation, the proportion of MGDG was much higher in the inner membrane than in the outer membrane. In contrast, the proportion of PC was higher in the outer membrane than in the inner membrane. The relationship between the lipid composition of the inner and outer membranes of the chloroplast envelope and freeze-induced membrane lesions is discussed.

Membrane destabilization resulting from freeze-induced dehydration is the primary cause of freezing injury in herbaceous plants such as winter cereals (Steponkus, 1984). In nonacclimated leaves and protoplasts injury at or below the temperature at which 50% of cells are lethally injured is a consequence of freeze-induced formation of the H_{II} phase in regions where the plasma membrane is brought into close apposition with various endomembranes (Gordon-Kamm and Steponkus, 1984). After 1 week of cold acclimation, freeze-induced formation of the H_{II} phase is precluded, and injury is associated with the fracture-jump lesion, which in freeze-fracture electron micrographs is characterized by localized deviations of the fracture plane in regions of the plasma membrane that are in close apposition to subtending lamellae (Fujikawa and Steponkus, 1990; Steponkus et al., 1993). During weeks 2 to 4 of cold acclimation of winter rye (*Secale cereale* cv Puma) (Fujikawa and Steponkus, 1990) and spring oat (*Avena sativa* cv Ogle) (Webb et al., 1994) the threshold temperature of the fracture-jump lesion decreased to lower temperatures. Because both freeze-induced formation of the H_{II} phase and the fracture-jump lesion are manifestations of the lyotropic phase behavior of the membrane lipids, it is expected that alterations in membrane lipid composition during cold acclimation influence the occurrence of these two freeze-induced lesions.

Previously, we demonstrated that the change in the incidence of the freeze-induced formation of the H_{II} phase is associated with alterations in the lipid composition of the plasma membrane that occur during cold acclimation (for review, see Steponkus et al., 1990, 1993). For example, severe dehydration resulted in the formation of the H_{II} phase in liposomes prepared from the total lipid extract of the plasma membrane isolated from nonacclimated rye leaves, but not in liposomes prepared from the total lipid extract of the plasma membrane isolated from cold-acclimated leaves (Cudd and Steponkus, 1988). Direct evidence for a causal role of specific alterations of the lipid composition in the incidence of freeze-induced formation of the H_{II} phase has come from membrane engineering studies, in which the participation of the plasma membrane in the freeze-induced formation of the H_{II} phase is precluded by artificial enrichment of the plasma membrane with di-unsaturated species of PC (Sugawara and Steponkus, 1990). In fact, the proportion of the di-unsaturated species of PC in the plasma membrane increased during cold acclimation (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994).

Although the involvement of alterations in the lipid composition of the plasma membrane in the occurrence of the fracture-jump lesion remains to be determined, there is an apparent relationship between the ratio of phospholipids to cerebrosides in the plasma membrane and the tempera-

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Abbreviations: DAG, diacylglycerol(s); DGDG, digalactosyldiacylglycerol(s); H_{II} phase, hexagonal II phase; ILA, interlamellar attachments(s); IMI, inverted micellar intermediate(s); MGDG, monogalactosyldiacylglycerol(s); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SQDG, sulfoquinovosyldiacylglycerol(s); TGDG, trigalactosyldiacylglycerol(s); TTGDG, tetragalactosyldiacylglycerol(s).

ture range over which the fracture-jump lesion occurs (Steponkus et al., 1993; Uemura and Steponkus, 1994). This is well documented with winter rye and spring oat, two cereals that differ widely in their freezing tolerance. In rye the ratio of phospholipids to cerebrosides increases continuously (from 2.23 to 4.12) during 4 weeks of cold acclimation as a result of an increase in the proportion of phospholipids and a decrease in the proportion of cerebrosides. In contrast, in oat the ratio increases after 1 week of cold acclimation (from 1.05 to 1.39) but remains relatively constant, with a lower value (1.52) than in rye, after 4 weeks of cold acclimation. These differences are associated with the observations that during cold acclimation there is a much larger decrease in the threshold temperature for the fracture-jump lesion in rye than in oat (Webb et al., 1994).

Both freeze-induced formation of the H_{II} phase and the fracture-jump lesion are interbilayer events and are most frequently observed in regions where the plasma membrane is brought into close apposition with the chloroplast envelope during freeze-induced cell dehydration (Webb et al., 1994; Uemura et al., 1995). Therefore, the plasma membrane is only one of the participating membranes, and it is very likely that the lipid composition of the chloroplast envelope, like that of the plasma membrane, also changes during cold acclimation. Thus, the altered incidence of both the freeze-induced formation of the H_{II} phase and the fracture-jump lesion are likely to be influenced by changes in the lipid composition of both the plasma membrane and the chloroplast envelope. The objective of this study was to characterize the lipid composition of the inner and outer membranes of the chloroplast envelope of rye leaves before and after cold acclimation. The results are discussed with reference to the relationship between membrane lipid composition and the occurrence of freeze-induced membrane lesions.

MATERIALS AND METHODS

Seeds of winter rye (*Secale cereale* L. cv Puma) were germinated on moist vermiculite and grown in a controlled environment chamber at 20°C/15°C day/night temperatures (16-h photoperiod; light intensity, 150 μE m^{-2} s^{-1} at soil level). Plants were irrigated with Hoagland nutrient solution three times a week. Nonacclimated plants were kept in this environment for 10 to 14 d. Cold acclimation was achieved by transferring 7-d-old plants to 13°C/7°C day/night temperatures (11.5-h photoperiod; light intensity, 150 μE m^{-2} s^{-1} at soil level) for 1 week and then to 2°C (8-h photoperiod; light intensity, 125 μE m^{-2} s^{-1} at soil level) for 4 weeks.

Isolation of Intact Chloroplasts

Purification

To obtain fractions enriched in the chloroplast envelope, intact chloroplasts were first isolated from rye leaves according to the method of Douce and Joyard (1980), with slight modifications. All procedures were performed at 0 to 4°C. Rye leaves were cut into small pieces, suspended in an isotonic homogenization medium (5 mL/g fresh weight leaves) that consisted of 50 mM Tricine/NaOH (pH 7.8), 2 mM EDTA, 0.1% (w/v) BSA, and either 0.43 M sorbitol for nonacclimated leaves or 0.83 M sorbitol for cold-acclimated leaves, and then homogenized with a polytron (Brinkman) at a medium speed for 10 s. The homogenates were filtered through four layers of cheesecloth and were then centrifuged at 1,500*g* for 5 min. The pellets containing the intact chloroplasts were suspended in the homogenization medium. Aliquots of the crude chloroplast suspension (12 mL) were layered on top of a discontinuous Percoll gradient (18 mL of 40% [v/v] and 6 mL of 70% [v/v] Percoll for nonacclimated leaves, or 18 mL of 35% [v/v] and 6 mL of 65% [v/v] Percoll for cold-acclimated leaves). All Percoll solutions contained 50 mM Tricine/NaOH (pH 7.8), 2 mM EDTA, 0.1% (w/v) BSA, and sorbitol (0.43 M for nonacclimated leaves and 0.83 M for cold-acclimated leaves). After the Percoll gradients were centrifuged at 10,000*g* for 10 min, the intact chloroplasts were collected at either the 40%/70% interface for nonacclimated leaves or the 35%/65% interface for cold-acclimated leaves. The chloroplast fraction was then diluted with a washing medium comprised of 10 mM Tricine/NaOH (pH 7.8), 4 mM $MgCl_2$, and sorbitol at a concentration of either 0.43 M for nonacclimated leaves or 0.83 M for cold-acclimated leaves and centrifuged at 3,300*g* for 5 min.

Purity Checks

Purity of the intact chloroplast fraction was determined by assaying for the presence of marker enzymes associated with other membranes according to the methods of Uemura and Yoshida (1983) and Yoshida et al. (1986). The marker enzymes determined in this study were Cyt *c* oxidase for mitochondria, NADH Cyt *c* reductase for the ER, Triton X-100-stimulated UDPase for the Golgi body, nitrate-sensitive ATPase for tonoplasts, and vanadate-sensitive ATPase for the plasma membrane. Chlorophyll was quantified after extraction with 80% (v/v) acetone, according to the method of Arnon (1949). Protein content was determined by a dye-protein binding method using BSA as a standard (Bradford, 1976).

Thermolysin Treatment of Intact Chloroplasts

The purified, intact chloroplasts of nonacclimated leaves were treated with thermolysin according to the method of Joyard et al. (1987). The intact chloroplasts (1 mg chlorophyll/mL) were incubated for 1 h at 4°C in a solution containing 0.43 M sorbitol, 10 mM Tricine/NaOH (pH 7.8), 1 mM $CaCl_2$, and 200 μg/mL thermolysin (Calbiochem) with gentle shaking. The digestion was stopped by the addition of EGTA at a final concentration of 10 mM and the suspension was then incubated for an additional 5 min. Control experiments were performed under the same conditions, except that EGTA was added to the incubation mixture when thermolysin was added to the mixture. After digestion, chloroplast suspensions were loaded onto 40% (v/v) Percoll containing 0.43 M sorbitol and 10 mM Tricine/NaOH (pH 7.8) and protease inhibitors (5 mM ϵ-aminocaproic acid,

1 mM benzamidine-HCl, and 1 mM PMSF) and then centrifuged at 10,000*g* for 10 min. The repurified chloroplasts were recovered as a pellet, resuspended in a medium containing 0.43 M sorbitol, 10 mM Tricine/NaOH (pH 7.8), and protease inhibitors, and centrifuged at 3,300*g* for 5 min. After washing twice more by resuspension and centrifugation, as above, the intact chloroplasts were used for the isolation of the chloroplast envelope, as described below.

Isolation of the Chloroplast Envelope

Purification of the Chloroplast Envelope

The chloroplast envelope fraction (a mixture containing the inner and outer membranes of the chloroplast envelope) was isolated from the intact chloroplasts after thermolysin treatment according to the method of Douce and Joyard (1980), with slight modifications. The intact chloroplasts were lysed in a hypotonic medium (10 mM Tricine/NaOH [pH 7.8] and protease inhibitors) for 30 min at 0°C, and the lysate (16 mL) was then loaded onto to a discontinuous Suc density gradient (10 mL of 0.6 M, 6 mL of 0.9 M, and 4 mL of 1.2 M Suc). All Suc solutions contained 10 mM Tricine/NaOH (pH 7.8) and protease inhibitors. After the Suc density gradients were centrifuged at 72,000*g* for 3 h, the chloroplast envelope membranes were recovered at the 0.6/0.9 M interface, diluted with the hypotonic medium, and centrifuged at 156,000*g* for 60 min.

Separation of the Inner and Outer Membranes of the Chloroplast Envelope

The inner and outer membranes of the chloroplast envelope were separated and purified from the intact chloroplasts according to the method of Block et al. (1983a). The intact chloroplasts that were purified by the Percoll gradient centrifugation were suspended in a hypertonic sorbitol solution (0.8 M for nonacclimated leaves and 1.3 M for cold-acclimated leaves) containing 10 mM Tricine/NaOH (pH 7.8) and 4 mM $MgCl_2$ and kept on ice for 10 min. After hypertonic treatment, which resulted in the contraction of chloroplasts and the separation of the inner and outer membranes of the chloroplast envelope, the intact chloroplast suspensions were passed through a Yeda press (Linca Scientific Instruments, Tel Aviv, Israel) at a pressure of 60 psi. After passage through the Yeda press, the tonicity of the suspensions was adjusted to 0.4 M sorbitol (0.43 osmolality) by the addition of a solution containing 10 mM Tricine/NaOH (pH 7.8) and 4 mM $MgCl_2$. Aliquots of the suspensions (12 mL) were then layered onto a discontinuous Suc gradient (8 mL each of 0.4, 0.65, and 1.0 M Suc containing 10 mM Tricine/NaOH [pH 7.8] and 4 mM $MgCl_2$) and centrifuged at 90,000*g* for 90 min. The outer and inner membranes of the chloroplast envelope banded at the 0.4/0.65 M and 0.65/1.0 M interfaces, respectively. These two fractions were collected, diluted with a solution of 10 mM Tricine/NaOH (pH 7.8) and 4 mM $MgCl_2$, and centrifuged at 156,000*g* for 60 min. The pellet, which contained either the inner or outer membrane of the chloroplast envelope, was suspended in the same medium and used immediately for lipid extraction.

Purity Checks

To determine the presence of thylakoid membranes in the purified chloroplast envelope fractions, chlorophyll was quantified according to the method of Arnon (1949). Protein content was determined by the method of Bradford (1976). The cross-contamination between the inner and outer membranes of the chloroplast envelope was not determined in the present study; the rationale for this is presented in "Discussion."

Lipid Analysis

Lipids were extracted from the purified chloroplast envelope membranes according to the procedure of Bligh and Dyer (1959). After separation of the neutral lipid, glycolipid, and phospholipid fractions with a silica Sep-Pak cartridge according to the method of Lynch and Steponkus (1987), each lipid fraction was subjected to TLC according to the method of Uemura and Steponkus (1994). The Gal content in galactolipids such as MGDG, DGDG, and SQDG was quantified according to the method of Dubois et al. (1956), with Gal as a standard. Sterol lipids and phospholipids were quantified as previously described (Uemura and Steponkus, 1994). DAG was quantified by the method of Snyder and Stephens (1958) with 1,2-dioleoylglycerol as a standard. The results shown are averages ± SD of two different determinations. For each determination, several different isolations of the chloroplast envelope were combined before lipid extraction to obtain a sufficient amount of lipids for quantitative determinations of each lipid class.

RESULTS

The chloroplast envelope membrane fraction that was isolated from rye leaves was of a very high purity. In the purified intact chloroplast fraction from which the chloroplast envelope was subsequently isolated, the activity of the marker enzymes associated with various membranes, such as Cyt *c* oxidase for mitochondria, NADH Cyt *c* reductase for the ER, and Triton X-100-stimulated UDPase for the Golgi body, was undetectable (Table I). Furthermore, the activity of ATPase was not inhibited by the addition of vanadate (an inhibitor for the plasma membrane-associated ATPase) or nitrate (an inhibitor for the tonoplast-associated ATPase). These results indicate that the isolated chloroplast envelope fraction was devoid of these membranes. In addition, the isolated chloroplast envelope, either the mixture containing the inner and outer membranes or the separated inner and outer membrane fractions, contained no detectable amounts of chlorophyll (Table II), indicating that there was no contamination by thylakoid membranes.

Although Douce et al. (1984) reported that intact chloroplasts isolated from spinach leaves must be first treated with a protease, thermolysin, to inactivate the galactolipid: galactolipid galactosyltransferase to determine the true

Table I. *Marker enzyme activities of intact chloroplast fraction isolated from nonacclimated rye leaves*

The intact chloroplast fraction of nonacclimated rye leaves was purified by a discontinuous Percoll density gradient centrifugation as described in "Materials and Methods." The activity of enzymes associated with various cellular membranes was assayed. The concentrations of vanadate as Na_3VO_4 and nitrate as KNO_3 when added in the ATPase assay are 100 μM and 50 mM, respectively.

Intact Chloroplast Fraction	Total Activity	Specific Activity
	μmol substrate/50 g fresh wt/h	*μmol substrate/mg protein/h*
Enzyme		
Cyt *c* oxidase	Not detected	Not detected
NADH Cyt *c* reductase	Not detected	Not detected
Triton X-100-stimulated UDPase	Not detected	Not detected
ATPase	4.589	0.095
+ Vanadate	4.385	0.091
+ Nitrate	4.987	0.103
	mg/50 g fresh wt	
Protein	48.37	
Chlorophyll	2.45	

lipid composition of the chloroplast envelope, there was no effect of thermolysin treatment on the lipid composition of the chloroplast envelope of rye leaves (Table III). There was very little difference in the proportion of each galactolipid and phospholipid class in the chloroplast envelope membranes isolated from the intact chloroplasts either treated or not treated with thermolysin. In addition, DAG and unnatural galactolipids such as TGDG or TTGDG, which are the products of the galactolipid:galactolipid galactosyltransferase, were not detected in the chloroplast envelope membranes isolated from chloroplasts without the thermolysin treatment. These results indicate that, unlike the results of Douce et al. (1984) with spinach, it is not necessary to treat chloroplasts of rye leaves with thermolysin to obtain the true lipid composition of the chloroplast envelope. Therefore, the thermolysin treatment was not used in the other experiments.

Previous reports have established that there is a substantial difference in the lipid composition of the inner and outer membranes of the chloroplast envelope of spinach (Block et al., 1983b) and pea (Cline et al., 1981). This large difference in the lipid composition also occurs in the chloroplast envelope of rye (Table IV). The inner membrane of the chloroplast envelope of rye that was grown under nonacclimating conditions (20°C/15°C day/night, 16-h photoperiod) contained high proportions of MGDG (47.9 mol% of the total lipids) and DGDG (31.3 mol%) and low proportions of PC (8.1 mol%), SQDG (6.6 mol%), and PG (4.0 mol%). The proportions of other phospholipids such as PI, PS, PE, and PA were approximately 1 mol% or less. As a result, the proportions of total galactolipids and phospholipids in the inner membrane were 85.8 and 14.3 mol%, respectively. In contrast, although the outer membrane contained a similar proportion of DGDG (30.0 mol%), the proportion of MGDG was much lower (20.1 mol%) and the proportion of PC was much higher (31.5 mol%) than that in the inner membrane. The proportions of SQDG, PE, PG, and PA in the outer membrane were similar to those in the inner membrane; however, the proportion of PS plus PI was higher in the outer membrane (4.5 mol%) than in the inner membrane (0.2 mol%). Consequently, the proportion of total galactolipids in the outer membrane (56.9 mol%) was significantly lower than that in the inner membrane (85.8 mol%), and the proportion of total phospholipids in

Table II. *Distribution of protein and chlorophyll in the chloroplast envelope fraction (a mixture of the inner and outer membranes) and the separated inner and outer membrane fractions isolated from nonacclimated rye leaves*

The chloroplast envelope fraction of nonacclimated rye leaves was isolated by hypotonic treatment of the intact chloroplasts; the inner and outer membrane fractions of the chloroplast envelope were isolated by a hypertonic treatment of the intact chloroplasts followed by passage through a Yeda press (see "Materials and Methods").

Fractionation Procedure	Chlorophyll		Protein	
	mg	*%*	*mg*	*%*
Experiment 1				
Intact chloroplasts	10.5	100.0	212.5	100.0
Chloroplast envelope fraction (a mixture containing the inner and outer membranes)	Not detected		0.223	0.10
Experiment 2				
Intact chloroplasts	12.2	100.0	256.2	100.0
Inner membrane fraction	Not detected		0.081	0.03
Outer membrane fraction	Not detected		0.048	0.02

Table III. *Effect of thermolysin treatment on the lipid composition of chloroplast envelope isolated from intact chloroplasts of nonacclimated rye leaves*

The chloroplast envelope fraction of nonacclimated rye leaves, a mixture containing the inner and outer membranes, was isolated by hypotonic treatment of the intact chloroplasts after treatment with thermolysin, as described in "Materials and Methods." The results shown are the average ± SD of two determinations.

Lipid	Nontreated	Thermolysin-Treated
	mol% of total lipids	
MGDG	35.5 ± 1.0	35.8 ± 0.6
DGDG	30.8 ± 1.0	30.7 ± 0.8
SQDG	6.3 ± 0.2	6.9 ± 0.3
TGDG	Not detected	Not detected
TTGDG	Not detected	Not detected
Total	[72.6]	[73.4]
PC	19.1 ± 1.1	18.7 ± 0.4
PE	0.8 ± 0.1	1.2 ± 0.2
PG	4.5 ± 0.4	4.0 ± 0.2
PI+PS	2.2 ± 0.1	2.3 ± 0.2
PA	0.9 ± 0.4	0.7 ± 0.1
Total	[27.5]	[26.9]
DAG	Not detected	Not detected

the outer membrane (43.2 mol%) was much higher than in the inner membrane (14.3 mol%).

After 4 weeks of cold acclimation (2°C constant, 8-h photoperiod), the lipid compositions of both the inner and outer membranes of the chloroplast envelope of rye leaves were significantly altered (Table IV). In the inner membrane, the proportion of MGDG decreased from 47.9 to 38.4 mol% and the proportion of DGDG increased from 31.3 to 39.3 mol%, with only a slight change in the proportion of PC (from 8.1 to 8.8 mol%). As with the inner membrane, the proportion of MGDG in the outer membrane decreased from 20.1 to 14.8 mol% and the proportion of DGDG increased from 30.0 to 39.9 mol%; however, the proportion of PC decreased (from 31.5 to 25.4 mol%). In both the inner and outer membranes, there were only small changes in the proportions of other lipids such as SQDG, PE, PG, PI plus PS and PA during cold acclimation.

DISCUSSION

Lipid Composition of Chloroplast Envelope of Rye

The lipid composition of the inner and outer membranes of the chloroplast envelope isolated from rye leaves has characteristics in common with the chloroplast envelope isolated from spinach and pea leaves. In spinach (Block et al., 1983b) and pea (Cline et al., 1981), the inner membrane contains a much higher proportion of MGDG and a lower proportion of PC than does the outer membrane; these large differences are also observed in the envelope of rye chloroplasts (Table IV). In all three species (spinach, pea, and rye), there is only a small difference in the proportion of DGDG in the inner and outer membranes. Furthermore, the results of the present study are consistent with previous studies of spinach and pea in that, in the chloroplast envelope, there are neither sterol lipids (such as free sterols, sterol esters, sterylglucosides, and acylated sterylglucosides), nor cerebrosides, both of which are major constituents of the plasma membrane and tonoplast (Yoshida and Uemura, 1986; Larsson et al., 1990; Uemura and Steponkus, 1994; Uemura et al., 1995). Collectively, the results indicate that the lipid composition of both the inner and outer membranes of the chloroplast envelope is conserved among the three diverse plant species—even between dicotyledonous (pea and spinach) and monocotyledonous (rye) species.

The lipid composition of the chloroplast envelope of rye leaves was not influenced by treatment of the intact chloroplasts with thermolysin (Table III). This result is quite different from the result with spinach leaves reported by Douce and colleagues (1984). They reported that it is necessary to treat the intact chloroplasts with thermolysin to inactivate the galactolipid:galactolipid galactosyltransferase to determine the true lipid composition of the enve-

Table IV. *Lipid composition of the inner and outer membrane of the chloroplast envelope isolated from nonacclimated and cold-acclimated rye leaves*

The inner and outer membrane fractions of the chloroplast envelope of rye leaves were isolated by a hypertonic treatment of the intact chloroplasts followed by passage through a Yeda press, as described in "Materials and Methods." The results shown are the average ± SD of two determinations.

Lipid	Inner Membrane		Outer Membrane	
	Nonacclimated	Cold-acclimated	Nonacclimated	Cold-acclimated
	mol% of total lipids			
MGDG	47.9 ± 0.4	38.4 ± 1.0	20.1 ± 0.3	14.8 ± 1.5
DGDG	31.3 ± 1.3	39.3 ± 2.0	30.0 ± 1.6	39.9 ± 2.5
SQDG	6.6 ± 0.8	5.9 ± 0.4	6.8 ± 0.3	6.9 ± 0.5
Total	[85.8]	[83.6]	[56.9]	[61.6]
PC	8.1 ± 0.3	8.8 ± 0.3	31.5 ± 1.8	25.4 ± 1.1
PE	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
PG	4.0 ± 0.0	5.4 ± 0.8	5.0 ± 0.3	5.6 ± 0.6
PI+PS	0.7 ± 0.2	0.7 ± 0.0	4.5 ± 0.2	4.9 ± 0.1
PA	1.3 ± 0.3	1.4 ± 0.1	1.5 ± 0.4	1.8 ± 0.2
Total	[14.3]	[16.6]	[43.2]	[38.3]

lope membranes. Galactolipid:galactolipid galactosyltransferase mediates the interlipid exchange of Gal of MGDG to form DAG, DGDG, and unnatural galactolipids such as TGDG and TTGDG during purification of the chloroplast envelope. This results in the underestimation of the amount of MGDG and the overestimation of the amount of DGDG. However, in the present study, there was very little effect of the thermolysin treatment on the lipid composition of the chloroplast envelope of rye. The reason for the difference between spinach and rye is not known; nevertheless, these results indicate that it is not necessary to treat the chloroplasts of rye leaves with thermolysin to determine the true lipid composition of the chloroplast envelope.

Although the inner and outer membrane fractions of the chloroplast envelope isolated from rye leaves in the present study are devoid of contamination by other membranes (Tables I and II), it is likely that there is a cross-contamination between the inner and outer membrane fractions. Cross-contamination between the inner and outer membranes of the chloroplast envelope has been reported in spinach and pea (Block et al., 1983a; Cline and Keegstra, 1983; Miquel and Dubacq, 1992). This is a consequence of contact sites between the inner and outer membranes of the chloroplast envelope that are present in situ (Cline et al., 1985), and which are also observed in the isolated chloroplast envelope fractions (Cline et al., 1985; Miquel and Dubacq, 1992). Immunochemical studies of the inner and outer membranes of the chloroplast envelope isolated from spinach leaves using the same method as used in the present study, with antibodies raised against polypeptides localized in either of these two membranes, have shown that on a protein basis, the outer membrane fraction contains approximately 10% of inner membrane proteins, whereas the inner membrane fraction contains approximately 20% of outer membrane proteins (Block et al., 1983a). With the chloroplast envelope isolated from pea leaves using a freeze/thaw method, densitometric analysis of polypeptides after SDS-PAGE revealed that the outer membrane fraction contains <5% of inner membrane proteins and the inner membrane fraction contains approximately 10% of outer membrane proteins (Cline and Keegstra, 1983; Miquel and Dubacq, 1992). Thus, cross-contamination between the inner and outer membrane fractions of the chloroplast envelope appears to be unavoidable regardless of the isolation procedure. Therefore, we did not determine the cross-contamination between the two membrane fractions in this study, and it is expected that the inner and outer membranes of the chloroplast envelope isolated from rye leaves in the present study have a purity similar to that reported in the literature, i.e. 80 to 90% purity of the inner membrane fraction and 90 to 95% purity of the outer membrane fraction.

Effect of Cold Acclimation on Lipid Composition and Cryostability of Chloroplast Envelope Membranes

The difference in the lipid composition of the inner and outer membranes of the chloroplast envelope is of special interest when considering freeze-induced formation of the H_{II} phase, which is the predominant freeze-induced lesion that occurs in leaves and protoplasts of nonacclimated seedlings at or below the temperature at which 50% of cells are lethally injured (Gordon-Kamm and Steponkus, 1984; Webb and Steponkus, 1993). In freeze-fracture electron micrographs, the H_{II} phase is most frequently observed in regions where the plasma membrane is brought into close apposition with the chloroplast envelope as a result of freeze-induced cell dehydration (Webb et al., 1994). Of the lipids that comprise the chloroplast envelope, MGDG has a high propensity to form the H_{II} phase, whereas DGDG and PC have a low propensity to form the H_{II} phase and would stabilize the bilayer (lamellar) configuration (Quinn and Williams, 1983; Gruner et al., 1985). In the chloroplast envelope isolated from nonacclimated rye leaves, the ratio of MGDG (nonbilayer forming lipid) to DGDG plus PC (bilayer-forming lipids) was substantially greater in the inner membrane (1.22) than in the outer membrane (0.33). This suggests that the inner membrane has a higher propensity to form the nonbilayer H_{II} phase than the outer membrane. In fact, a preliminary ^{31}P-NMR spectroscopy study of fully hydrated mixtures (20% [w/w] lipids) of MGDG:DGDG:SQDG:PC in proportions similar to those in the inner and outer membranes of rye chloroplast envelope showed different phase behavior (M. Uemura and P.L. Steponkus, unpublished results). The lipid mixture in proportions similar to the inner membrane (50:30:5:10 mol ratio) underwent a lamellar-to-nonlamellar phase transition between 15 and 20°C. In contrast, the lipid mixture in proportions similar to the outer membrane (20:30:5:30 mol ratio) remained in the lamellar phase over the range of 0 to 70°C. Because the lamellar-to-nonlamellar phase transition temperature of lipids decreases with decreasing hydration, it is expected to occur at subzero temperatures after freeze-induced dehydration. Collectively, given that freeze-induced formation of the H_{II} phase is an interbilayer event and is most frequently observed to involve the plasma membrane and the chloroplast envelope, it is likely that the propensity of the inner membrane of the chloroplast envelope to form the H_{II} phase during freeze-induced dehydration will determine the freezing temperature (hydration) at which freeze-induced formation of the H_{II} phase will first occur.

After cold acclimation, freeze-induced formation of the H_{II} phase does not occur and the fracture-jump lesion is the predominant freeze-induced lesion at any injurious temperature (Fujikawa and Steponkus, 1990; Webb and Steponkus, 1994). We have previously proposed that the fracture-jump lesion is the result of the formation of ILA between the plasma membrane and endomembranes (primarily the chloroplast envelope) when they are brought into close apposition as a result of freeze-induced cell dehydration (Steponkus et al., 1993). Under conditions of severe dehydration, removal of water from the lipid headgroups results in an increased lateral packing pressure in the acyl domain relative to that in the head group region, which results in the development of a bending energy (Gruner 1989a, 1989b). At low water contents, the bending energy is minimized by the formation of the H_{II} phase.

However, freeze-induced formation of the H_{II} phase does not occur in cold-acclimated leaves; instead, injury is associated with the fracture-jump lesion. Theoretical studies by Siegel (1986a, 1986b, 1986c, 1987) provide a possible explanation for the difference in the manifestation of freezing injury. Siegel has suggested that there is a common structural intermediate in the formation of both the H_{II} phase and ILA: the IMI that forms between two closely apposed bilayers. Whether the IMI are converted to either the H_{II} phase or ILA is dependent on the intrinsic curvature of the constituent monolayers; monolayers with a high intrinsic curvature form the H_{II} phase, whereas those with a low intrinsic curvature form ILA.

With this working hypothesis, both the freeze-induced formation of the H_{II} phase and the fracture-jump lesion are associated with the formation of IMI between the plasma membrane and the chloroplast envelope; however, alterations in membrane lipid composition that alter the intrinsic curvature of the monolayers will determine whether the IMI are converted to the H_{II} phase (in nonacclimated tissues) or ILA (in cold-acclimated tissues). In the plasma membrane of rye, an increase in the proportion of PL and a decrease in the proportions of cerebrosides and free sterols during cold acclimation alter the desorption characteristics of the bilayers such that a greater amount of water is retained on the membrane surface at a given osmotic pressure, which is a direct function of the freezing temperature, and decrease the intrinsic curvature of the constituent monolayers. In the chloroplast envelope of rye, a decrease in the proportion of MGDG and an increase in the proportion of DGDG during cold acclimation would have a similar effect (i.e. an increase in the hydration of the inner and outer membranes of the chloroplast envelope and a decrease in the intrinsic curvature of the monolayers). Consequently, after cold acclimation a lower freezing temperature must be imposed to remove water from the membrane surface and to bring the chloroplast envelope into close apposition with the plasma membrane. The lower temperature, however, is apparently below the lamellar-to-H_{II} phase transition temperature because the H_{II} phase was not observed in cold-acclimated tissues at any injurious temperature. Although the extent of dehydration is sufficient to result in the formation of IMI between the plasma membrane and the chloroplast envelope, the decrease in the intrinsic curvature of the constituent monolayers leads to formation of ILA and subsequent fusion of the plasma membrane and the chloroplast envelope.

In summary, the results of the present study demonstrate that there are substantial differences in the lipid composition of the inner and outer membranes of the chloroplast envelope of rye leaves and that the inner membrane of the chloroplast envelope has a higher propensity to form the nonlamellar H_{II} phase than the outer membrane. During cold acclimation, the lipid composition of both the inner and outer membranes was altered such that the stability of the lamellar configuration of the membranes is expected to be increased. Thus, alterations in the lipid composition of both the chloroplast envelope and the plasma membrane during cold acclimation are associated with alterations in the incidence of freeze-induced formation of the H_{II} phase and the fracture-jump lesion and, hence, increase freezing tolerance.

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