Plant Physiol. (1985) 79, 756–759
0032-0889/85/79/0756/04/$01.00/0

# Effect of Frost Hardening on Lipid and Fatty Acid Composition of Chloroplast Thylakoid Membranes in Two Wheat Varieties of Contrasting Hardiness[1]

Received for publication March 8, 1985 and in revised form June 29, 1985

LÁSLÓ VIGH[2], IBI HORVÀTH[2], PHILIP R. VAN HASSELT, AND PIETER J. C. KUIPER*
*Department of Plant Physiology, University of Groningen, P. O. Box 14, 9750 AA Haren (Gn) The Netherlands*

## ABSTRACT

**Lipid and fatty acid composition of chloroplast thylakoid membranes was determined in two varieties of wheat (*Triticum aestivum* L.), the hardy *Miranovskaja* and the sensitive *Penjamo*. Plants were grown at room temperature or under frost hardening conditions (1.5°C). Changes in lipid and fatty acid composition of the isolated thylakoids could be related to the temperature dependence of light-stimulated proton uptake. Changes in the thylakoid phospholipids upon hardening of the two varieties did not show any direct relation with low temperature tolerance of light-dependent $H^+$ uptake; neither did changes in phospholipid fatty acid chain lengthening to 20 and 22 C-atoms in combination with increased desaturation up to 6 double bonds. Increased low temperature tolerance of light-induced $H^+$ uptake by hardening was correlated with the following glycolipid changes: maintained glycolipid level, a proportionally increased digalactosyl diglyceride fraction, a decrease in thylakoid monogalactosyl diglyceride, increased sulfolipid fatty acid chain lengthening (20 and 22 C-atoms), and increased sulfolipid desaturation (4–6 double bonds). We suggest that the above mentioned changes in glycolipids have adaptive value for low temperature tolerance of light-dependent proton uptake.**

Frost hardiness of plants depends on the physical structure of plant cell membranes: properties of the plasmalemma will determine to a very large extent death or survival of plant cells under freezing conditions (16). It seems likely that also in other organelles, such as chloroplasts, damage may occur during freezing (13). Chloroplasts may contribute to the freezing tolerance of the plasmamembrane by the provision of metabolic energy and material such as sugars and lipids. For these reasons a study of the role of chloroplasts in freezing tolerance of plants is appropriate (17).

Functioning of chloroplasts will at least partly be dependent upon the thylakoid lipid composition. Several intriguing questions remain to be answered. Upon hardening of the plants the thylakoid galactolipid levels (MGDG[3] and DGDG) decreased proportionally or remained unaffected in some species/varieties (frost-sensitive wheat variety [17]; pea and spinach [1]), whereas in the thylakoids of other species an increased DGDG/MGDG ratio was observed upon hardening (frost-resistant wheat variety, [17]; pine, [12]). Taking into account the different physical structures of DGDG and MGDG (14), analysis of a possible adaptive value of the observed changes for survival and functioning of chloroplasts under freezing conditions seems warranted.

Another physical factor in functioning of chloroplasts in hardened plants is the phase behavior of the chloroplast lipids. Of the chloroplast lipids, only phosphatidylglycerol exhibits phase transitions at room temperature (chilling-sensitive plants only; [11]). Changes in chloroplast lipid composition, induced by hardening the plants, may be accompanied by changes in phase transitions of chloroplast membranes and a possible adaptive value of such changes should be evaluated.

In this paper we report on the lipid and fatty acid composition of the thylakoid membranes of two wheat varieties of contrasting hardiness. The results are discussed with reference to data on the functioning of chloroplasts of the same varieties (17).

## MATERIALS AND METHODS

**Growth Conditions and Hardening Procedure.** Seeds of two wheat varieties (*Triticum aestivum* L.), the frost-sensitive *Penjamo* and the hardy variety *Miranovskaja*, sown in soil, were germinated at a day/night temperature regime of 20/12°C, 16 h d-length, for 6 d. The hardening program was subsequently started by lowering the temperature gradually during 3 weeks, followed by a continuous period of 4 weeks at 1.5°C, with the same light regime. After finishing the hardening, the leaves were harvested for thylakoid isolation.

For the isolation of thylakoids the procedure of Lineberger and Steponkus (7) was used. Lipids were extracted as described earlier (4) and separation was done by two-dimensional TLC chromatography (15). Fatty acid methyl esters were prepared by transesterification of the isolated lipids in methanol, containing 5% HCl at 80°C in sealed ampules for 1 h. Analysis of fatty acid methyl ester was carried out by GLC on a column with SP-2340 (Supelco) coating. Identification of extralong-chain fatty acids of 20 and 22 C-atoms was done by a combination of the hydrogenation procedure and interpretation of the observed retention times of the fatty acid in question. Two fatty acids had to be listed as unknown and await identification by GC-MS.

Determination of thylakoid lipid composition and fatty acid composition of each thylakoid lipid was done on preparations of leaves of plants from three control and hardening experiments. Lipid composition of the thylakoids of plants from the three experiments was very similar, showing 10% variation; in one

[1] Supported by a grant from the Netherlands Organization for the Advancement of Pure Research, Z. W. O. to L. Vigh.

[2] Present address: Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.

[3] Abbreviations: MGDG, monogalactosyl diglyceride; DGDG, diagalactosyl diglyceride; PC, phosphatidyl choline; PG, phosphatidyl glycerol; SL, sulfolipid.

experiment the values for phosphatidylglycerol were 20% lower than in the other two experiments. Fatty acid composition of specific lipids also was similar for preparations from plants of each growth experiment, with 15 to 20% variation. In the "Results" section a single growth and hardening experiment has been presented. Within a single growth experiment, variation in lipid composition and fatty acid composition of lipids did not exceed 10%.

In every experiment, low temperature tolerance of the light-dependent proton uptake of the thylakoid sample was measured as well as cold tolerance of the leaves (17).

## RESULTS

Light-dependent proton uptake of the thylakoids of both wheat varieties was determined. Hardening of the plants resulted in increaed low temperature tolerance of light-stimulated $H^+$ uptake in *Miranovskaja* but not in *Penjamo*. Actual data of these experiments are given in Vigh (17).

**Phospholipids.** The level of PC in the thylakoids increased after hardening of the frost sensitive *Penjamo* (Table I), in agreement with numerous observations in the literature. On the contrary, in the thylakoids of the hardy *Miranovskaja* the level of PC was decreased by the hardening treatment. Clearly, genetic differences for the level of PC in thylakoids are indicated for wheat. The increased level of PC in *Penjamo* was accompanied by changes in fatty acid composition: decreased 16:0 and 16:1, increased 18;0, decreased 18:1, 18:2, and 18:3 and a large increase in C20 and C22 polyunsaturated fatty acids, indicating stimulation of fatty acid chain lengthening in combination with increased desaturation of these extralong-chain fatty acids. The effect of hardening of *Miranovskaja* on fatty acid composition of thylakoid PC was quite similar: disappearance of 18:2 and 18:3 and replacement by C20 and C22 polyunsaturated fatty acids, again indicating chain lengthening. Part of the reduced levels of 18:2 and 18:3 may be due to transfer of these fatty acids to thylakoid glycolipids, especially DGDG.

Table I. *Phospholipid Composition (mol % of Polar Lipids) and Fatty Acid Composition of Phospholipids (weight %) from Thylakoids of Hardened and Unhardened Wheat Plants of Miranovskaja (Resistant) and Penjamo (Sensitive)*

Results from one out of three growth experiments are presented.

| Variety: | Miranovskaja | | Penjamo | |
|---|---|---|---|---|
| Growth Condition | Unhardened | Hardened | Unhardened | Hardened |
| PC | 5.5 | 4.0 | 4.5 | 7.5 |
| PG | 8.4 | 8.1 | 10.1 | 13.8 |
| | | Phosphatidyl choline | | |
| Fatty acid | | | | |
| 16:0 | 31.9 | 17.0 | 27.6 | 21.2 |
| 16:1 | 1.1 | 3.5 | 3.4 | tr[a] |
| 18:0 | 1.4 | 6.4 | 1.5 | 5.3 |
| 18:1 | 7.9 | 7.1 | 7.4 | tr |
| 18:2 | 28.7 | 8.5 | 21.4 | 3.5 |
| 18:3 | 20.3 | 4.2 | 27.8 | 4.2 |
| 20:2 | | | | 5.7 |
| 20:3 | 2.2 | 13.8 | 2.3 | 15.5 |
| U[b] | | | | 17.0 |
| 20:4 | 4.8 | 21.3 | 6.3 | 16.9 |
| 22:4 | 1.7 | 18.1 | 2.3 | 10.6 |
| | | Phosphatidyl glycerol | | |
| 16:0 | 13.2 | 14.2 | 9.7 | 20.3 |
| 16:1 (3-t) | 28.4 | 8.9 | 33.5 | 5.5 |
| 18:0 | 1.4 | 4.3 | 0.4 | 19.8 |
| 18:1 | 1.9 | 5.3 | 1.1 | 7.5 |
| 18:2 | 5.7 | 14.9 | 3.6 | 8.1 |
| 18:3 | 32.1 | 12.3 | 48.1 | 1.6 |
| 20.2 | | | 0.8 | 2.4 |
| 20:3 | 3.3 | 12.7 | | 1.0 |
| U | | | | 14.6 |
| 20:4 | 3.8 | 19.0 | 2.8 | 3.4 |
| 20:5 | 4.7 | tr | | 2.9 |
| 22:4 | 5.5 | 8.4 | | 0.8 |
| U | | | | 2.6 |
| 22.5 | | | | 1.7 |
| 22:6 | | | | 8.0 |

[a] Trace. [b] Unknown.

The level of PG in thylakoids was slightly increased by hardening plants of the sensitive *Penjamo*. No effect of hardening of *Miranovskaja* was observed. Hardening of both varieties resulted in a dramatic increase of polyunsaturated fatty acids of 20 and 22 C-atoms in thylakoid PG at the expense of 16:1 (3-t) and 18:3. The levels of saturated fatty acids (16:0 and 18:0) tended to increase in hardened plants, especially in plants of *Penjamo*. In conclusion, fatty acid chain lengthening was strongly stimulated by the hardening treatment, while in *Penjamo* the desaturation activity seemed to be slightly inhibited. Other phospholipids were absent (PE) or only present in traces.

**Glycolipids.** Hardening the plants strongly increased the level of sulfolipid in thylakoids of *Penjamo* (Table II); in *Miranovskaja* only a slight increase was observed. Dramatic changes in fatty acid composition occurred in thylakoids from hardened plants, practically all fatty acids were increased in length to 20 or 22 C-atoms in thylakoids of *Miranovskaja* and to a lesser extent in *Penjamo*. The elevated levels of saturated fatty acids in the thylakoids of the frost-sensitive *Penjamo* again indicated reduced desaturation activity as a reaction upon hardening. In both varieties the level of MGDG was decreased upon hardening, the decrease being most pronounced in *Miranovskaja*. The level of linolenic acid, 18:3, was increased by hardening the plants, especially in *Miranovskaja*, indicating a higher desaturation activity in hardened *Miranovskaja* plants.

In *Penjamo* the level of thylakoid DGDG was decreased upon hardening, in proportion to the observed decrease in MGDG. As a result, the ratio of the two glycolipids remained unaffected. In the thylakoids from hardened *Miranovskaja* the level of DGDG was increased to the same extent as MGDG was decreased, indicating stimulation of galactosylation of MGDG. Fatty acid composition of DGDG was not affected by hardening of *Penjamo* and in thylakoids of hardened *Miranovskaja* stimulation of desaturation was evident as increased level of linolenic acid (18:3) at the expense of palmitic acid (16:0).

## DISCUSSION

Four weeks of hardening at 1.5°C increased the low temperature tolerance of the light-dependent proton uptake of the thylakoids of the wheat variety *Miranovskaja*. In thylakoids from unhardened and hardened seedlings, light-dependent proton uptake was reduced to 80% at −6 and −10°C, respectively. In thylakoids from *Penjamo* ΔpH was reduced to 80% at −6°C, independent of the hardening procedure (17). As expected, the hardening treatment resulted in a greater frost tolerance of *Miranovskaja* than of *Penjamo*, the temperature at which 50% of the leaf cells were killed, being respectively −16 and −8°C.

Because of the genetic differences for low temperature tolerance of light-dependent proton uptake by the thylakoids, corresponding changes in lipid and fatty acid composition of the thylakoids may be related to changed low temperature tolerance of light-dependent proton uptake. Lipid and fatty acid changes in thylakoids from *Penjamo*, in which no appreciable changes in

Table II. *Glycolipid Composition (mol % of Polar Lipids) and Fatty Acid Composition of Glycolipids (Weight %) from Thylakoids of Hardened and Unhardened Wheat Plants of Miranovskaja (Resistant) and Penjamo (Sensitive)*

Results from one out of three growth experiments are presented.

| Variety: | Miranovskaja | | Penjamo | |
|---|---|---|---|---|
| Growth Condition | Unhardened | Hardened | Unhardened | Hardened |
| MGDG | 58.6 | 44.4 | 54.5 | 45.4 |
| DGDG | 17.3 | 31.0 | 23.9 | 19.7 |
| Sulfolipid | 10.2 | 12.5 | 7.0 | 14.1 |
| | | Monogalactosyl diglyceride | | |
| Fatty acid | | | | |
| 16:0 | 2.6 | 0.7 | 2.1 | 6.8 |
| 16:1 | tr[a] | 0.3 | tr | 0.3 |
| 16:2 | 1.1 | 0.8 | 2.9 | 0.7 |
| 16:3 | 4.0 | tr | 4.1 | tr |
| 18:0 | 1.1 | tr | 1.0 | tr |
| 18:1 | 1.5 | tr | 1.1 | tr |
| 18:2 | 4.5 | 1.5 | 3.5 | 1.5 |
| 18:3 | 85.2 | 96.7 | 85.3 | 90.7 |
| | | Digalactosyl diglyceride | | |
| 16:0 | 15.5 | 5.0 | 8.9 | 7.8 |
| 16:1 | tr | 0.5 | 1.1 | 0.3 |
| 16:2 | tr | 1.6 | tr | tr |
| 16:3 | tr | 0.1 | tr | 1.5 |
| 18:0 | 2.3 | tr | 0.8 | 0.5 |
| 18:1 | 1.6 | 1.0 | 0.9 | 1.0 |
| 18:2 | 3.4 | 1.1 | 2.2 | 1.8 |
| 18:3 | 77.2 | 90.7 | 86.1 | 87.1 |
| | | Sulfolipid | | |
| 16:0 | 23.6 | 13.7 | 30.0 | 37.3 |
| 16:1 | 1.5 | 2.6 | 5.3 | 5.3 |
| 18:0 | 0.9 | 2.1 | 8.1 | 5.9 |
| 18:1 | 1.2 | tr | 4.7 | tr |
| 18:2 | 3.8 | 3.7 | 4.5 | tr |
| 18:3 | 63.9 | 5.3 | 45.1 | 12.4 |
| 20:2 | tr | 6.3 | | |
| 20:3 | 1.1 | 10.3 | 1.1 | |
| U[b] | tr | 8.7 | | |
| 20:4 | 2.4 | 17.4 | 4.7 | 39.0 |
| 20:5 | | 13.1 | 0.9 | |
| 22:4 | 1.5 | 16.8 | 1.5 | |

[a] Trace. [b] Unknown.

low temperature tolerance of light-dependent proton uptake by hardening are noted, serves in this case as a control: the observed changes in lipid and fatty acid composition merely reflect a change in lipid enzymic activity without significance for temperature dependence of light-dependent proton uptake or they may represent degradative processes. Considering the tolerance of wheat seedlings to a prolonged period at 1.5°C, the latter explanation does not seem likely. Changes in lipids of the thylakoids of frost-tolerant *Miranovskaja* by hardening (which are not observed in *Penjamo*) may contribute to low temperature tolerance of light-dependent $H^+$ uptake.

Differences between the two varieties, as far as the effect of hardening on thylakoid galactolipids are concerned, were striking: in *Penjamo* the total glycolipid level was decreased and the proportions of both galactolipids remained unchanged as was also observed in pea and spinach (1). In *Miranovskaja* the total thylakoid galactolipid level was maintained during hardening and the DGDG fraction was proportionally increased, as was also observed in pine (12). A higher portion of DGDG (lamellar phase lipid) might contribute to increased low temperature tolerance of light-dependent $H^+$ uptake. It would be interesting to test whether hardening of spinach, pea, and pine would result in changes of low temperature tolerance of light-dependent proton uptake, in correspondence with the observed changes in thylakoid glycolipids. At the moment it seems hazardous to state that the reduced level of MGDG (hexagonal phase-2 structure) itself would contribute to increased low temperature tolerance of light-dependent proton uptake in *Miranovskaja*. The same may be said about the increased level of linolenic acid upon hardening in MGDG (both varieties) and DGDG (*Miranovskaja*). Most likely, increased desaturation activity during hardening may explain this result.

Changes in the thylakoid PC and PG upon hardening the plants do not show any direct relation with either low temperature tolerance of light-dependent proton uptake, nor with frost resistance of the plant cells. Possibly increased levels of PC upon hardening of *Miranovskaja* are found at other sites in the plant cells since the leaves of hardening *Miranovskaja* have much higher PC content than those of hardened *Penjamo* (3). It may be argued that under freezing conditions PC from a cytoplasmic pool is transported to the plasmalemma to replace plasmalemma PC which has been degraded by freezing-induced phospholipase D-activity (5).

The data do not support the hypothesis that 16:1 (3-t) fatty acid in thylakoid PG is essential for functioning of the chloroplast under low temperature conditions. The lowered levels of 18:2 and 18:3 in thylakoid PC and PG upon hardening of the wheat seedlings do not relate to the role these fatty acids are given in chilling-resistant plants (9).

A remarkable fatty acid chain lengthening of phospholipids to 20 or 22 C-atoms in combination with increased desaturation of these fatty acids to 4, 5, and 6 double bonds is observed in hardening plants of both varieties. No genetic differences are evident. Possibly such changes may relate to other thylakoid functions than light-dependent proton uptake or reflect stimulated chain lengthening activity and increased desaturation activity during hardening. Increased elongation and desaturation of fatty acids may be ascribed to a longer availability of substrate for fatty acid elongation and desaturation due to inhibition of transfer of acyl groups from the acyl carrier protein to phospholipids (2). Murata *et al.* (10) suggest that extralong-chain saturated fatty acids observed in leaf phosphatidyl serine represent precursors of epidermal lipids. Genetic differentiation for fatty acid chain lengthening in combination with increased desaturation was evident for thylakoid SL. Chain lengthening was much more pronounced in thylakoids from hardened *Miranovskaja* than in those from hardened *Penjamo*. Such extralong-chain fatty acids in the thylakoid SL fraction could contribute to low temperature tolerance of the light-dependent proton uptake; SL itself is the most effective lipid in protection of chloroplast coupling factor to low temperature damage (8). The above observation also is in agreement with data on early spring plants, which show a direct relation between SL esterified with fatty acids of 20 or more C atoms and growth of the plant at low temperature (6). Further interpretation of the adaptive value of the thylakoid glycolipid changes in low temperature tolerance of light-dependent proton uptake is only possible when the presence of these lipids in the thylakoid proton channels has been established.

*Acknowledgment*—Secretarial assistance of Mrs. Jannie Nuijten-Tjalkes is gratefully acknowledged.

## LITERATURE CITED

1. CHAPMAN DJ, J DE-FELICE, J BARBER 1983 Influence of winter and summer growth conditions on leaf membrane lipids of *Pisum sativum* L. Planta 157:

218–223
2. HARWOOD JL 1984 Effects of the environment on the acyl lipids of algae and higher plants. *In* P-A Siegenthaler, W Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 543–550
3. HORVÀTH I, L VIGH, A BELEA, T FARKAS 1980 Hardiness dependent accumulation of phospholipids of wheat cultivars. Physiol Plant 49: 117–120
4. HORVÀTH I, L VIGH, T FARKAS, LI HORVÀTH, D DUTITS 1981 Effect of choline chloride on fatty acid chain ordering in membranes of wheat (*Tritium aestivum* L. cv. *Miranovskaja* 808). Planta 153: 476–480
5. KUIPER PJC 1984 Lipid metabolism of higher plants as a factor in environmental adaptation. *In* P-A Siegenthaler, W Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 525–530
6. KUIPER PJC, B STUIVER 1972 Cyclopropane fatty acids in relation to earliness in spring and drought tolerance in plants. Plant Physiol 49: 307–309
7. LINEBERGER RD, P STEPONKUS 1980 Effects of freezing on the release and inactivation of chloroplast coupling factor. Cryobiology 17: 486–494
8. LIVNE A, E RACKER 1969 Partial resolution of the enzymes catalyzing photophosphorylation. V Interaction of coupling factor 1 from chloroplast with ribonucleic acid and lipids. J Biol Chem 244: 1332–1338
9. MURATA N, K KURISU 1984 Fatty acid compositions of phosphatidyl glycerol from plastids in chilling-sensitive and chilling-resistant plants. *In* P-A Siegenthaler, W. Eichenberger, eds, Structure, Function and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 551–554
10. MURATA N, N SATO, N TAKAHASHI 1984 Very-long-chain saturated fatty acids in phosphatidyl serine from higher plant tissues. Biochim Biophys Acta 795: 147–150
11. MURATA N, J YAMAYA 1984 Temperature-dependent phase behavior of phosphatidyl glycerols from chilling-sensitive and chilling-resistant plants. Plant Physiol 74: 1016–1024
12. ÖQUIST G 1982 Seasonally induced changes in acyl lipids and fatty acids of chloroplast thylakoids of *Pinus sylvestris*. Plant Physiol 69: 869–875
13. PALTA JP, PH LI 1978 Cell membrane properties in relation to freezing injury. *In* PH Li, A Sakai, eds, Plant Cold Hardiness and Freezing Stress. Academic Press, New York, pp 49–71
14. QUINN PJ, WP WILLIAMS 1983 The structural role of lipids in photosynthetic membranes. Biochim Biophys Acta 737: 223–266
15. ROUSER G, S FLEISCHER, A YAMAMOTO 1970 Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipid phosphorous analysis of spots. Lipids 5: 494–496
16. STEPONKUS PL 1984 Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol 35: 543–584
17. VIGH L 1982 Adaptation of thylakoid membranes of wheat seedlings to low temperature. *In* JFGM Wintermans, PJC Kuiper, eds, Biochemistry and Metabolism of Plant Lipids. Elsevier, Amsterdam, pp 401–414