Lipids in Rye Seedlings in Relation to Vernalization

Lawrence W. Thomson and Saul Zalik
Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada

Received for publication February 14, 1973

ABSTRACT

Increasing the chilling time from 1 to 8 weeks decreased the time to heading of winter rye (Secale cereale var. Sangaste) to approximate that of the spring variety (Prolific). On a dry weight basis, the total phospholipid content of the embryos was higher in Sangaste but declined in both varieties during chilling. The proportions of the individual phospholipid components were similar for both varieties and showed similar responses during the 8-week chilling period. Phosphatidylethanolamine declined and phosphatidic acid increased in both varieties during the treatment.

During the initial 3 weeks, an increased accumulation of linolenic acid and a corresponding decline in linoleic acid occurred for all the lipid components and then remained relatively stable. The glycolipids were more unsaturated than the phospholipids; however, the amount of linolenate was approximately doubled in both during the treatment. In general, the fatty acid content of the respective lipid classes were similar for both varieties.

Vernalization and photoperiodism are two phenomena in which external conditions influence plant development by inducing the reproductive growth phase. Vernalization is defined as a chilling effect which either enhances or enables flower differentiation. Photoperiodism is a light effect which also influences flower differentiation and the heading processes which follow. The photo-effect is known in part to be due to a transmittable stimulus from the leaf to the apical regions. Despite the numerous studies on vernalization (6, 15), no clear explanation has emerged. Most of the studies have been on winter cereals due, perhaps, to the economic importance of these crops and because their seeds are receptive to the chilling stimulus when germinated in the dark, thus enabling the photo-effect to be separated from the chilling effect.

It is known that the amount of unsaturated fatty acids in plants increases when they are grown at low temperatures (8–10), and it was found that the mitochondrial lipids of cold-tolerant plants were more unsaturated than those from sensitive plants (13). From in vivo (5) and in vitro (11, 12, 16) studies, as well as studies with model systems (20), it was suggested that both the unsaturation level of the fatty acid moieties and the polar end groups of the complex lipids influence the physical properties of the membranes and the activities of some enzymes associated with them.

Previous studies on winter cereals attempted to relate lipid changes to the vernalization process (17). Although noticeable differences were found, both the spring and winter varieties showed similar trends in their polar and neutral lipids and the constituent fatty acids. The analyses were based on whole seedlings as were those of De La Roche et al. (2), who studied changes in the phospholipid components during cold treatment of winter wheat. Since excised embryos can be vernalized (15), it is possible that in these studies changes in embryo lipids were masked by those of the endosperm. To investigate the possibility that vernalization affects membrane characteristics and on the assumption that this might be reflected in changes in the polar lipids, embryos of spring and winter rye seedlings were analyzed, at intervals during vernalization, for individual phospholipid components and their constituent fatty acids.

MATERIALS AND METHODS

Plant Material and Growing Conditions. Two varieties of Secale cereale were used: Sangaste winter rye, which exhibits a vernalizing response, and Prolific spring rye, which does not show an enhancement in flowering when subjected to a corresponding chilling period. The seeds of both varieties were surface sterilized with 2.6% sodium hypochlorite for 10 min, rinsed in glass distilled water, then soaked for 12 hr in the dark at 24 ± 3°C. The seeds were then transferred to a Plexiglas germination box which was layered with moist filter paper and germinated in the dark for a further 36 hr. Seeds which had not germinated after this period were discarded. The box with the germinated seeds was placed in a dark room at 4 ± 2°C. At weekly intervals, samples of both varieties were collected, and the embryos were separated from the endosperm in a room at 4°C with a green safelight. The embryos, including scutellum, roots, and shoots, were frozen in liquid nitrogen, lyophilized, and stored at −20°C until required. At the same time, to relate the chilling period with the time to heading, some intact seedlings of each variety were planted in a growth chamber at 20°C under 1100 ft-c of continuous light.

To compare seedlings grown without cold treatment and those exposed to vernalizing temperatures, seeds of both varieties were surface sterilized, soaked for 12 hr, and grown in the dark at 24 ± 3°C for 4 days. The growth of the plants after 4 days at this temperature approximated the 3- to 4-week growth stage of the cold-treated seedlings.

Lipid Extraction. Lipids were extracted from the freeze-dried embryos by homogenizing the tissue three times, for 30 sec each, in chloroform-methanol (2:1, v/v), which contained 10⁻⁴ M PCMB (26), using a polytron with a 20ST generator (Brinkmann Instruments). A total of 90 ml of solvent per 0.3

Abbreviations: PCMB: p-chloromercuribenzoate; PC: phosphatidyl choline; PE: phosphatidyl ethanolamine; PG: phosphatidyl glycerol; PA: phosphatic acid; DGGD: digalactosyl diglyceride; MGDG: monogalactosyl diglyceride; UGL: unidentified galactolipid; 2,7-DCF: 2',7'-dichlorofluorescein; 16:0, palmitate; 18:0, stearate; 18:1, oleate; 18:2, linoleate; 18:3, linolenate.

1 This work was supported by a grant to Saul Zalik from the National Research Council of Canada.
The nonlipid contaminants were removed by adding 1 g Sephadex G-25 (coarse) and 1 ml of water to the chloroform-methanol extract using a slight revision of the method devised by Williams and Merrilees (25). The revision involved the addition of water to swell the Sephadex, since lyophilized samples were used. The suspension was taken to dryness under vacuum at room temperature. Sephadex with the sample was resuspended in chloroform and evaporated under vacuum and again resuspended in chloroform. The suspension was poured into a chromatographic column (1 cm i.d.) with the end drawn to a capillary. The lipids were washed from the Sephadex with 100 ml of chloroform followed by 10 ml of chloroform-methanol (2:1, v/v), which removed the polar lipids as determined by monitoring with TLC. The column eluate was concentrated to dryness under vacuum and made to 5 ml in chloroform.

Aliquots were taken for phosphorus (1) and sugar (19) determinations. Although nonlipid contaminants were still present in the eluate, they remained close to the origin when the polar lipids were separated by TLC.

**Separation of Polar Lipids.** The polar lipids were separated by two-dimensional TLC (14) on glass plates (20 × 20 cm) coated with a layer of 0.3 mm Silica Gel HR (Merck). A 0.5-ml aliquot containing about 2 mg of total lipid was applied under a stream of nitrogen to each plate. The solvent systems were chloroform-methanol-7 N ammonium hydroxide (65:25:4, v/v/v) for the first dimension, followed by chloroform-methanol-acetic acid-water (85:15:10:3) in the second. The first dimension solvent was removed under vacuum.

**Identification of Lipids.** The plates were sprayed lightly with 50% sulfuric acid and charred at 180 °C for 15 min. Specific color tests and comparison with chromatograms of authentic samples, as well as published chromatograms (7, 14), were used to identify the lipid components.

**Phospholipid Determinations.** The spots were detected with iodine vapor and scraped into glass test tubes. The phosphorus was quantitated using the method described by Bartlett (1). Single plates gave adequate samples of individual phospholipids for analyses. As Silica Gel HR does not interfere with the phosphorus assay, elution of the lipid from the absorbent was not required, but after color development the samples were centrifuged to pellet the absorbent, and the absorbance of the solution was read at 830 nm with a Beckmann DK-1 spectrophotometer.

**Fatty Acid Determination.** The separated lipids were detected by spraying the plates with 2% 2,7-DCF in 95% ethanol (18, 21) and viewing under UV. Duplicate plates were required to supply a sufficient sample of individual lipids for fatty acid analysis, except for PC and neutral lipid for which one plate was adequate. Esterification was achieved by adding 2 ml of methanol and 0.1 ml of concentrated sulfuric acid to the tubes containing the sample and adsorbent and refluxing at 70 °C for 2 hr. This method was used routinely, since upon comparison with a series of the samples esterified in screw-capped vials, sealed under nitrogen, the results were virtually identical, indicating oxidation had not occurred. Following esterification, 3 ml of water were added, and the methyl esters were extracted with three washings of petroleum ether. The petroleum ether was evaporated under nitrogen, and the methyl esters were taken up in methanol.

The fatty acid methyl esters were analyzed on an Aerograph Model 200 gas chromatograph equipped with a hydrogen flame ionizing detector. A coiled stainless steel column (2.7 m × 3 mm) packed with 20% ethylene glycol adipate on Anakrom SD 60/70 mesh, P (Analabs), was used with nitrogen as carrier and a column temperature of 195 °C. The injector temperature was 240 °C. The fatty acids were quantitated by triangulation and identified by matching their retention times with standards (Analabs).

**RESULTS**

Plants of the unvernalized Sangaste headed when grown under continuous illumination at 20 °C; however, they required at least 200 days. An increase in the chilling time of from 1 to 8 weeks for Sangaste was accompanied by a corresponding decrease in the time required for heading, from 200 days to only 55 days. The spring variety showed no enhancement due to the chilling treatment and headed at about 45 days regardless of the length of chilling.

**Lipids Identified.** The lipid spots revealed by charring of the developed TLC plates with sulfuric acid are shown in Figure 1, which is representative of the lipid separations obtained for both varieties at all the growth stages analyzed.

Spots 1, 2, 4, 5, 6, and 9 were identified as phospholipids by spraying the plates with molybdenum reagent (3). A trace of phosphorus also appeared around spot 10. The Dragendorff test (24) identified spot 2 as a choline containing lipid and ninhydrin spray (22) indicated spot 5 contained a free amino group. Chromatography of authentic samples confirmed that spots 1, 2, and 5 were PI, PC, and PE, respectively. Comparisons with lipid patterns in the published chromatograms of Nichols (14) and Galliard (7) suggested that spots 4 and 9 were PG and PA, respectively, and provided further support for the identity of spots 1, 2, and 5. Spot 6 was not identified.

Spots 3, 7, 8, 11, 12, and 13 gave a purple color characteristic of glycolipids and sterols during early color development when the plates were charred. Also with the orcinol-sulfuric acid spray (23) they gave the characteristic blue color of glycolipids and sterols. Comparisons with the lipid patterns in the published chromatograms of Nichols (14) and Galliard (7) suggested that spot 3 was DGDG and either spot 12 or 13 was MGDG. Galliard (6) was unable to identify a component with a similar migration pattern as spot 11 and referred to it as an unidentified galactolipid. Spots 3, 11, 12, and 13 all showed characteristic hexose absorption peaks at 490 nm when analyzed by the method of Dubois et al. (4) as modified by Roughton and Batt (19). Spots 7 and 8 may have been cerebrosides (6) but were not analyzed.

Spot 10 did not appear when the chromatogram was exposed to iodine vapor or to 2,7-DCF and was not further identified. However, as a trace of phosphorus was evident at the edge of spot 10 nearest to the PA spot, it was included in the phosphorus assay.

**Phosphorus and Sugar Content.** The total phosphorus and sugar of the lipid extracts for both varieties sampled during the treatment period expressed on the dry weight of the embryo are shown in Table I. Sangaste contained more total phosphorus in the extract than Prolific; however, a gradual decline was evident for both varieties throughout the treatment period. Initially, Sangaste had a higher sugar content in the lipid extract, but it was not consistently higher throughout the treatment period.

**Phospholipids.** Although a higher total phospholipid content was evident for Sangaste (Table I), the percentages of PI, PE, PG, and unknowns 6 and 10 were similar for both varieties and showed little variation during the chilling period (Table...
II). The PC and PA content also showed a similar response for both varieties except at 1 week. There was a gradual, almost linear, decline of PC during the first 7 weeks (46-31% for Prolific and 44-30% for Sangaste), while PA showed a corresponding increase (13-21% for Prolific and 14-24% for Sangaste).

The phospholipid composition of the embryo tissue following growth at 24°C for 4 days is shown as an inset in Table II. There was little difference between the varieties grown at room temperature; however, differences were evident between the two growing temperatures when seedlings of comparable growth stages were analyzed. PI was higher in both varieties grown at 24°C in comparison to 4°C (16% versus 7-9%), whereas PA was much lower (3-4% versus 12-18%).

Fatty Acid Content. The changes in the fatty acid content within the lipid classes during the treatment period are shown in Figures 2 to 11. The amounts of the individual fatty acids are expressed as a percentage of their total in the sample. The major fatty acids detected were 16:0, 18:1, 18:2, and 18:3. Stearic acid was present in trace amounts in some samples but could be quantitated only in spot 11. Short chain fatty acids containing less than 16 carbon atoms appeared in trace amounts in the neutral fraction and to a lesser extent in the glycolipid fractions. They were not present in sufficient amount to enable quantitation. Palmitoleic (16:1) appeared as a small peak immediately after 16:0 in the PG and PE fractions in both varieties after the 3rd week of cold treatment.

For all of the lipid classes analyzed from both varieties during the chilling period, the most marked changes were for 18:3 and 18:2. The percentage of 18:3 increased during the initial 3 to 4 weeks of chilling with a corresponding decrease in the percentage of 18:2. In general, a higher unsaturation level was evident in the glycolipid fractions (Figs. 8-10) than in the phospholipid fractions (Figs. 3-7), however, the amount of 18:3 was approximately doubled in both during the chilling period.

The fatty acid composition of the individual phospholipids differed during the treatment period. In PI and PG (Figs. 3

\[ \text{FIG. 1. Two-dimensional TLC on Silica Gel HR of polar lipids from Sangaste embryo tissue after 3 weeks of cold treatment. The solvent system for the first dimension was chloroform-methanol-7 N NH}_4\text{OH (65:25:4), followed by chloroform-methanol-acetic acid-water (85:13:10:3) in the second dimension. The chromatogram was charred following spraying with 50\% sulfuric acid and is typical of the separations obtained for both varieties at the growing stages analyzed. The spots were identified as: 0, origin; 1, PI; 2, PC; 3, DGDG; 4, PG; 5, PE; 6, 7, 8, and 10, unidentified; 9, PA; 11, 12, and 13 were considered as unidentified glycolipids, 11 was analyzed separately, whereas spots 12 and 13 were combined; 14, neutral lipid.} \]
and 5), 16:0 was a major fatty acid in both varieties during all stages. The initial increase in 18:3 and corresponding decline in 18:2 was more pronounced in Sangaste than in Prolific for PI and PG. In PC and PE (Figs. 4 and 6), 18:2 was the predominant fatty acid. During the initial 3 weeks, 18:3 increased by approximately 15% in PC and 20% in the PE fraction, representing an increase of approximately 2-fold in the PA fraction (Fig. 7). 18:3 became the major fatty acid at the 2-week period and also showed an approximate 2-fold increase. For fatty acid analysis of the unidentified glycolipids (spots 11, 12, and 13), spot 11 was analyzed separately (Fig. 10), whereas spots 12 and 13 were combined (Fig. 9). In the glycolipid fractions analyzed (Figs. 8–10), 18:3 was the most predominant fatty acid, except in the UGL fraction of Figure 10 prior to chilling. The percentage of 18:3 increased from approximately 40 to 75% in DGDG during the initial 4 weeks for Sangaste, and from approximately 50 to 70% for Prolific (Fig. 8). Likewise, in the UGL fraction (Fig. 10), the amount of 18:3 increased more in Sangaste. The variations in the fatty acid content of the neutral lipid fraction (Fig. 11) were similar to those of the total lipid fraction (Fig. 2), but the initial increase in unsaturation appeared 1 week later.

The double bond index was calculated according to the method outlined by Lyons et al. (13), in which the summation of the weight percentage of each acid is multiplied by the number of double bonds it contains per molecule and divided by 100. The double bond index for the total lipids in both varieties was about 1.73 at 0 weeks, it rose to 1.80 at 1 week, and leveled off at about 2.0 by 3 weeks. The corresponding values for the total phospholipids were 1.58, 1.75, and 1.80, and those for the glycolipids calculated from Figures 8 and 9 only were 1.98, 2.12, and 2.21.

Discussion

Unfortunately, means of direct investigation of physical and physiological changes in plant membranes during the course of vernalization are not available. Therefore, we thought that analysis of changes in the lipid components of spring and winter rye exposed to vernalization temperatures might reveal varietal differences associated with their growth habit.

An influence of the polar portion of lipid membrane properties has been indicated from physical studies on lipid films (20), and Kimelberg and Papahadjopoulos (11) suggested that specific phospholipids can be associated with specific enzyme activities. In the present study, the only changes noted in the polar lipid composition of the rye seedlings, which might have influenced membrane associated activities, was the decline in PC and the corresponding increase in PA (Table II). The possibility that this trend was due to enzymatic transesterification during isolation was minimized by the addition of PCMB (26) to the extraction solvent. The differing amounts of their respective fatty acids (Figs. 4 and 7) also suggest the major source of PA was not the direct transesterification of PC during growth at low temperature. However, the decline in PC and the accompanying increase in PA was similar in the spring and winter varieties.

The increased unsaturation of all lipid classes during initial chilling and attainment of maximum unsaturation by approximately the 3rd week may have reflected an increased plasticity of the membranes (5, 13) and is in keeping with the view that the fatty acid composition affects the physiological characteristics of membranes (4, 11, 12, 16). But since both varieties exhibited similar changes in their lipid components, these

Table I. Phosphorus and Sugar Content of Total Lipid Extracts from Embryo Tissue of Rye Seedlings Sampled at Intervals during the Vernalization Period

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Phosphorus Content</th>
<th>Galactose Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sangaste (µg/g tissue dry wt.)</td>
<td>Prolific (µg/g tissue dry wt.)</td>
</tr>
<tr>
<td>0</td>
<td>1081.0</td>
<td>899.7</td>
</tr>
<tr>
<td>1</td>
<td>966.1</td>
<td>681.5</td>
</tr>
<tr>
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<td>586.4</td>
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<td>305.3</td>
</tr>
<tr>
<td>6</td>
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<td>292.4</td>
</tr>
<tr>
<td>7</td>
<td>304.5</td>
<td>286.6</td>
</tr>
</tbody>
</table>

Table II. Phospholipids in Embryo Tissue of Rye Seedlings Sampled at Weekly Intervals during the Vernalization Period and Following Growth at 24°C for 4 Days

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Lipid Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
</tr>
<tr>
<td>0</td>
<td>S</td>
</tr>
<tr>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
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<tr>
<td>7</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>S</td>
</tr>
</tbody>
</table>

1 Visually, the growth of these seedlings was the same as those grown for 3 weeks at 4°C.
Figs. 2 to 11. Percentage of fatty acids in lipid fractions isolated from embryo tissue of Sangaste (San) and Prolific (Pro) sampled at weekly intervals during the chilling period. Seedlings grown for 4 days at 24 C were visually like those grown for 3 weeks at 4 C. Values for 4 C; 16:0 (---); 18:0 (++); 18:1 (---); 18:2 (----); 18:3 (--); values for 24 C; 16:0 (●); 18:1 (○); 18:2 (∗); 18:3 (*). The fatty acids shown in Fig. 9 were assayed from the combined contents of spots 12 and 13 denoted in Fig. 1.
changes cannot be considered unique to vernalization but may only represent a general hardening process.

Acknowledgement—We thank Barry Zytaruk for his technical assistance.

LITERATURE CITED