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RAPID CHANGES IN SOYBEAN ROOT MEMBRANE LIPIDS WITH ALTERED TEMPERATURE*

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Key Word Index—*Glycine max*; Leguminosae; soybean roots; phospholipids; fatty acids; mitochondria; plasmalemma; temperature.

Abstract—In soybean roots, as temperature was increased between 15° and 30°, palmitic and stearic acids increased, and oleic, linoleic, and linolenic acids decreased in both plasmalemma and mitochondrial membrane fractions. As temperature was decreased, the reverse trend occurred. Membranes in both the mitochondria and plasmalemma responded with a significant shift in fatty acid composition in 48 hr, often in 24 hr. Mitochondria responded more quickly than the plasmalemma, particularly at the lower temperature.

INTRODUCTION

Lipid composition in plant tissue is apparently in a constant state of flux, responding not only to external stimuli such as temperature but also to internal processes such as maturation. Previous studies have frequently ignored the possibility that differences in lipid composition may be due to a single membrane species such as the plasmalemma or mitochondria. Kennan *et al.* [13] compared the lipid composition of mitochondria and plasmalemma from oat (*Avena sativa*) roots and showed the compositions to be distinctly different. Mitochondria were higher in total phospholipid and free fatty acids but lower in triglycerides. There were also differences in individual phospholipids (18.2% phosphatidylcholine in plasmalemma cf. 9.9% in mitochondria) and in the percentage of unsaturated fatty acids (mitochondria had twice as much). Reported high levels of phosphatidic acid, 41.3 and 13.9% in the mitochondria and plasmalemma, respectively, are suggestive of phospholipid degradation probably occurring during the membrane separation process.

In a variety of biological systems, lipid composition has been studied in relation to growth at a single temperature [2–8]. Thus, tissues have been observed to contain a higher degree of saturated fatty acids at higher temperatures and a higher degree of unsaturated fatty acids at lower temperatures. Prominent shifts in two of the principal fatty acids, namely linoleic and linolenic acids, were generally involved. But no reports could be found on higher plants dealing with continuous shifts in lipid composition as a function of shifts in temperature. In addition, total lipid measurements run the risk of masking subtle shifts in the fatty acid composition of individual lipid components. Such may have been the case when Wilson and Crawford [9] could not demonstrate a difference in leaf fatty acid content between four chill-resistant and chill-sensitive plant species. Similarly, de la Roche *et al.* [10] could not show a difference in total lipid and total fatty acid content in winter wheat (*Triticum aestivum*) seedlings grown at 2° or 24°. In the phospholipid fraction, they found a higher total phospholipid and linolenic acid content of seedlings grown at 2°. In these studies and others, constant temperatures were utilized and shifts in lipid composition from one temperature regime to another were either not performed or inferred from the data.

In addition to separate analysis of membrane fractions, the dynamics of lipid compositional changes have not been well documented, particularly with respect to temperature-induced changes in the composition of the plasmalemma lipids. Therefore, the present investigation was undertaken to examine two specific membrane sources, the plasmalemma and mitochondria, and to characterize the dynamic nature of the fatty acid composition while the membranes were being subjected to shifts in temperature.

RESULTS AND DISCUSSION

Five major fatty acids were present in the membrane fractions of soybean roots: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3). Trace amounts of palmitoleic (16:1), arachidic (20:0), and behenic (22:0) acids were occasionally observed. Purification of the plasmalemma from other cell contaminants was verified by electron microscopy using a phosphotungstic–chromic acid stain specific for plant plasmalemma [11]. Quantitative analysis of membrane sections revealed a plasmalemma purity in excess of 70%, sufficient for analysis, although other material present may have affected the results.

Raising or lowering the temperature between 15° and 30° resulted in rapid measurable responses in the fatty acid compsoition of both the plasmalemma and mito-

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Table 1. Influence of temperature shift and time of harvest on the fatty acid composition of soybean root plasmalemma phospholipids

Growth temperature	Treatment temperature	Sampling Time (hr)	Fatty acid composition 16:0	18:0	18:1 (% of total wt)	18:2	18:3
15°	22°	0	26.5	17.5	10.0	26.2	19.8
		24	27.9	14.2	14.0	25 7	18.2
		48	36.7	17 0	11 4	17 5	17.5
22°	30°	0	30.1	16.0	8.5	24.5	20.7
		24	32.4	16.8	8.9	20 2	21.7
		48	33.7	18 2	8.4	20 5	19.1
30°	22°	0	37.2	14.3	12.7	23.0	12.5
		24	30.1	13.7	12.5	27 4	16.2
		48	24.8	11.8	8.9	29.0	25.6
22°	15°	0	30.1	16 0	8.5	24.5	20 7
		24	28.4	16.6	10.5	24 4	20.0
		48	26.5	14.3	10.2	24.7	24.3

chondria (Tables 1 and 2). Moving the plants to treatment temperatures higher than the temperature during growth caused an increase in the percentage of saturated fatty acids, palmitic acid, and to a lesser extent, steric acid with time, while the percentage of unsaturated fatty acids, oleic, linoleic and linolenic acids decreased. The trend was reversed as treatment temperature decreased. This relationship between temperature and fatty acid composition supports previous investigations concerning other plant species [10, 12–15], bacteria [2], fungi [16] and animals [1, 6, 7]. In contrast to the steady state conditions used in previous studies, the present study demonstrates the rapidity of membrane response to fluctuations in temperature. Although the response to temperature in the present investigation is shown to be measurable within 24 hr, roots of younger plants might be expected to exhibit a quicker response due to a higher proportion of actively growing root initiates.

In contrast to the study by Keenan *et al.* [13] which reported distinct differences between mitochondria and plasmalemma of oats, no marked differences were seen in the fatty acid composition between the plasmalemma and mitochondria of soybeans (Tables 1 and 2) even though separation techniques were similar. Lipase activity may have been high enough even at pH 7.2 used by Keenan *et al.* [13] to cause the differences seen. The high levels of phosphatidic acid, a phospholipid degradation product, tend to support this conclusion. Angelo and Altschul [17] and Galliard [18] reported lipase activity maxima and free fatty acid-stimulated enzymic deacylation of lipids in the pH range 6.2–7.2. Inclusion of BSA and a high pH in the homogenizing medium minimizes the deacylation process.

An unsaturation ratio (UR) was calculated as the % wt contributed by linoleic plus linolenic acids divided by the percent weight contributed by palmitic acid to obtain some measure of a temperature-induced shift in the balance between saturated and unsaturated fatty acids (Table 3). These particular fatty acids may represent opposing ends of the portion of the biosynthetic pathway in consideration and changes in their overall weights would reflect shifts toward saturation or unsaturation. Shifts in UR permit a membrane to retain its fluidity at a particular temperature [3, 19, 20]. Plasmalemma phospholipids (Table 3) exhibited a significant decrease in UR within 48 hr when subjected to a rise in temperature. When the temperature was increased from 15° to 22°, a significant decrease in unsaturation was noted within 24 hr.

Table 2. Influence of temperature shift and time of harvest on the fatty acid composition of soybean root mitochondria phospholipids

Growth temperature	Treatment temperature	Sampling time (hr)	Fatty acid composition 16:0	18:0	18:1 (% of total wt)	18:2	18:3
15°	22°	0	24.0	16.4	10.3	28.7	20.5
		24	25.7	15.2	14.1	29.2	15.7
		48	31.6	11 3	12.7	27.5	16.8
22°	30°	0	22.8	17 1	11.6	24.4	24.0
		24	31.4	17.6	10.8	20.1	19.9
		48	29.9	21.1	8.5	21 1	19.9
30°	22°	0	29.1	12.0	11.6	29.8	17.3
		24	29.2	12.0	9.6	27.0	22.2
		48	23 7	10.9	9.3	26.7	29.3
22°	15°	0	22 8	17.1	11.6	24.4	24.0
		24	21.6	12.7	4.6	30 8	30.1
		48	18.6	12.8	5.9	29.8	32.9

Table 3. Unsaturation ratios* of fatty acids from soybean root plasmalemma phospholipids

Sampling time (hr)	Growth/Treatment temperature† 15°/22°	22°/30°	30°/22°	22°/15°
0	2.11 c	1.50 b	0.94 a	1.50 a
24	1.76 b	1.31 ab	1.49 ab	1.57 a
48	1.41 a	1.18 a	2.24 b	1.56 a

* Unsaturation ratio is calculated as (%18:2 + %18:3)/(%16:0).

† Means followed by the same letter within a column do not differ significantly at the 5% level by Duncan's Multiple Range Test.

However, when the temperature was increased from 22° to 30°, 48 hr was required for a significant response. Decreasing the treatment temperature increased the degree of unsaturation. A significant response was exhibited within 48 hr when the temperature was shifted from 30° to 22°. However, shifting the temperature from 22° to 15° did not alter the degree of unsaturation, at least within the 48 hr observation period, although trends were evident in the fatty acid composition (Table 1).

The mitochondrial phospholipids responded similarly (Table 4). Increasing the temperature significantly reduced the degree of unsaturation within 24 hr. Decreasing the temperature, significantly increased the degree of unsaturation; however, 48 hr was required. In contrast to the plasmalemma, when the temperature was lowered from 22° to 15°, a marked increase in unsaturation occurred after 48 hr. One possible explanation may be that the mitochondria are more active metabolically at the lower temperature than the plasmalemma and, hence, would respond more rapidly. The mitochondria had a higher degree of unsaturation (Tables 3 and 4), an observation not readily apparent in Tables 1 and 2. Higher UR values at lower temperatures allow for greater membrane lateral compressibility and extensibility [21], thus facilitating insertion of new membrane components with little increase in membrane surface area [22]. It would also provide the necessary flexibility for protein carrier kinetics in the transport of solutes across the membrane. The quicker response by mitochondria might be explained on the basis of the synthesis and transfer of membrane lipids to the various membranes. Kagawa *et al.* [23] and Morre [24] demonstrated a time lapse in incorporation of choline-[^{14}C] as a phospholipid precursor into lecithin of the endoplasmic reticulum first, followed in order by dictyosomes, mitochondria and plasma membrane. Consideration of this labeling sequence and the low temperature utilized in the present investigation (which is near the minimum temperature at which soybeans can be successfully grown) might account for the lack of response seen in the plasmalemma within the 48-hr observation period.

Table 4. Unsaturation rations* of fatty acids from soybean root mitochondria phospholipids

Sampling time (hr)	Growth/Treatment temperature† 15°/22°	22°/30°	30°/22°	22°/15°
0	2.05 c	2.18 b	1.67 a	2.18 a
24	1.74 b	1.30 a	1.72 ab	2.85 ab
48	1.40 a	1.50 ab	2.37 b	3.50 b

* Unsaturation ratio = (%18:2 + %18:3)/(%16:0).

† Means followed by the same letter within a column do not differ significantly at the 5% level by Duncan's Multiple Range Test.

The data presented emphasize the importance of examining individual cell membranes as well as examining the continuous response of membranes to fluctuations in temperature. The mitochondria and plasmalemma utilized in this study were derived from whole root samples. Had actively growing root initiates only been used, a more rapid response (less than 24 hr) might have been seen.

EXPERIMENTAL

Germination procedure and temperature treatment. Seeds of soybean [*Glycine max* (L.) Merr., cv 'Hark'] were planted in quartz sand in 946 ml wax-coated paper cups with drainage holes. Seeds were germinated at constant 15°, 22° or 30° with a 16-hr daylength in controlled environment chambers. Illumination was provided by incandescent and fluorescent bulbs with the light intensity between chambers ranging from 15 to 20 klx. At each temp, seedlings were grown to the second trifoliolate stage at which time they were subjected to a shift in temp. Treatments consisted of constant temp (no shifts) or a shift to the next highest or lowest temp; i.e. a shift from 15° to 22°, from 22° to 15° or 30°, or from 30° to 22°. The plants were then harvested 0, 24 and 48 hr. after treatment. Plants were watered daily as required and fertilized once with Hoagland's No. 1 soln 2–3 weeks after planting. Data presented are the means of 3 expts with 3 replications each.

Membrane separation. Plasmalemma and mitochondria membranes were obtained according to a modified procedure of ref. [25]. Fresh root tissue (30 g) was homogenized by grinding in a mortar and pestle for 90 sec in 30 ml of ice-cold medium of 0.25 M sucrose, 3 mM EDTA, 50 mM tricine (*N*-Tris(hydroxymethyl)methyl glycine), and 1% (w/v) BSA (fatty acid free) (pH 7.8). The homogenate was strained through 4 layers of cheesecloth and centrifuged at 13000 *g* for 15 min at 2°. The 13000 *g* pellet containing mitochondria was resuspended in homogenizing medium and centrifuged at 2500 *g* for 10 min to remove cell walls and other large cellular fragments. The resulting supernatant was then pelleted at 13000 *g* for 15 min, the pellet rinsed and suspended in deionized H_2O and repelleted at 13000 *g* for an additional 15 min. The resulting mitochondria were then held at −15° for further analysis. The supernatant containing plasmalemma from the original 13000 *g* centrifugation was further centrifuged at 80000 *g* for 30 min. The pellet was then resuspended in 2 ml 20% (w/w) sucrose containing 1 mM $MgSO_4$ and 1 mM Tris-MES (2-*N*-morpholino ethane sulphonic acid), pH 7.8. The suspension was layered onto a discontinuous sucrose gradient consisting of 28 ml of 45% (w/w) sucrose and 8 ml of 34% (w/w) sucrose. The sucrose solns each contained 1 mM $MgSO_4$ and 1 mM Tris-MES, pH 7.8. The gradient tubes were centrifuged for 2 hr at 95000 *g* in a swinging bucket rotor (Beckman SW27 rotor). The plasmalemma were then obtained from the 34% to 45% interface, diluted in deionized H_2O and pelleted at 80000 *g* for 10 min. The plasmalemma samples were held at −15° for further analysis.

Extraction and analysis of phospholipids. The frozen membrane samples were lyophilized and the lipids extracted by a modified procedure of ref. [26]. All solvents used contained 0.1 mg/l antioxidant BHT (2,6-ditert-butyl-*p*-cresol). Ten ml of $CHCl_3$–MeOH methanol (2:1) (C/M) was added to the lyophilized tissue and the samples shaken in a water bath at 33° for 30 min. The extract was filtered (Whatman No. 42) into a second tube and the residue re-extracted twice with 5 ml C/M

by shaking in a H_2O bath for 15 min. The filtered extracts were combined and washed with 0.2 vols of 0.9% NaCl soln [27] in a tube stirrer for 1 min. After the mixture settled, the upper phase was discarded and the lower phase washed twice with 0.2 vols of $CHCl_3$–MeOH–H_2O (3:48:47) containing 0.9% NaCl soln. The lower phase containing the lipids was then taken to dryness under N_2 at 33° and the residue dissolved in 50 µl $CHCl_3$. The lipids were then applied to TLC plates (20 × 20 cm) precoated with 0.25 mm Si gel GF. The phospholipids were separated from the remaining lipids in Me_2CO–HOAc–H_2O (100:2.1) compared with published R_f values [14] and verified with known phospholipid standards. Glycolipid contamination as indicated by orcinolsulfuric acid spray [28] was minimal. The phospholipid band was scraped from the plates, extracted with 2 ml C/M followed by 1 ml MeOH, and the resultant soln taken to dryness under N_2 at 33°. Fatty acid Me esters were prepared according to a modified method of ref. [29]. 0.5 N methanolic KOH (1 ml) was added to the dried sample followed by boiling for 5 min. After the tubes had cooled, 1 ml of 14% BF_3–MeOH was added and the samples boiled for an additional 2 min. One drop of satd NaCl was then added and the Me esters extracted × 3 with 1 ml hexane. The extracts were combined, dried under N_2 and the residue taken up in 50 µl hexane for GLC analysis. Fatty acid composition was determined by FID using a 1.83 m × 2 mm id glass column packed with 12% stabilized DEGS on Anakrom ABS and operated at 170°. N_2 was the carrier gas. Peak identification and quantification was performed by a computer-interfaced system using authentic fatty acid Me ester standards. Data were analyzed as a completely randomized design.

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