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CHANGE IN CITRUS LEAF LIPIDS DURING FREEZE-THAW STRESS

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Key Word Index—*Citrus*; *Poncirus*; *Rutaceae*; hybrid; parentage; leaf lipids; cold hardening; fatty acids; freeze stress; phosphatidylcholine; triacylglycerol.

Abstract—Fatty acids in the leaves of the citrus hybrid B5-9-68, a BC_1 progeny [(*Citrus paradisi* × *Poncirus trifoliata*) × *C. sinensis*], were compared with those in *P. trifoliata* and *C. sinensis*. Total lipid and triacylglycerol fatty acid profiles of the cold hardened hybrid were similar to the profiles of the hardy *P. trifoliata* and different from the profiles of the less hardy *C. sinensis*. When subjected to a freeze regime, the unhardened hybrid lost 22% leaf fatty acids during the freeze stage and 13% during the thaw stage. Linolenic acid accounted for 98% of the fatty acid decrease. Degradation was greatest in phosphatidylcholine (89%), phosphatidylglycerol (83%), monogalactosyldiglyceride (79%) and digalactosyldiglyceride (50%). Phosphatidic acid increased 4-fold over the two stress stages. Total leaf fatty acids during the freeze-thaw regime increased 12% in the cold hardened hybrid. Three molecular species of triacylglycerol which were rich in linolenic acid increased in the hardened hybrid during the freeze-thaw regime. The increase in highly unsaturated triacylglycerol species under freeze-thaw stress suggests that triacylglycerol has a role in maintaining the fluidity of biomembranes during freezing conditions.

INTRODUCTION

Cold hardening of citrus trees to increase protection from freeze damage results in qualitative and quantitative changes in leaf lipids [1–3]. Accumulation of triacylglycerol and linoleic acid are the major changes. The accumulation is greater in cold-tolerant than in more sensitive cultivars. Many plants respond to cold hardening regimes through increases in phospholipids [4–9], some at the expense of triacylglycerol [5, 6]. Fatty acid desaturation generally occurs to a greater extent in plants grown at low temperatures (cold-hardened) [4, 10] and in cold-tolerant plant varieties [11–13]. Other workers, however, have failed to find a positive correlation between fatty acid unsaturation and cold tolerance [6, 14–16].

Changes in plant lipids under freeze-thaw stress have been reported for a number of plants [17–22]. Generally, degradation of cell membranes results in lower levels of phosphatidylcholine and higher levels of phosphatidic acid. The increase in phosphatidic acid has been negatively correlated with the cold hardiness of rye and wheat cultivars [20]. Crown and root tissue of wheat during the first six hours after thawing showed a rapid degradation of polar lipids and increases in diacylglycerols, unesterified fatty acids and triacylglycerols [22]. In rape leaves, frost hardening promoted an increase in phospholipids after freezing [21]. For our study, we have selected a BC_1 hybrid [(*C. paradisi* × *P. trifoliata*) × *C. sinensis*] (B5-9-68) which has the cold tolerance of *P. trifoliata* and also is a desirable dessert-type fruit resembling *C. sinensis* [23]. Our objective was to determine the effect of freeze-thaw stress on the lipids of the hardened and non-hardened hybrid. These effects may suggest a protective role for the lipids produced during cold hardening of the citrus plant.

RESULTS AND DISCUSSION

Inherited chemical markers

Profiles of hardened (H) citrus leaves (Table 1) showed typical hardening effects [1, 2], e.g. an increase in linoleic acid (18:2) and a decrease in oleic acid (18:1). Previously we observed that the increase in 18:2 with hardening related to the degree of cold tolerance of the citrus variety [1–3]. In this study, the increases in 18:2 with hardening were: hybrid, 6.4 fold; *P. trifoliata*, 5.4 fold; and *C. sinensis*, 1.7 fold. These data indicate that the hybrid B5-9-68 has similar characteristics to *P. trifoliata* in accumulation of 18:2. The level of 18:2 was 50% greater in H hybrid than H *P. trifoliata*; however cold hardiness in citrus depends on a number of factors. Thus, we cannot at this time, based on 18:2 levels, assign greater hardiness to the hybrid than to *P. trifoliata*. Hardening increased total fatty acids in the hybrid 67.9%, in *P. trifoliata* 33.8% and decreased the level in *C. sinensis* 5.8%. Fatty acids associated with triacylglycerol (TAG) as percentage of total lipid were: hybrid, 39.6%; *P. trifoliata*, 33.9%; and *C. sinensis*, 10.9%. This last value for succory sweet orange compares with the value of 13.5% for Valencia sweet orange [2]. The other two values are greater than the value for sour orange seedling, the value of the hardiest citrus variety reported to date [2]. From these data hybrid B5-9-68, a hardy-type citrus cultivar, should be useful in studying the effect of freeze-thaw stress on leaf lipids.

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Table 1. Effect of cold hardening on major fatty acids in leaves of a citrus hybrid, B5-9-68 and two of its parentage

	μg FA/g fr. wt					
	Unhardened			Hardened		
Fatty acid	*P. trifoliata*†	B5-9-68‡	*C. sinensis*§	*P. trifoliata*†	B5-9-68‡	*C. sinensis*§
Total lipid						
16:0	1582	1810*	1486	1654***	2232***	1288***
18:0	340***	200**	136***	362	340***	166***
18:1	612*	752	800*	290**	492***	222**
18.2	478**	614	584*	3038***	4544***	1580***
18:3	3712	3628	3372	3584**	4154***	2750***
Total	6724	7004	6378	8928***	11762***	6006***
Triacylglycerol						
16:0	100	59	78	429**	754***	153***
18:0	15	8	10	85*	121**	9**
18·1	64	36	52	143***	251***	49***
18:2	59	24	25	1494**	2597***	280***
18:3	74	33	40	879	931**	162**
Total	312	160	205	3030**	4654***	653***

Within unhardened or hardened leaves, levels of each acid in: †*P. trifoliata* vs (B5-9-68); ‡(B5-9-68) vs *C. sinensis*; §*C. sinensis* vs *P. trifoliata* were significantly different (* = $p < 0.1$, ** = $p < 0.05$), *** = $p < 0.01$) tested with Student's *t* test

Lipid degradation

The polar lipids of unhardened (UH) leaves differed in their susceptibility to degradation by freeze-thaw stress (Table 2). Over the two stages phosphatidylcholine (PC) decreased 89%, phosphatidylglycerol (PG) 83%, monogalactosyldiglyceride (MGDG) 79%, digalactosyldiglyceride (DGDG) 50%, phosphatidylinositol (PI) (including sulpholipid) 44%, and phosphatidylethanolamine (PE) 28%. Polar lipids of wheat were also degraded by freeze-thaw stress [22]. Acyl products of degradation increased during the two stage stress. Phosphatidic acid (PA) increased 4-fold during the freeze stage and an additional 31% during the thaw stage.

Cold hardening increased the level of fatty acids in citrus leaves as observed in previous studies [1–3] and the first part of this study (Table 1). The increase for the hybrid was 3197 μg/g or 39.3% (Table 2). The increases were 1894% in triacylglycerol (TAG), 77% in FFA, 72% in PE and 56% in DAG while decreases were observed in PG (53%) and MGDG (32%). A decrease in MGDG with cold hardening was previously observed with Valencia on sour orange [2].

Lipids in leaves which had been cold-hardened showed a great deal less change over the stress stages than observed with UH leaf lipids (Table 2). Only PC (32%) and PE (19%) were degraded noticeably during the freeze stage. During the thaw stage fatty acids increased 14% (Table 2). Fatty acids associated with MAG, SE, PC, FFA, DAG and PE increased from 37 μg/g (MAG) to 244 μg/g (DAG) while acids in TAG increased 918 μg/g. Similar PC and PE responses to freeze-thaw stress were observed in rape leaves [21]. PA was the major degradation product of freeze-thaw stress of H plants as was observed in UH plants. PA was the major degradation product of PC in rye and wheat [9, 22]. PA formation paralleled the order of frost sensitivity of both species and cultivars [9].

Table 3 shows the changes in fatty acids in various lipids resulting from the freeze-thaw stress. Of the 4867 μg/g decrease in total fatty acid in UH tissue, 65% was due to 18:3 and only 9.5% to 18:2. These data indicate that 18:3 rich lipids in UH tissue are more susceptible to degradation than 18:2 lipids. Only 2039 μg/g or 42% of the 4867 μg/g decrease in total fatty acids in UH leaves under stress was accounted for in the acid increases in PA (53.6%), DAG (17.0%), FFA (7.7%), TAG (6.1%) and other neutral lipids. Loss of 18:3 and 18:2 acids in freeze-damaged wheat tissue without recovery in unesterified fatty acid fraction suggested rapid lipoxygenase action on FFA liberated by the hydrolases [22]. Thus, lipoxygenase action may account for the 58% net loss of fatty acids in UH leaves under stress. In hardy leaves the 694 μg decrease was shared by 18:3 and 18:2. Hardy winter wheat lost similar amounts of 18:3 and 18:2 from a lethal frost [22]. The glycolipids (MGDG and DGDG) accounted for most of the loss in 18:3 in both H and UH tissues. In H leaves, saturated and 18:2 acids were lost preferentially from PC.

PA and neutral lipids accounted for the increases in fatty acids in H and UH leaves under freeze-thaw stress. Although the total increases in fatty acids were similar in H and UH leaves, 18:3 accounted for 50% of the increase in H leaves and only 19% in UH leaves. About 71% of the increase in UH leaves was in non-polyunsaturates. TAG accounted for most of the increase in 18:3 in H leaves. Freeze-thaw stress increased 18:3 level in TAG 5-fold more than 18:2 level. This enrichment in 18:3 is in contrast to TAG increased by hardening where the 18:2 level increased about 2.25 times more than 18:3 level (Table 1). These data indicate that in hardened leaves freeze-thaw stress stimulated preferential synthesis of TAGs containing predominately 18:3. Separation and quantitation of the various TAG species illustrates this

Table 2. Levels of major fatty acids in leaves of citrus hybrid, B5-9-68 at prefreeze (PF), frozen (F) and thawed (T) stages of stress regime

	μg FA/g fr. wt					
	Unhardened			Hardened		
	PF	F†	T†	PF‡	F†	T†
Total lipid	8140	6350**	5312*	11337**	11094	12705**
Phospholipids						
PC	1082	251***	117**	1127	770**	885**
PE	591	663*	354**	1015**	826*	1051**
PG	645	331***	109**	302**	445**	327**
PI	325	256**	183*	423*	419	498**
PA	250	1264***	1342	267	704**	821**
Glycolipids						
MGDG	2772	1303***	574**	1885**	1769	1648*
DGDG	1571	1140**	783	1629*	1679	1529**
Neutral lipids						
TAG	186	222**	310*	3708***	3477	4395**
DAG	275	427**	622*	428**	514*	758***
MAG	75	82	175**	112*	81*	118**
FFA	98	75**	255**	173**	115**	308***
SE	270	336**	488*	268	295	367**

Phospholipids are: phosphatidyl—choline, PC; ethanolamine, PE; glycerol, PG; inositol, PI; PA, phosphatidic acid. Glycolipids are mono- and di- galactosyldiglycerides, MGDG and DGDG. Neutral lipids are mono-, di- and tri- acylglycerols, MAG, DAG, TAG. Sterol ester, SE; free fatty acids, FFA.

†Fatty acid levels of this lipid were significantly different (* = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$) between F vs PF and F vs T stages of stress.

‡Fatty acid levels of this lipid at the PF stages in unhardened and hardened leaves were significantly different (* = $p < 0.1$, ** = $p < 0.5$, *** = $p < 0.01$).

Table 3. Effects of freeze-thaw stress on fatty acid levels of hybrid B5-9-68

	μg/g fr. wt							
	Total		18:3		18:2		Other*	
	UH	H	UH	H	UH	H	UH	H
Total lipid								
Prefreeze level	8140	11337	4266	4390	748	3729	3126	3677
—Decreases	−4867	−694	−3148	−266	−460	−288	−1259	−140
—Increases	+2039	+2062	+381	+1033	+217	+430	+1441	+599
TAG increase	+124	+687	+11	+555	+15	+110	+98	+22
	% of above total lipid changes in specific lipids							
MGDG	−45.2	−34.1	−62.3	−82.7	−34.3	−13.5	−6.3	+3.7
DGDG	−16.2	−14.4	−18.9	−17.3	−8.3	−6.9	−12.2	−24.3
PC	−19.8	−34.9	−9.9	+2.9	−26.7	−57.6	−42.0	−75.7
PG	−11.0	+1.2	−3.7	+1.9	−14.6	+0.5	−28.1	+0.5
PA	+53.6	+26.9	+57.0	+13.5	+69.1	+53.0	+50.3	+31.2
TAG	+6.1	+33.3	+2.9	+53.7	+6.9	+25.6	+6.8	+3.7
Other NL†	+40.3	+27.6	+40.1	+16.9	+21.3	+20.5	+42.9	+51.2

*Includes 3t-16:1, 16:0, 18:0 and 18:1.

†Includes DAG, FFA, MAG and SE.

preference (Table 4). The three molecular species, trilinolenin (LnLnLn), linoleyldilinolenin (LnLnL) and linolenyldilinolein (LnLL) accounted for 72% of the TAG increase.

The high triacylglycerol (TAG) levels in hardened leaves may be incidental to protection of the phospholipids from degradation after freeze-thaw stress. However, enrichment of TAG with linolenic acid during

Table 4. Effect of freeze thaw stress on levels of triacylglycerol species in hardened citrus leaves

Carbon No.	Triacylglycerol species: Major species	Minor constituents‡	μg TAG/g fresh weight (s.d.)*: PF§	F	T	Change† (s.d.)
36	LnLnLn‖		57 (6)	88 (7)	260 (20)	+203 (18)
38	LnLnL		295 (27)	337 (29)	582 (28)	+287 (34)
40	LnLL		633 (39)	582 (24)	821 (32)	+188 (44)
40	LnLnP	(LnLnO)	177 (20)	201 (19)	225 (13)	+48 (21)
42	LLL		584 (16)	501 (26)	580 (62)	−4 (28)
42	LnLP	(LnLO + LnLnS)	589 (14)	587 (43)	653 (31)	+64 (29)
44	LLO	(LnOO)	95 (14)	80 (9)	91 (15)	−4 (10)
44	LLP		628 (34)	556 (30)	599 (52)	−29 (36)
44	LnPP	(LnLS + LnOP)	84 (15)	81 (21)	60 (25)	−24 (20)
46	LOP	(LOO + LLS)	182 (12)	140 (26)	162 (12)	−20 (13)
46	LPP	(LnOS + LnPS)	163 (15)	143 (15)	136 (20)	−27 (19)

*Standard deviation in triplicate HPLC analyses.
†Difference between PF and T stages.
‡Minor triacylglycerol species in HPLC peak.
§Prefreeze, frozen and thawed stages of stress.
‖Ln, Linolenate; L, linoleate; P, palmitate; O, oleate; S, stearate—constituent of molecule (location of acid on glycerol moiety not determined).

recovery of tissue from freeze-thaw stress suggests TAG may be involved in phospholipid protection as well as recovery. TAG is a known lipid component of cell membranes of poplars [5]. We have not as yet attempted to determine whether TAG is a component of membranes in citrus leaves. The great increase in TAG with cold-hardening accompanied with its further increase under freeze stress suggests, however, that TAG is a membrane component and, as suggested by Yoshida for poplar bark [5] may be involved in TAG-phospholipid interconversion under low temperatures. Further studies are being conducted to determine the possible role of TAG in freeze protection of citrus.

EXPERIMENTAL

Trees, hardening and freeze regimes. Hybrid B5-9-68 originated as follows: Duncan grapefruit (*Citrus paradisi*) was pollinated by 'Gotha Road' trifoliate orange (*Poncirus trifoliata*). Flowers of the mature progeny were pollinated by 'Succory' orange (*C. sinensis*). Hybrid B5-9-68, 'Gotha Road' and 'Succory' were grown as seedlings in pots in a greenhouse under general horticultural conditions described in previous cold hardening studies [1, 2]. After eight months the three citrus cultivars were grafted to sour orange rootstock (*C. aurantium*) and continued to be cultivated in a greenhouse. In the hybrid-parentage experiment six 12-month-old trees within each of the three citrus cultivars were selected for uniformity in physical health and growth. Three of these were left in the greenhouse an additional four weeks (UH), and the other three were cold hardened for four weeks in an environmental chamber as in previous studies (2 weeks—12 hr light at 70°/12 hr dark at 50° then 2 weeks—12 hr light at 60°/12 hr dark at 40°) [1, 2]. In the freeze study conducted two months later, 12 additional B5-9-68 plants were selected for uniformity. Six of these plants were cold hardened for four weeks [1, 2] while the UH plants were left in the greenhouse. Then 4 H and 4 UH plants were placed in an environmental chamber and the temperature lowered 5°/hr to −6.7° and held for 2 hr at which time 2 UH and 2 H plants were sampled (frozen stage). The chamber temperature was raised 5°/hr to +4.4° and the 2 UH and 2 H remaining plants transferred to a greenhouse and kept for 4 hr at 25–30° before being sampled (thawed stage).

Lipid extraction and fractionation. In both studies leaves from the entire tree were washed and blotted dry. After the main vein was removed the leaves were cut into small pieces and 15 g samples taken. Samples were in triplicate in the hybrid-parentage study and duplicate in the freeze study. Enzymes were deactivated by boiling the sample in methanol for 10 min. All samples after enzyme deactivation were stored in MeOH at −60° until extracted with $CHCl_3$–MeOH (2:1) within two weeks [1, 2]. In the parentage study duplicate 0.5 ml aliquots of the total lipid samples were reduced to approximately 0.2 ml under N_2. TAG in these samples were isolated by TLC with $CHCl_3$ as solvent [24]. In the freeze study five TLC solvent systems were used to resolve all 12 lipids analysed for their fatty acid profiles. Each system resolved from one to five of these 12 acyl lipids. The systems and lipid (or fraction) isolated were: (1) hexane–Et_2O–HOAc (79:10:1) resolved SE and TAG from a mixture of neutral and polar lipids. (2) Heptane–Et_2O–HOAc (75:25:4) resolved SE, TAG, FFA, DAG and MAG from polar lipids. (3) Acetone resolved neutral lipid mixture from MGDG, DGDG and phospholipid mixture. (4) $CHCl_3$–MeOH–7 N NH_4OH (65:30:4) resolved neutral lipid–MGDG mixture from FFA–PG mixture, PE–DGDG mixture, PC–PI mixture and PA. (5) $CHCl_3$–Me_2CO–MeOH–HOAc–H_2O (65:85:20:20:10) resolved neutral lipid–MGDG mixture from PA, DGDG–PE–PG mixture, PC and PI. All lipid samples were analysed for possible minor lipid constituents by spotting samples along with standards on TLC plates at a series of concentrations, developing the plates in the above solvent systems and spraying the plates with specific lipid sprays. Sulphoquinovosyldiglyceride was detected as a minor constituent in the PI fraction. Cardiolipin, phosphatidylserine, lysophospholipids and lysoglycolipids were not detected.

For the TAG HPLC analyses the duplicate lipid samples of the prefreeze hardened leaves remaining from the fatty acid analyses were combined and $CHCl_3$ removed under vacuum at 30°. Likewise hardened-freeze and hardened-thawed samples were

combined and concentrated. TAGs from the three samples were first isolated from polar lipids on 10 g silica gel columns eluted with 100 ml hexane–Et_2O (5:1) and subsequently isolated from other neutral lipids by prep. TLC on 1000 μ silica gel plates developed with hexane–Et_2O (5:1). Purity and absence of degradation were monitored by TLC and GLC analyses.

Lipid analyses. Aliquots of total lipid, lipid fractions and individual lipids were determined as fatty acid methyl esters (FAMEs) prepared by methanolysis with NaOH–BCl_3–MeOH [1–3] with methyl heptadecanoate as internal standard. FAMEs were prepared from bands scraped from TLC plates without prior removal of silica gel. FAMEs were analysed by FID-GC using glass columns packed with 10% SP-1000 on Gas Chrom Q [1]. In the parentage study, total FAs included only the five major C_{16}–C_{18} fatty acids while in the freeze study 3-*trans*-hexadecenoic was also included. Palmitoleic, a major component of SE, was not always resolved from palmitic. Thus, this acid is reported as 16:0 in Tables 1–3. The amounts of FAs in each of the 12 lipids were calculated as percentages of total lipid FA. These percentages were obtained from the GLC analyses of the three to six acyl lipid bands obtained from the five TLC systems. Values for the five or six FAs that comprise these lipid's total FA were calculated from the mean relative percentages of the FAs in the 12 lipids. The three samples of TAG (PF, F, T) were analysed by HPLC as reported in a previous study using the ternary gradient system of acetonitrile, methylene chloride and methyl-*t*-butyl ether [3]

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