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PHOSPHOLIPID, GALACTOLIPID, AND STERYL LIPID COMPOSITION OF APPLE FRUIT CORTICAL TISSUE FOLLOWING POSTHARVEST $CaCl_2$ INFILTRATION

G. A. PICCHIONI,* A. E. WATADA,† W. S. CONWAY,† B. D. WHITAKER† and C. E. SAMS‡

Department of Agricultural Sciences, Technology and Education, Louisiana Tech University, Ruston, LA 71272, U.S.A.; †Horticultural Crops Quality Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.; ‡Department of Plant and Soil Science, University of Tennessee, Knoxville, TN 37901, U.S.A

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Key Word Index—*Malus domestica*; Rosaceae; 'Golden Delicious'; senescence; lipid catabolism; sterol conjugation; plastid integrity; fruit softening; postharvest quality; HPLC.

Abstract—Fruit firmness and membrane lipid composition were evaluated in outer cortical tissue of 'Golden Delicious' apples, which were pressure-infiltrated with distilled water, or 2 or 4% $CaCl_2$ solutions at harvest. After six months storage at 0°, fruit were held at 20° for 1, 7 or 14 days and then evaluated. During storage at 0°, firmness had decreased by 20% in water-infiltrated fruit, but by only 6% in fruit infiltrated with 4% $CaCl_2$. During the span of 7 to 14 days at 20°, firmness decreased more rapidly in water-infiltrated fruit compared with $CaCl_2$-infiltrated fruit. Reductions in specific phospholipids (primarily phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) occurred after transfer to 20°, but were largely independent of the infiltration treatment. Steryl glycosides, acylated steryl glycosides, monogalactosyldiacylglycerol and digalactosyldiacylglycerol concentrations were positively correlated to $CaCl_2$ concentration of infiltration solutions, and a large, transient increase (35–37%) in acylated steryl glycoside concentration occurred during the first 7 days at 20° in $CaCl_2$-infiltrated fruit. In contrast, acylated steryl glycosides decreased by 19% in water-infiltrated fruit over the same time interval. Overall, the results indicate that $CaCl_2$ infiltration may delay galactolipid breakdown, increase the rate of sterol conjugation, and thus affect membrane organization and function during postharvest life of apple fruit.

INTRODUCTION

Membrane lipid alterations are thought to be a central factor in the senescence of harvested commodities [1, 2]. The importance of Ca^{2+} in cell membrane structure and function has long been recognized [3, 4], and it has been suggested that Ca^{2+} serves in the regulation of fruit ripening and senescence through interactions with cell membranes [5, 6]. Apple fruit with low Ca^{2+} concentrations are particularly prone to physiological disorders [7]. Increasing the Ca^{2+} content of apples has been shown to alleviate physiological storage problems [8, 9], reduce losses due to decay-causing organisms [10], and maintain fruit firmness and quality [11–13]. These studies have demonstrated that a positive correlation exists between Ca^{2+} concentration in treatment solutions, tissue Ca^{2+} content, fruit firmness and fruit quality.

Preharvest and postharvest Ca^{2+} applications to apple fruit can forestall the increases in cell membrane microviscosity [14] and cell membrane permeability [7, 15, 16] that are both characteristic of senescing apple fruit [17–19]. Ultrastructural evaluation has revealed a greater tendency for plastid membrane degradation during low temperature storage in low-Ca^{2+} compared with high-Ca^{2+} apple fruit [20].

Changes in the proportions and composition of membrane lipids are associated with membrane alterations during ripening of apple fruit [18, 21]. Direct and indirect evidence indicates that membranes of excised apple tissue cells [22, 23] or of isolated microsomes [24] respond to Ca^{2+}-containing solutions *in vitro*, but the relevance of these findings to responses of whole fruit has been questioned [25].

There have been several reports involving changes in membrane lipids in cortical tissue during ripening of whole apple fruit, [21, 26, 27], but none providing data on the effects of postharvest Ca^{2+} infiltration on membrane lipid composition. The theory that cell turgor reduction (through altered permeability) is partially accountable for losses in fruit firmness [28–30] demonstrates the significance of membranes in affecting the storage life and quality of apple fruit. The effect of Ca^{2+} on fruit firmness may be turgor-dependent, mediated through the stabilizing properties of Ca^{2+} on membranes

*Author to whom correspondence should be addressed.

[31]. Our objectives for this study were to investigate the effects of postharvest $CaCl_2$ infiltration of apples on firmness and on the concentrations of phospholipid, galactolipid, and steryl lipid classes in 'Golden Delicious' fruit cortical tissue.

RESULTS

Fruit firmness and Ca^{2+} concentration

At harvest, fruit firmness averaged 88.1 ± 1.8 N. During 6 months of cold storage, firmness decreased by 20% in fruit infiltrated with distilled water (0% $CaCl_2$), but by only 6% in fruit infiltrated with 4% $CaCl_2$ (day 1 at 20° in Fig. 1). Firmness measurements recorded on an identical number of fruit lots following five months at 0° (as described in Experimental for 6-month lots) and up to seven days at 20° did not differ from the values in Fig. 1 (data not shown).

Following removal from low temperature storage, firmness decreased significantly during the 14-day period at 20° but not at a constant rate. For $CaCl_2$-infiltrated fruit (both 2 and 4% $CaCl_2$ treatments), no softening occurred between one and seven days at 20°, whereas firmness of the water-infiltrated fruit had decreased by a further 8% during this time (Fig. 1). Between seven and 14 days at 20°, firmness of all fruit decreased to a similar degree, but remained greater in $CaCl_2$-infiltrated fruit. The 2 and 4% $CaCl_2$ infiltration treatments increased fruit Ca^{2+} concentration over 3-fold and 10-fold, respectively, above water-infiltrated fruit (Table 1).

Lipid class analyses

Glycolipid and phospholipid chromatograms (HPLC) enabled the identification and quantitation of nine polar lipid classes. Phosphatidlyserine, phosphatidylglycerol, sulpholipids and cerebrosides were present in insufficient quantities to allow detection. Digalactosyldiacylglycerol (DGDG; present in the phospholipid fraction rather than the glycolipid fraction) eluted near the void volume; this peak was well resolved from the phospholipid peaks. The calibration and quantitation of DGDG were thus completed in conjunction with the phospholipid chromatograms. Total phospholipid mass (TPL) was estimated both by lipid-P determination (data not shown) and by totalling the masses of individual phospholipids calculated after integration of HPLC peaks. Lipid-P values averaged 77.9 ± 3.6% of HPLC values; both methods were well correlated ($r = 0.96$ among 48 individual phospholipid fractions).

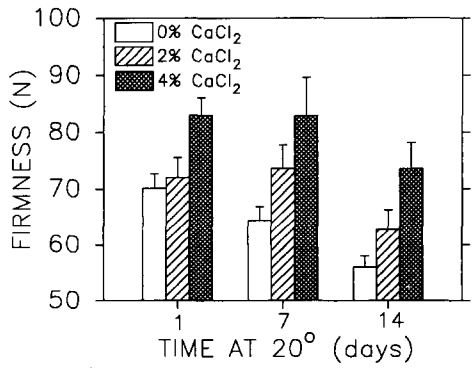

Fig. 1. Flesh firmness of 'Golden Delicious' apple fruit. Fruit were infiltrated with deionized water (0% $CaCl_2$) or with $CaCl_2$-containing solutions at harvest, stored for 6 months at 0°, and held at 20° for 1, 7 or 14 days. Each value is the mean ± s.d. of four, 5-fruit replicates. Significant F-tests: $P < 0.01$ (days at 20° and $CaCl_2$ infiltration treatment).

Table 1. Calcium concentration in outer cortical tissue of 'Golden Delicious' apple fruit following one day at 20° (fruit treated and handled as described in Fig. 1)

$CaCl_2$ soln (% w/v)	Ca concn (mg kg^{-1} dry weight)
0	201 ± 15
2	655 ± 246
4	2187 ± 119

F-test significant at $P < 0.01$.
Each value is the mean ± s.d. of four, 5-fruit replicates.

Concentrations of all glycolipid classes changed significantly with time at 20° and were increased by $CaCl_2$ infiltration (Table 2). Steryl glycoside (SG) concentrations were greater than those of acylated steryl glycosides (ASG). The inverse changes in ASG concentration between water and $CaCl_2$-infiltrated fruit resulted in an interaction between days at 20° and $CaCl_2$ infiltration (see footnote of Table 2). Between one and seven days at 20°, ASG levels increased by 35–37% in $CaCl_2$-infiltrated fruit, but then decreased by 48–53% between seven and 14 days. By contrast, ASG concentrations in water-infiltrated fruit decreased by 19% between one and seven days at 20°, with no further change between seven and 14 days. Changes in ASG concentrations of noninfiltrated fruit were identical to those of water-infiltrated fruit, although the concentrations in noninfiltrated fruit averaged 30% less (data not shown).

Unlike the changes in ASG, changes in SG concentrations with time at 20° were similar among the infiltration treatments. In all infiltrated fruit, SG concentrations increased by 14–23% between one and seven days at 20°, then decreased by 7–9% thereafter. On average, SG concentrations of fruit infiltrated with 4% $CaCl_2$ were 12 and 7% greater, respectively, than those in fruit infiltrated with water and 2% $CaCl_2$.

DGDG and monogalactosyldiacylglycerol (MGDG) were present in a mass ratio of between 1.5 and 1.7 one day after transfer of fruit to 20°. This ratio decreased with further time at 20° as a result of substantial reductions in DGDG concentration (average net loss of 56% during the 14-day period at 20°). Following one day at 20°, MGDG and DGDG concentrations were 32% and 14% greater, respectively, in high-$CaCl_2$ fruit compared with water-infiltrated fruit, but this difference was less apparent by seven and 14 days at 20°. MGDG concentrations decreased by 18–21% between seven and 14 days at 20°,

Table 2. Glycolipid class concentrations in outer cortical tissue of 'Golden Delicious' apple fruit (treated and handled as described in Fig. 1)

$CaCl_2$ soln (% w/v)	Glycolipid concn (mg 100 g^{-1} dry wt)* ASG	SG	MGDG	DGDG
1 day at 20°				
0	21.2 ± 4.4	47.7 ± 3.1	39.5 ± 5.8	66.2 ± 2.4
2	25.2 ± 6.6	50.5 ± 1.2	44.9 ± 4.4	71.3 ± 7.1
4	29.4 ± 5.3	56.7 ± 6.2	52.1 ± 7.9	75.5 ± 7.3
7 days at 20°				
0	17.2 ± 0.7	58.6 ± 1.1	41.8 ± 1.3	41.3 ± 2.3
2	34.5 ± 7.7	61.7 ± 5.7	44.6 ± 4.8	43.1 ± 5.0
4	39.6 ± 11.8	64.7 ± 5.0	45.0 ± 4.4	43.7 ± 3.4
14 days at 20°				
0	17.6 ± 2.0	54.6 ± 1.7	34.1 ± 2.3	31.5 ± 3.0
2	16.3 ± 1.0	56.4 ± 5.0	35.1 ± 4.8	33.1 ± 5.3
4	20.6 ± 3.8	58.7 ± 2.7	35.5 ± 2.6	28.6 ± 1.1

*Abbreviations used as follows: ASG, acylated sterol glycosides; SG, sterol glycosides; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

Significant F-tests: days at 20°, $P < 0.01$ (all classes); $CaCl_2$ infiltration treatment, $P < 0.01$ (ASG, SG), $P < 0.05$ (MGDG, DGDG); days × infiltration interaction, $P < 0.01$ (ASG).

Each value is the mean ± s.d. of four, 5-fruit replicates.

during which time all fruit had similar concentrations. Overall, the net reduction in MGDG concentration through the 14-day period at 20° averaged 23%. Thus, a differential rate of net loss occurred among the two galactolipid classes.

Following the cold storage period, the proportions of individual phospholipid classes changed very little (data not shown), with phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) averaging 58, 22 and 14%, respectively, of the TPL mass. Nonetheless, concentrations of all phospholipids decreased significantly when fruit were moved to 20° (Table 3).

Although there was an indication that TPL concentrations of fruit treated with 4% $CaCl_2$ were greatest for as long as seven days at 20°, only phosphatidic acid (PA) concentrations were significantly affected by the infiltration treatment. PA represented 4–8% of TPL mass and changed little during the last seven days at 20°, but its concentration was inversely related to the $CaCl_2$ treatment concentration. Throughout the 14-day period at 20°, PA averaged 19.1, 15.2 and 12.7 mg 100 g^{-1} dry wt for 0, 2 and 4% $CaCl_2$ treatments, respectively.

Loss of TPL at 20° was greatest in fruit infiltrated with 4% $CaCl_2$ on either an absolute basis or as a percentage reduction below the day one value. The percentage reduction in TPL during the first seven days at 20° ranged from 8.5 to 16.8 and was quite variable for all treatments (Table 3). Following 14 days, there was nearly double the loss in TPL (on a percentage reduction basis) in fruit from the 4% $CaCl_2$ treatment compared with the other infiltration treatments. Between one and 14 days at 20°, high-Ca^{2+} fruit lost 114.6 ± 36.9 mg TPL per 100 g dry wt of tissue as opposed to only 58.1 ± 13.6 mg per 100 g and 57.5 ± 16.6 mg per 100 g for 0 and 2% $CaCl_2$ treatments, respectively.

Sitosterol represented the dominant free sterol of the total free sterol pool (TFS), while campesterol was only a minor constituent (Table 4). Free stigmasterol, cholesterol, as well as steryl esters, were typically below detection limits. $CaCl_2$ infiltration had no effect on individual free sterol concentrations, TFS or the TFS:TPL ratio. On average, TFS concentrations and the TFS:TPL ratio increased by 8 and 26%, respectively, between one and seven days at 20°. However, these values subsequently decreased by an average of 14 and 4%, respectively, between seven and 14 days at 20°.

DISCUSSION

The results reported in this study agree with earlier findings which demonstrated that, in similar experimental conditions, the rapid softening of 'Golden Delicious' fruit in storage can be delayed by postharvest $CaCl_2$ infiltration treatments [12, 32, 33]. Although the processes which govern apple fruit softening in storage are not fully understood [28], the reductions in galactolipid and phospholipid concentrations and the increase in TFS:TPL ratio which occurred at 20° suggest membrane degradative reactions. These processes are known to occur during senescence and fruit ripening [19, 34, 35].

Pronounced degradation of galactolipids in the flesh of ripening apple fruit is probably associated with selective breakdown of plastid membranes [21], where DGDG and MGDG are most abundant in the plant cell [36, 37]. De Barsy *et al.* [38] showed that a 43% loss of plastid

Table 3. Phospholipid class concentrations, total phospholipid (TPL), and the reduction in TPL (expressed as percentage decrease below day 1 value) in outer cortical tissue of 'Golden Delicious' apple fruit (treated and handled as described in Fig. 1)

$CaCl_2$ soln (% w/v)	Phospholipid concn (mg 100 g^{-1} dry wt)*						% Reduction in TPL
	PE	PI	PA	PC	LPC	TPL	
1 day at 20°							
0	60.4 ± 1.4	45.8 ± 2.0	22.1 ± 2.1	161.4 ± 7.3	2.2 ± 0.4	291.9 ± 8.3	—
2	64.4 ± 4.0	46.8 ± 6.4	18.0 ± 2.1	170.8 ± 9.6	2.0 ± 0.3	302.1 ± 19.7	—
4	72.7 ± 9.2	55.4 ± 10.2	14.6 ± 1.3	183.5 ± 17.3	3.0 ± 0.5	329.2 ± 36.2	—
7 days at 20°							
0	55.4 ± 4.0	37.9 ± 4.0	17.2 ± 2.4	154.9 ± 8.4	1.6 ± 0.3	267.0 ± 13.2	8.5 ± 5.2
2	53.2 ± 8.1	39.8 ± 7.6	13.0 ± 3.0	153.4 ± 20.7	2.0 ± 0.4	261.4 ± 37.3	13.8 ± 7.8
4	61.2 ± 5.8	34.6 ± 2.4	12.6 ± 2.4	162.0 ± 6.7	2.0 ± 0.5	272.4 ± 14.1	16.8 ± 5.6
14 days at 20°							
0	53.4 ± 3.4	25.2 ± 0.9	17.8 ± 0.9	136.2 ± 3.9	1.1 ± 0.5	233.8 ± 8.1	19.8 ± 4.2
2	54.5 ± 6.8	32.2 ± 4.1	14.5 ± 3.5	142.6 ± 14.6	0.8 ± 0.4	244.6 ± 25.9	19.1 ± 5.6
4	49.2 ± 4.0	22.4 ± 3.4	10.9 ± 3.1	131.4 ± 4.8	0.8 ± 0.6	214.6 ± 9.3	34.2 ± 7.9

*Abbreviations used as follows: PE, phosphatidylethanolamine; PI, Phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

Significant F-tests: days at 20°, $P < 0.01$ (all classes and TPL); $CaCl_2$ infiltration treatment, $P < 0.01$ (PA).

Each value is the mean ± s.d. of four, 5-fruit replicates.

Table 4. Free sterol concentration, total free sterol concentration (TFS), and total free sterol:total phospholipid mole ratio (TFS:TPL) in outer cortical tissue of 'Golden Delicious' apple fruit (treated and handled as described in Fig. 1)

$CaCl_2$ soln (% w/v)	Sterol concn (mg 100 g^{-1} dry wt)			TFS:TPL ratio
	Sitosterol	Campesterol	TFS	
1 day at 20°				
0	65.7 ± 0.6	1.0 ± 0.1	66.8 ± 0.6	0.42 ± 0.01
2	74.4 ± 8.7	1.1 ± 0.1	75.6 ± 8.8	0.46 ± 0.03
4	72.3 ± 12.9	1.1 ± 0.2	73.4 ± 13.1	0.42 ± 0.04
7 days at 20°				
0	77.9 ± 2.2	1.3 ± 0.1	79.1 ± 2.3	0.53 ± 0.01
2	75.1 ± 8.0	1.2 ± 0.2	76.3 ± 8.1	0.56 ± 0.08
4	76.0 ± 8.8	1.3 ± 0.2	77.3 ± 9.0	0.53 ± 0.04
14 days at 20°				
0	65.3 ± 1.3	1.1 ± 0.1	66.4 ± 1.4	0.51 ± 0.02
2	67.6 ± 6.0	1.2 ± 0.2	68.8 ± 6.2	0.51 ± 0.02
4	63.2 ± 3.4	1.1 ± 0.1	64.3 ± 3.5	0.55 ± 0.03

Significant F-tests: days at 20°, $P < 0.01$ (sitosterol, TFS, TFS:TPL ratio), $P < 0.05$ (campesterol).

Each value is the mean ± s.d. of three, 5-fruit replicates.

volume occurs in cells of 'Golden Delicious' flesh after only three months of storage in air at 1°. The plastid envelope is visibly altered in low-Ca^{2+} fruit [20], and the fact that both MGDG and DGDG concentrations were greater in $CaCl_2$-treated fruit upon transfer to 20° (Table 2) indicates that $CaCl_2$ treatment may have delayed plastid degradation during low temperature storage. This finding may be important for both the chloroplast and amyloplast, which share similar morphological and metabolic functions [39] as well as envelope lipid profiles [40]. For example, loss of green coloration during ripening of 'Golden Delicious' fruit is delayed by Ca^{2+} infiltration treatments [7, 41]. We also observed greater retention of green coloration by $CaCl_2$-infiltrated fruit following low temperature storage (data not shown). Likewise, the ripening-related increase in starch hydrolysis of apple fruit [42, 43] is presumably accelerated by amyloplast membrane degradation. In potato tuber, recent evidence has suggested that increases in free sugar concentrations during senescence are the result of peroxidative attack on amyloplast membranes, which may be curbed in the presence of Ca^{2+} [44].

Collectively, these findings may indicate that a possible role of applied Ca^{2+} in delaying apple fruit ripening is

through preservation of the integrity of plastid membranes, thus delaying catabolic pathways associated with decompartmentation processes, such as starch and chlorophyll breakdown.

In plant cells, free and conjugated sterols are thought to occur primarily in the plasma membrane [45, 46], and sterol conjugation may be an important determinant of membrane organization and function [47, 48]. In addition, free sterol (FS) glycosylation occurs on the plasma membrane [49] and is believed to be tightly coupled to the SG acylation process to form ASG [50]. These results indicate that the net synthesis of SG and particularly ASG in apple fruit following low temperature storage may be dependent on the fruit Ca^{2+} content.

It has recently been postulated that membrane sterols comprise a major proportion of plasma membrane lipid components, and that sterol conjugates are involved in stress acclimatization at the plasma membrane level [46 and R. Moreau, personal communication]. The maintenance of plasma membrane integrity (and thus cell turgor), if dependent on SG and ASG accumulation, could possibly delay fruit softening, since part of the softening process is believed to result from turgor loss [29, 31]. Very little is known about the role of plant sterols in determining membrane properties [45], thus further study is necessary to verify this possibility.

The changes in phospholipid concentrations are difficult to interpret, since their actual sites within the cells cannot be determined with the extraction procedure used. $CaCl_2$ infiltration did not reduce the rate of TPL loss following cold storage, rather TPL reduction appeared to be greatest in high-Ca^{2+} fruit. The fact that these fruit maintained greatest firmness following cold storage indicates that TPL content may not in itself be the only membrane-associated component related to apple fruit firmness.

In disrupted plant tissues, phospholipase-D is known to hydrolyse membrane-bound phospholipids into PA [51]. Thus, even though $CaCl_2$ infiltration appeared to reduce the concentration of PA following cold storage, part of the measured PA may have arisen during the extraction process.

Although $CaCl_2$ infiltration did not affect TFS levels or the TFS:TPL ratio following six months cold storage, we have confirmed increases in TFS concentrations of water-infiltrated apple fruit which do not occur in $CaCl_2$-infiltrated fruit during 3-month cold storage periods (unpublished data). Research is in progress to evaluate the effects of $CaCl_2$ infiltration on TPL and TFS levels during and after apple fruit cold storage periods.

In conclusion, the results demonstrate the involvement of $CaCl_2$ in senescence-related membrane lipid changes of apple fruit. The fact that galactolipid breakdown was delayed in $CaCl_2$-treated fruit is consistent with the known stabilizing effects of Ca^{2+} on cell (plastid) membranes. The influence of $CaCl_2$ infiltration on conjugated sterol concentrations may imply an effect on structure and function of the plasma membrane. The significance of the increased levels of SG and ASG in $CaCl_2$-infiltrated fruit and the possible Ca^{2+}-dependence of enzymes involved in sterol gylcosylation and acylation of apple fruit need to be elucidated.

EXPERIMENTAL

Test material and postharvest handling. 'Golden Delicious' fruit were harvested at the preclimacteric stage from a commercial orchard. The apples were randomized into 12 equal lots of 20 fruit. One day after harvest, each of three, 3-lot groups were pressure infiltrated (103 kPa; 3 min) with distilled water (control) or with $CaCl_2$ (99% dihydrate salt, Sigma), at rates of 2 or 4%. The remaining 3 lots were not infiltrated.

Following infiltration, fruit were allowed to air dry, placed in tray-packed boxes with perforated polyethylene bags as liners, and stored in air at 0° and 85 ± 5% RH for 6 months. After the 0° storage period, fruit were moved to a 20° room for 1, 7 and 14 days, at which time fruit firmness, Ca^{2+} concns and lipid classes were determined as described below.

Firmness evaluation. Fruit firmness was measured on 20 individual fruit per treatment (four, 5-fruit replicates) using an electronic pressure tester (EPT-1; Lake City Technical Products) set in the Magness–Taylor mode and interfaced to a personal computer [33]. The instrument had an 11-mm tip which penetrated to a depth of 8 mm. Two measurements were made on opposite, equatorial sides of each fruit with the peel removed.

Sample prepn and analyses. Immediately following firmness measurements, the peel and outer 1–2 mm of flesh of the whole fruit was removed using a mechanical peeler and was discarded. The next 2–3 mm of flesh (cortical) tissue was removed with the peeler and used for Ca^{2+} and lipid class analyses. After removal from the fruit, the tissue was freeze-dried and ground to provide a total dry wt of 5–10 g per sample. Each sample was made up of the flesh of 5 fruit and 3–4 samples were analysed per treatment.

For Ca^{2+} analysis (for the 1-day period at 20° only), 1-g subsamples of the dried tissue were ashed, dissolved in 2N HCl, and filtered. Ca^{2+} concns were determined as described by Sams and Conway [12] using a plasma emission spectrometer (Model 61, Thermo Jarrell Ash Corp.).

Total lipid extracts were obtained using methods described earlier [52]. Briefly, 500-mg subsamples were homogenized in $CHCl_3$–MeOH (2:1) with three, 15-sec bursts of a Polytron homogenizer. Homogenates were filtered through a sintered glass funnel and the tissue residue was re-extracted with $CHCl_3$–MeOH (2:1). The combined filtrates were washed sequentially with 0.85% NaCl and MeOH–H_2O (1:1). The $CHCl_3$ phase containing total lipids was dried under N_2 and redissolved in 1 ml $CHCl_3$.

To separate neutral lipids, glycolipids and phospholipids, we used the method of Glass [53] with slight modifications. The total lipid extract was passed through a single silica sep-pak cartridge (Waters) to sequentially elute two neutral lipid frs, then glycolipids and phospholipids as follows: 3 ml $CHCl_3$ to precondition the

cartridge; sample applied in 1 ml $CHCl_3$ followed by 4 ml $CHCl_3$ to elute pigments and steryl esters; 8 ml of 9:1 $CHCl_3$–Me_2CO to elute free sterols (FS) and pigments of greater polarity; 8 ml Me_2CO to elute the glycolipids ASG, SG and MGDG; and 12 ml of 9:1 MeOH–H_2O to elute the phospholipids PE, PI, PA, PC and LPC (lysophosphatidylcholine). Using this procedure, DGDG eluted entirely with the phospholipid rather than the glycolipid fr. Each fr. was dried under N_2, redissolved in 1 ml $CHCl_3$ (neutral lipids and glycolipids) or 1 ml of 1:1 $CHCl_3$–MeOH (phospholipids), and stored under N_2 at −70° until the time of analysis.

Prior to HPLC analyses (see below), aliquots of the glycolipid and phospholipid frs were dried under N_2 and redissolved in HPLC mobile phase (1:1 mixt. of *iso*-PrOH–hexane for glycolipids and a 58:40:2 mixt. of *iso*-PrOH–hexane–H_2O for phospholipids). Samples were then passed through a 0.2-μm PTFE membrane filter (Gelman Sciences) using a gas-tight syringe. The syringe and filter were washed twice with mobile phase to completely recover the held-up sample vol. Combined filtrates were dried under N_2, taken up in a final mobile phase vol. of 200 μl, and a sufficient vol. was transferred to autosampler vials for 100-μl injections (see below).

Total lipid-P was determined on duplicate, 20-μl aliquots of the phospholipid frs using the method of Ames [54] and assuming an average M_r of 750 [55]. FS in the second neutral lipid fr. (see above) were pptd with digitonin and analysed by capillary GLC–FID [56] using lathosterol (cholest-7-en-3β-ol; Sigma) as an int. standard (10 μg added to total lipid extract before fractionation). The carrier gas (He) flow rate and oven temp. were maintained constant at 1 ml min^{-1} and 260°, respectively. The injector temp. was 300° and the detector temp. 350°.

Component classes in the glycolipid and phospholipid frs were sepd using a recently developed HPLC method [57, 58]. Independent injections (100-μl) were made for each fr. and sepn was achieved on a 10 cm × 3 mm Chromsep LiChrosorb Si 60 (5-μm) silica cartridge system (Chrompack). The HPLC system included a WISP 712 programmable injector and 600E multisolvent delivery system (Waters). To begin an analysis, the injector initialized solvent gradient delivery and data acquisition on a personal computer. Maxima 820 software (Dynamic Solutions) was used for peak integration and determination of lipid class concns.

Glycolipid and phospholipid classes were quantified using a Varex IIA evaporative light scattering detector (Varex Corp.). The detector N_2 flow rate was maintained constant at 45 mm (2.5 l min^{-1}) and the drift tube temp. at 90° throughout all analyses. Calibration curves (for external standardization) were generated for each set of analyses by injecting a concn series of commercial lipid standards. Glycolipid standards (MGDG, DGDG, ASG and SG) were purchased from Matreya, Inc. and phospholipid standards (PE, PI, PA, PC, and LPC) from Sigma. HPLC-grade solvents (*iso*-PrOH and hexane) were obtained from Mallinckrodt. Water used for the phospholipid gradient (see below) was purified using a Nanopure II system (Barnstead), then passed through 0.22-μm cellulose filters (Nitroplus) before use.

The mobile phase flow rate was maintained constant at 0.5 ml min^{-1} and the composition reported by Moreau *et al.* [57] and Letter [58] was modified to optimize sepn. Glycolipids were eluted using linear gradients without water, beginning with a mixt. of 5:95 (*iso*-PrOH–hexane) to 20:80 in 15 min, to 40:60 in 10 min, a 25-min hold, a reverse to the starting solvent in 5 min, and a 40-min hold for re-equilibration. Phospholipids were eluted with a mobile phase of *iso*-PrOH–hexane–H_2O using a concave gradient starting from 58:40:2 to 52:40:8 in 20 min, a 40-min hold, a reverse linear gradient to the starting mixt. in 15 min, and a 40-min hold for re-equilibration.

Statistical analyses. The experiment was completely randomized and subjected to analysis of variance using the General Linear Models Procedure (SAS Institute) for a 3 × 3 factorial (0, 2 and 4% $CaCl_2$ infiltration treatments × 1, 7 and 14 days at 20° following 6 months of 0° storage). Where significant, *F*-tests for main effects (days at 20°, $CaCl_2$ infiltration treatment, and days × infiltration interaction) are noted in the presentation of the data. Each observation is reported as the mean ± s.d. of four, 5-fruit replicates (3 replicates only for FS and TFS:TPL mole ratio). Results involving non-infiltrated fruit were excluded from statistical analyses. Unless noted, results did not differ significantly from water-infiltrated fruit ($P < 0.05$).

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