Plant Physiol. (1992) 100, 1656-1660
0032-0889/92/100/1656/05/$01.00/0

Received for publication April 3, 1992
Accepted July 27, 1992

Delay of Membrane Lipid Degradation by Calcium Treatment during Cabbage Leaf Senescence[1]

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ABSTRACT

Cabbage leaf discs (*Brassica oleracea* L., Capitata group) were floated adaxial side up in 0, 0.05, or 0.25 M $CaCl_2$ solutions at 15°C for 14 d in the dark. To assess whether the delay of senescence by calcium treatment involved protection of membrane lipids, chlorophyll and protein content and the lipid composition of the membranes were determined during incubation. Chlorophyll and protein content decreased with time, in correlation with a reduction in the amount of phospholipids. The degree of unsaturation of phospholipids and free fatty acids decreased, whereas the ratio of sterol to phospholipid increased. The proportions of phospholipid classes did not change during senescence. The catabolism of phospholipids was delayed by 0.05 M calcium, but accelerated by 0.25 M, as compared to the untreated control. Based on the levels of the lipid intermediates, phospholipase D, phosphatidic acid phosphatase, lipolytic acyl hydrolase, and lipoxygenase appeared to be involved in the breakdown of phospholipids during senescence. Phospholipase D and phosphatidic acid phosphatase may be directly influenced by calcium. The calcium treatment apparently did not affect the activity of acyl hydrolase. Lipoxygenase, responsible for the peroxidation of the polyunsaturated fatty acids, was probably indirectly influenced by calcium. We conclude that the delay of senescence of cabbage leaf discs by calcium treatment involved protection of membrane lipids from degradation.

Loss of cell membrane integrity is characteristic of senescence in plants (21). This is evident from progressive ultrastructural deterioration and from increased leakage of solutes (16).

Reduced membrane PL[3] content during senescence is an indication of membrane breakdown, as shown for senescing carnation petals (8, 22). Senescence can be delayed by calcium treatment (19), either by preharvest application (5) or by postharvest dip or vacuum infiltration (18). However, some references cited in the review paper by Ferguson (7) report acceleration of senescence. Although calcium treatment of fruits and vegetables has been shown repeatedly to delay senescence (7), the specific mode of action of calcium is not clear. Calcium, which plays an important role in many physiological processes, binds extracellulary to membrane PL. In this way, calcium maintains membrane integrity and controls membrane-associated functions (9). However, an increase in cytosolic calcium may also stimulate lipolytic enzyme activity and accelerate membrane deterioration (21).

In the present study, we investigated whether the delay of senescence of cabbage leaf discs by calcium was related to the protection of membrane lipids from degradation during senescence.

MATERIALS AND METHODS

Plant Material and Environmental Conditions

Cabbage (*Brassica oleracea* L., Capitata group) was obtained from the central warehouse of a local food distribution chain (Provigo, Québec, QC, Canada). Leaf discs measuring 1 cm in diameter were excised from the interveinal primary leaf with a cork borer. About 25 discs were floated adaxial side up in 10 mL of 0, 0.05, or 0.25 M $CaCl_2$ solution in 125-mL Erlenmeyer flasks. The levels of calcium were chosen after preliminary tests showed delay of Chl degradation below 0.07 M and acceleration above 0.15 M. The discs were incubated in the dark at 15 ± 1°C for 14 d.

Chl and Protein Determination

Total Chl was determined by the method of Arnon (2). Protein content was determined as described by Lowry et al. (10).

Lipid Extraction and Analysis

At the end of the incubation, the discs were fixed in boiling water for 3 min to inactivate endogenous phospholipases. Total lipids were extracted from the tissue using the procedure of Bligh and Dyer (3). The lipids in the chloroform phase were separated by TLC on 250-μm silica gel G plates (Fisher Scientific Co., Ottawa, ON). Acetone:acetic acid:water (100:2:1, v/v) was used to separate the PL from the galacto-

[1] This research was supported by the Conseil des Recherches en Pêche et Agro-alimentaire du Québec and the Natural Sciences and Engineering Research Council of Canada. Contribution No. 263 from the Centre de Recherches et de Développement sur les Aliments, Agriculture Canada, Saint-Hyacinthe. Contribution No. 61 from the Centre de Recherches en Horticulture, Université Laval.

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[3] Abbreviations: PL, phospholipid; DAG, diacylglycerol; LOX, lipoxygenase; FA, fatty acid; PUFA, polyunsaturated fatty acid; FFA, free fatty acid; PA, phosphatidic acid; FS, free sterol.

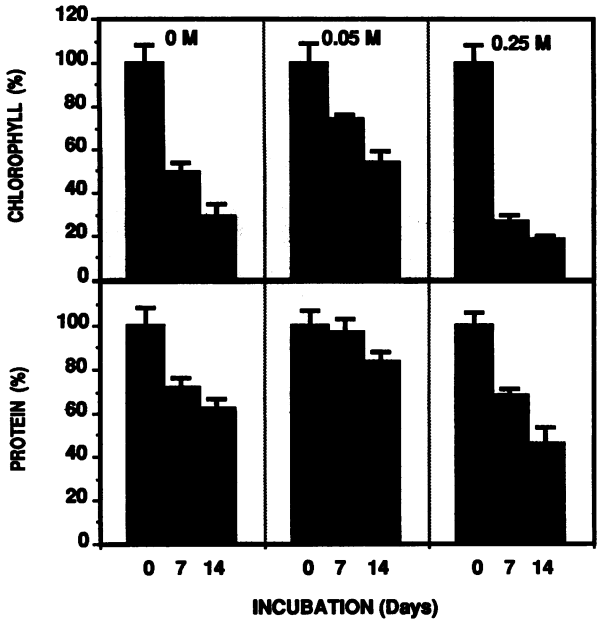

Figure 1. Change with time in Chl and protein content of cabbage leaf discs floated in 0, 0.05, and 0.25 M $CaCl_2$ solutions at 15°C during 14 d in the dark. Values are means ± SD for $n = 3$ to 4.

lipids, hexane:diethyl ether:acetic acid (80:20:1, v/v) was used to separate the neutral lipids, and chloroform:methanol:acetic acid:water (80:15:15:3.5, v/v) was used to separate the PL. The lipids were visualized briefly in iodine vapors and identified using authentic standards (Sigma).

The area corresponding to each lipid class on the TLC plate was scraped into a test tube and transmethylated directly onto the silica gel with 14% (w/v) BF_3 in methanol (13). For quantitative determination of FA, a known amount of heptadecanoate (C17:0) was added as an internal standard. Methyl esters of FA were analyzed by GLC on a 30-m capillary DB 225 column (J & W Scientific, Rancho Cordova, CA) as described by Makhlouf et al. (12). FS were silylated directly on the silica gel (6) and assayed by GLC (Hewlett-Packard model 5890A, Mississauga, Canada) using cholestane as a standard. Sterol trisilyl derivatives were separated by GLC on a 25-m ULTRA 1 capillary column (Hewlett-Packard). The PL content was also determined by phosphorus analysis (1). An average PL mol wt of 750 was assumed for calculation of the PL content.

LOX Assay

LOX activity was determined spectrophotometrically at 234 nm (11). The standard assay mixture contained 1.5 mM linoleic acid and 0.5% (v/v) Tween 20 in 50 mM Pipes buffer (pH 7). A 0.5-mL aliquot of the extract was added to 2 mL of reagent in a cuvette.

Statistical Analysis

Most analyses were carried out in quadruplicate. Analysis of variance was performed following a split-plot design in which the replications were arranged in randomized complete blocks (20). Means were compared with planned contrasts from the GLM procedure of the SAS statistical package (SAS Institute, Cary, NC).

RESULTS

Chl and protein content of leaves decreased significantly with time ($P \leq 0.001$) (Fig. 1), with Chl faster than protein. Treatment with 0.05 M $CaCl_2$ delayed the loss of Chl and protein, whereas 0.25 M $CaCl_2$ accelerated it. PL and FS content were measured to determine whether the changes in Chl and protein content were associated with an alteration in membrane lipid composition.

Total PL content declined during incubation for all treatments (Fig. 2). The PL-FA analysis confirmed the results of the lipid phosphorus assay. The rate of decline in PL level was less at 0.05 M $CaCl_2$, but greater at 0.25 M ($P \leq 0.001$) than in the control. The more important PL were phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol (44, 37, 11, and 8%, respectively). The proportions of the PL classes did not change significantly during incubation ($P \leq 0.05$), indicating that each class of PL declined at a similar rate.

The total amount of sterols on a fresh weight basis showed no significant change under any of the treatments during incubation. The loss of PL from the membranes was reflected in a shift in the ratio of FS to PL (Table I) ($P \leq 0.001$). The ratio, which increased significantly for the control and even more at 0.25 M $CaCl_2$, hardly changed at 0.05 M and was closely correlated with the loss of Chl and protein ($r = 0.94$ and 0.82, respectively).

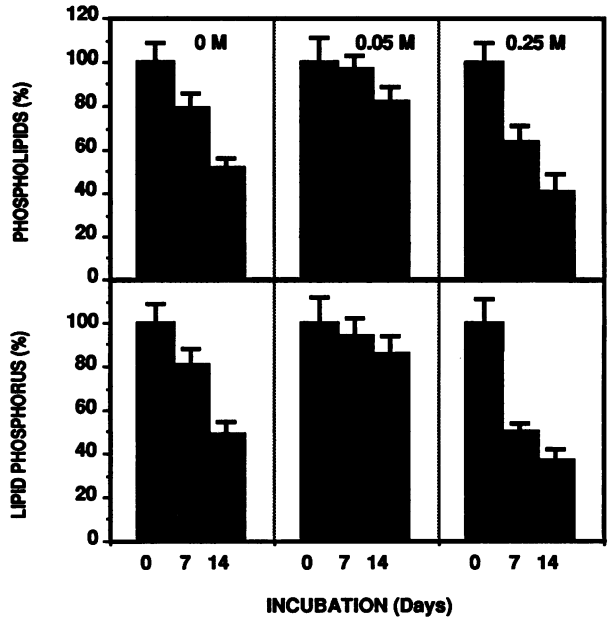

Figure 2. Change with time in phospholipids and lipid phosphorus content of cabbage leaf discs floated on 0, 0.05, and 0.25 M $CaCl_2$ solutions at 15°C during 14 d in the dark. Values are expressed as percentages of the value at day 0 ± SD for $n = 3$ to 4. Values (μg/g fresh weight) at day 0 were 523 ± 32.2 μg for PL and 513 ± 24.2 μg for lipid phosphorus.

Table I. *Change with Time in the Ratio of FS to PL (μg/μg) in Cabbage Leaf Discs Floated on 0, 0.05, and 0.25 M $CaCl_2$ Solutions at 15°C for 14 d in the Dark*

Values are means ± SD for $n = 3$ to 4.

Calcium Concentration	Incubation (d)		
	0	7	14
M		*FS/PL (μg/μg)*	
0	0.14 ± 0.01	0.16 ± 0.01	0.25 ± 0.03
0.05	-	0.14 ± 0.03	0.16 ± 0.02
0.25	-	0.20 ± 0.02	0.32 ± 0.06

Table II shows the FA composition of the PL and FFA fractions. The PL were rich in linolenic acid, and their ratio of PUFA to saturated FA (mol%), 2.09, was greater than that of the FFA, 0.41. Loss of PUFA from both fractions during incubation was reflected by a decrease in the ratio of PUFA to saturated FA (Fig. 3; Table III). The decrease was greater at 0.25 M $CaCl_2$ for the FFA fraction and less at 0.05 M for both fractions ($P \leq 0.05$) than in the control.

The loss of PUFA from FFA and their low level in the FFA fraction suggested LOX involvement in lipid breakdown. When assayed, LOX specific activity indeed steadily increased in cabbage leaf discs with incubation in the control and in 0.25 M $CaCl_2$ (Fig. 4). The increase was less at 0.05 M ($P \leq 0.001$). The large increase in LOX specific activity was only partly accounted for by the extensive loss of protein during senescence.

To establish the pathway of PL breakdown, the levels of the degradation products of PL were determined during incubation (Fig. 5). PA, DAG, and FFA levels rose with time under the three treatments. PA and DAG production was affected by the calcium concentration in the medium. The 0.05 M $CaCl_2$ reduced the production of PA and DAG during incubation, whereas 0.25 M $CaCl_2$ slightly enhanced their production. Levels of FFA increased in an essentially linear fashion throughout incubation, but they were apparently not affected by the calcium treatments.

Table II. *FA Composition and Ratio of PUFA to Saturated FA (mol%) of the PL and FFA Fractions of Cabbage Leaf Discs*

Values are expressed as mean ± SD for $n = 3$ to 4.

Fatty Acid	Fraction	
	PL	FFA
	mol%	
14:0		1.1 ± 0.3
16:0	25.2 ± 1.3	52.1 ± 1.6
16:1	1.1 ± 0.3	1.6 ± 0.4
16:3		2.1 ± 0.1
18:0	4.1 ± 0.2	4.3 ± 0.3
18:1	8.4 ± 1.1	15.6 ± 1.3
18:2	17.6 ± 0.6	9.4 ± 0.8
18:3	43.6 ± 1.3	13.8 ± 2.1
PUFA/S	2.09 ± 0.09	0.41 ± 0.06

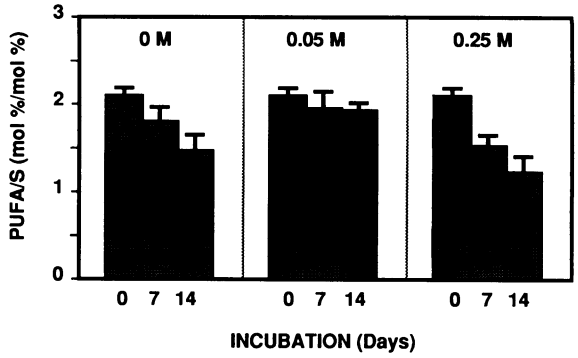

Figure 3. Change with time in the ratio of PUFA to saturated FA of PL in cabbage leaf discs floated on 0, 0.05, and 0.25 M $CaCl_2$ solutions at 15°C during 14 d in the dark. Values are means ± SD for $n = 3$ to 4.

DISCUSSION

A characteristic feature of senescence is membrane deterioration due to lipid degradation and the ensuing destabilization of the bilayer (8). The protection of cell membrane integrity by calcium during senescence (21) has been explained by the ability of calcium to bind to membrane PL and, in this way, to stabilize the membrane and to control membrane-associated functions (18).

Our results indicate that senescence of cabbage leaf discs, reflected by loss of Chl and protein, was delayed by calcium application, as reported previously by Poovaiah and Leopold (19) for *Rumex* leaves.

The involvement of membrane lipid breakdown in cabbage leaf disc senescence was indicated by several markers of lipid degradation during incubation: reduced PL content, larger ratio of FS to PL, increase in the level of the PL degradation products PA, DAG, and FFA, and loss of PUFA from PL and FFA. The levels of these markers of membrane lipid degradation changed in parallel with Chl and protein breakdown, common markers for leaf senescence. Protection of the membranes from lipid degradation by 0.05 M calcium was indicated by a decrease in most of these changes. The present results, based on the levels of intermediate products of PL breakdown, should be interpreted in the context of a steady-state equilibrium between synthesis and degradation.

In contrast, senescence and the associated membrane lipid degradation were accelerated by 0.25 M calcium. It is, there-

Table III. *Change with Time in the Ratio of PUFA to Saturated FA (mol%) in the FFA Fraction of Cabbage Leaf Discs Floated on 0, 0.05, and 0.25 M $CaCl_2$ Solutions at 15°C during 14 d in the Dark*

Values are means ± SD for $n = 3$ to 4.

Calcium Concentration	Time (d)		
	0	7	14
M		*PUFA/S (mol%)*	
0	0.41 ± 0.04	0.26 ± 0.01	0.23 ± 0.01
0.05		0.40 ± 0.03	0.34 ± 0.01
0.25		0.26 ± 0.01	0.21 ± 0.02

fore, important to control calcium concentration to avoid physiological injury to the cells. The contradictory results cited by Ferguson (7) may be due to supraoptimal levels of calcium salt.

The increase of PA, DAG, and FFA indicates that PL breakdown during senescence was mediated by several enzymes in sequence: phospholipase D, phosphatidic acid phosphatase, and lipolytic acyl hydrolase, in agreement with recent studies (e.g. ref. 23). The constancy of the proportions of the PL classes during incubation shows that the different PL were degraded at similar rates. The loss of PUFA from the PL fraction further confirms previous reports that the polar head-groups may have less influence on PL degradation than the FA composition of the molecular species (4). The marked rise in the relatively saturated FFA content, the progressive decrease in degree of unsaturation of the FFA, and the increasing LOX specific activity are ample evidence that LOX was also involved in membrane lipid breakdown during senescence.

Calcium protected the membranes from lipid degradation probably through several mechanisms. Calcium can stabilize the plasmalemma by binding to the negatively charged headgroups of PL, which become less prone to degradation by lipolytic enzymes (18, 21). The undegraded lipid bilayer prevents calcium from passively entering the cytosol and facilitates the pumping of calcium outside of the cytoplasm by membrane-associated Ca^{2+}-ATPase. A low calcium level in the cytosol is essential for the normal functioning of cell metabolism. When the calcium concentration of the cytosol increases, calcium interferes with normal biochemical activities by activating or deactivating numerous enzymes, either directly or indirectly, through various mechanisms involving a change in protein conformation, protein phosphorylation, or interaction with calmodulin (17).

Our results indicate that calcium treatment influenced the activity of lipolytic enzymes during incubation. Slower liberation of PA and DAG at 0.05 M calcium (Fig. 5) and slower decrease in PL content (Fig. 2) are indirect evidence for lower activity of phospholipase D and PA phosphatase. The decrease in the level of PUFA in the FFA fraction, a sign of LOX activity, was also delayed by 0.05 M calcium, suggesting lower LOX activity, which was confirmed by enzyme essay

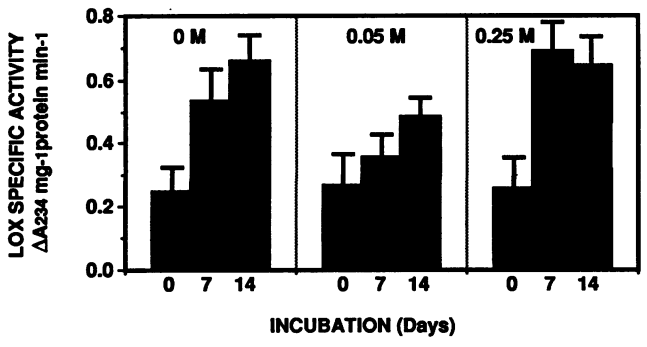

Figure 4. Changes with time of LOX activity of cabbage leaf discs floated in 0, 0.05, and 0.25 M $CaCl_2$ solutions at 15°C during 14 d of incubation in the dark. Values are means ± SD for $n = 3$ to 4.

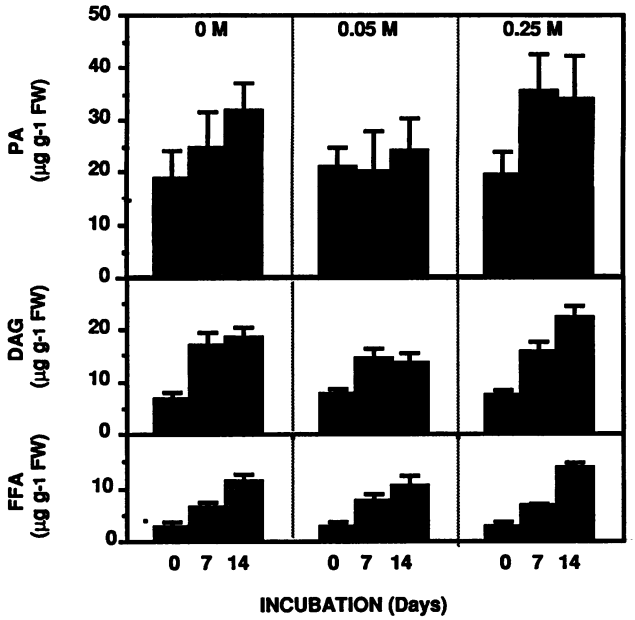

Figure 5. Change with time in PA, DAG, and FFA content of cabbage leaf discs floated on 0, 0.05, and 0.25 M $CaCl_2$ solutions at 15°C during 14 d in the dark. Vertical lines show average SD for $n = 3$ to 4.

(Fig. 4). Lipolytic acyl hydrolase was apparently not affected by calcium (Fig. 5), although the changes in FFA levels may have been masked by the corresponding degradation of PUFA by LOX and by the effect of the availability of the substrate DAG on the activity of lipolytic acyl hydrolase.

The accumulation of enzymic breakdown products of PL in 0.25 M $CaCl_2$ may be due to stimulation of lipolysis following entry of calcium into the cytoplasm (14, 15). Accelerated lipid degradation may be an aspecific response to salt stress. However, calcium is likely to be involved through stress-induced entry into the cytosol. The activation of the lipolytic enzymes will enhance the perturbation of the plasmalemma structure, which will in turn allow entry of more calcium, and membrane lipid degradation will become autocatalytic. The extra- and intracellular action of calcium are thus closely integrated and difficult to distinguish.

In conclusion, the present paper brings evidence that the delay of senescence and of membrane lipid degradation in cabbage leaf discs by a 0.05 M calcium treatment involved protection of membrane lipids from enzymic degradation. In contrast, a supraoptimal 0.25 M calcium concentration accelerated senescence and membrane lipid breakdown.

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