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Increases in Cytosolic Ca^{2+} in Parsley Mesophyll Cells Correlate with Leaf Senescence[1]

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The ability to maintain the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) at homeostatic levels has been examined during leaf senescence in detached parsley (*Petroselinum crispum*) leaves. Fluorescence ratiometric imaging of mesophyll cells isolated from parsley leaves at various senescence stages and loaded with the Ca^{2+} indicator fura-2 has revealed a distinct elevation of $[Ca^{2+}]_{cyt}$, which was positively correlated with the progress of leaf senescence. This initial increase of $[Ca^{2+}]_{cyt}$, which was first observed in cells isolated from 3-d-senescent leaves, occurred 1 d before or in parallel with changes in two established senescence parameters, chlorophyll loss and lipid peroxidation. However, the $[Ca^{2+}]_{cyt}$ elevation followed by 2 d the initial increase in the senescence-associated proteolysis. Whereas the $[Ca^{2+}]_{cyt}$ of nonsenescent cells remained at the basal level, the elevated $[Ca^{2+}]_{cyt}$ of the senescent cells was a long-lasting effect. Experimental retardation of senescence processes, achieved by pretreatment of detached leaves with the cytokinin benzyladenine, resulted in maintenance of homeostatic levels of $[Ca^{2+}]_{cyt}$ in cells isolated from 3-d-senescent leaves. These observations demonstrate for the first time to our knowledge a correlation between elevated $[Ca^{2+}]_{cyt}$ and the process of senescence in parsley leaves. Such senescence-associated elevation of $[Ca^{2+}]_{cyt}$, which presumably results from a loss of the cell's capability to extrude Ca^{2+}, may serve as a signal inducing subsequent deteriorative processes.

Senescence is a deteriorative process that is associated with a wide range of biochemical changes. Most notable in green leaves is the decrease of Chl content, which is accompanied by a parallel degradation of polar lipids from the matrix of the thylakoid membrane (Thomas and Stoddart, 1980; Thomas, 1986; Meir and Philosoph-Hadas, 1995) and by proteolysis (Thimann, 1985; Philosoph-Hadas et al., 1994). One of the factors that may participate in the regulation of leaf senescence is $[Ca^{2+}]_{cyt}$ (Leshem, 1987), the regulation of which is considered an essential cell function in all eukaryotes (Bush, 1995).

Cellular Ca^{2+} homeostasis is normally maintained at submicromolar levels by an ensemble of membrane-associated Ca^{2+} transport pumps, secondary transporters, and ion channels (Hepler and Wayne, 1985; Bush, 1995). Stimulus-induced changes in $[Ca^{2+}]_{cyt}$ may be transient, sustained, or oscillatory, and the time required for full response varies from a few seconds to several hours (Bush, 1995). A transient increase in $[Ca^{2+}]_{cyt}$ may result either from a decrease in plasma membrane Ca^{2+}-ATPase activity (Paliyath and Thompson, 1988) or from the opening of Ca^{2+} channels in the cell membranes (Schroeder and Hedrich, 1989).

Previous studies of various systems have indicated that $[Ca^{2+}]_{cyt}$ is involved in the regulation of senescence and ripening processes in plants. It was previously hypothesized (Leshem et al., 1984; Leshem, 1987) that deterioration of the membranes during leaf senescence could be linked to phospholipid catalytic processes induced by a Ca^{2+}-calmodulin complex. Further evidence for the involvement of $[Ca^{2+}]_{cyt}$ in the processes leading to senescence was reported for leaves of cowpea (Savithramma and Swamy, 1989), oat (Dreier, 1990), rice (Huang et al., 1990; Chou and Kao, 1992), corn (Huang and Kao, 1992a, 1992b), and parsley (*Petroselinum crispum*) (S. Philosoph-Hadas, R. Sabato, R. Ronen, S. Lurie, and S. Meir, unpublished data). In addition, the loss of Ca^{2+} transport capability has been shown to be an early event in the senescent stages of carnation flowers, which is likely to result in higher than normal levels of $[Ca^{2+}]_{cyt}$ and therefore the facilitation of senescence (Paliyath and Thompson, 1988). In tomato and apple fruits stage-specific changes in Ca^{2+}-dependent protein phosphorylation, which disappear completely at the onset of ripening and senescence, have been observed (Poovaiah and Reddy, 1987).

It is therefore reasonable to assume that senescence may generate an increase in $[Ca^{2+}]_{cyt}$, which through amplitude modulation stimulates turnover of membrane phospholipids, leading irrevocably to cell death. Such senescence-induced elevations of $[Ca^{2+}]_{cyt}$ have never been observed, even though their elucidation in terms of timing, kinetics, and magnitude are essential for a complete characterization of the signal transduction pathway (Hepler and Wayne, 1985). Because of the difficulties in measuring

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Abbreviations: $[Ca^{2+}]_{cyt}$, concentration of cytosolic Ca^{2+}; Chl, chlorophyll; CPW, cell and protoplast wash; MDA, malondialdehyde; TBA, thiobarbituric acid.

$[Ca^{2+}]_{cyt}$ in plant cells, only a few examples have been reported in early works (Thomas, 1982; Williamson and Ashley, 1982). A crucial step has been the development of techniques for measuring free $[Ca^{2+}]_{cyt}$ in single living plant cells using fluorescent dyes (Grynkiewicz et al., 1985; Bush and Jones, 1990). With this technique the $[Ca^{2+}]_{cyt}$ is measured directly in single living cells by fluorescence ratio-imaging microscopy (Tsien et al., 1985; McAinsh et al., 1990; Callaham and Hepler, 1991; Miller et al., 1992; Read et al., 1992; Pierson et al., 1994). This system is capable of measuring physiological $[Ca^{2+}]_{cyt}$ ranging from 30 to 3000 nM (Grynkiewicz et al., 1985).

In the present study we examined the prospect that senescence-related elevations of $[Ca^{2+}]_{cyt}$ may occur at early stages of the artificial senescence process in parsley leaves, in which senescence was induced by dark incubation of detached leaves. For this purpose, a method was developed for the isolation of living cells from nonsenescent and senescent detached parsley leaves. Such $[Ca^{2+}]_{cyt}$ increases were demonstrated, using fluorescence ratio-imaging microscopy, in single mesophyll cells in relation to changes in typical senescence parameters measured in whole leaves. This senescence-related response was characterized in terms of timing, kinetics, and magnitude and in relation to the external manipulation of $[Ca^{2+}]_{cyt}$ by loading the cells with Ca^{2+} and by pretreating the leaves with the senescence retardant BA. These studies provide direct evidence for the elevation of $[Ca^{2+}]_{cyt}$ during the senescence process in detached parsley leaves.

MATERIALS AND METHODS

Plant Material and Senescence Treatments

Experiments were performed with fresh, greenhouse-grown parsley (*Petroselinum crispum*) leaves, harvested at maturation. The detached bunches (including stems and leaves) were placed in perforated polyethylene bags containing two sheets of tissue paper to absorb excess moisture and allowed to senesce in the dark at 22°C for 6 d. Senescence parameters (contents of Chl, amino acids, and lipid peroxidation products) were assayed periodically as described below in leaf blades detached from the bunches of petioles at different senescence stages. In parallel, mesophyll cells were isolated from the leaves at the corresponding stages of senescence.

Plant growth regulators were applied as a pretreatment to detached mature, green, fully expanded leaf blades of parsley that had been freshly harvested from the greenhouse. The leaves were rinsed with distilled water and dried; the leaf blades were subsequently detached from their petioles and placed in Petri dishes (five to eight leaves/dish) floating on 3 mL of the appropriate solutions. Three concentrations of GA_3 or BA (10^{-6}, 10^{-5}, and 10^{-4} M) were used in the presence of 0.01% Tween 20 (as a surfactant) and 50 μg/mL chloramphenicol (to avoid contamination). Leaves were kept on the hormone solutions for 24 h at 22°C in darkness and then transferred to new Petri dishes on filter papers (Whatman no. 1) moistened with 3 mL of distilled water and chloramphenicol (50 μg/mL). Four Petri dishes were used for each incubation period. The various leaves incubated in the Petri dishes were allowed to senesce in darkness at 22°C for another 5 d, and their senescence parameters (contents of Chl and amino acids) were assayed periodically. Mesophyll cells were isolated from leaves treated with 10^{-5} M BA following 3 d of dark senescence. Experiments were repeated at least three times, with similar results. The results presented are from individual and representative experiments.

Chl and Amino Acid Determination

Chl and amino acids were extracted from samples of cut leaf segments (0.5 g) by boiling them for 30 min in 10 mL of 80% (v/v) ethanol, as previously described (Philosoph-Hadas et al., 1991; Meir et al., 1992). Chl was subsequently quantified by determining the A_{645} and A_{663} according to the method of Arnon (1949) and expressed as milligrams of Chl per gram fresh weight. The ethanolic extract was then used for the determination of amino acids by the ninhydrin method (Yemm and Cocking, 1954), adapted to microquantities suitable for ELISA plates (Philosoph-Hadas et al., 1991). A standard Met curve was included in every plate, and each point was repeated three or four times.

Extraction and Determination of Lipid Peroxidation Products

TBA-reactive substances representing lipid peroxidation products were extracted as described previously (Meir et al., 1992) by homogenization of 0.5 g of tissue in 5 mL of solution containing 20% TCA and 1.5 mM EDTA. TBA-reactive substances including aldehydes and MDA were assayed using the TBA test (Kosugi and Kikugawa, 1985), as modified by Meir et al. (1992). One milliliter of 0.67% TBA was added to 3-mL aliquots of the supernatant and the solution was incubated at 100°C for 1 h. The solution was then cooled and centrifuged for 10 min at 8000 rpm. The volume of the resultant supernatant was made up to 10 mL with distilled water, and the A_{455} and A_{532} of the colored reaction product were determined for aldehydes and MDA, respectively. Three replicates were used for each assay.

Isolation of Mesophyll Cells

A method was developed for the isolation of single mesophyll cells from parsley leaves at three different senescence stages: nonsenescent green leaves obtained immediately after harvest, yellowish-green leaves obtained following 3 d of dark incubation, and yellow leaves obtained following 6 d of dark incubation. In one experiment (Table I) mesophyll cells were also isolated from leaves at their initial senescence stages following 1 or 2 d of dark incubation. The method developed was based on that used for the isolation of leaf protoplasts (Power et al., 1976). Parsley leaves (0.25 g) were shredded into approximately 1-mm strips with a sharp razor blade. The leaf strips were suspended in 15 mL of 0.5% pectinase solution (Macerozyme R-10, Yakult Pharmaceutical Ind. Co., Tokyo, Japan)

Table I. *Effect of leaf senescence stage on $[Ca^{2+}]_{cyt}$ of successfully injected cells*

Mesophyll cells were isolated from leaves at various senescence stages and their $[Ca^{2+}]_{cyt}$ was monitored as described in "Materials and Methods." Values in parentheses represent the percentages of cells with either basal or high $[Ca^{2+}]_{cyt}$ of total measured cells at each senescence stage.

Stage of Leaf Senescence	No. of Measured Cells	Cells with Basal $[Ca^{2+}]_{cyt}$		Cells with High $[Ca^{2+}]_{cyt}$	
		No.	$[Ca^{2+}]_{cyt}$	No.	$[Ca^{2+}]_{cyt}$
d			μM		μM
0	58	58 (100%)	0.1–0.2	0	–
1	20	20 (100%)	0.1–0.2	0	–
2	20	20 (100%)	0.1–0.2	0	–
3	76	45 (60%)	0.1–0.2	31 (40%)	0.5–1.0
6	42	4 (10%)	0.1–0.2	38 (90%)	0.5–3.0

in CPW medium containing 9% (w/v) mannitol and the following inorganic salts: 0.2 mM KH_2PO_4, 1 mM KNO_3, 0.1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 1 M KI, and 0.1 M $CuSO_4 \cdot 5H_2O$, adjusted to pH 5.8 (Power et al., 1976).

Uptake of the enzyme solution was enhanced by using gentle (500 mm Hg) vacuum infiltration for 5 min. The flasks were agitated on a reciprocal shaker at 25°C at a speed of 120 strokes/min for 15 min, followed by filtration through a 70-μm nylon mesh to collect leaf pieces. Another 15 mL of enzyme solution in CPW medium was added to the leaf strips for 30 min for a second round of enzyme maceration and then filtered through the nylon mesh. The filtered suspension containing the isolated cells was rinsed by two cycles of centrifugation for 3 min at 3000 rpm in a fixed-angle centrifuge (Sorvall) in CPW medium to remove any remaining enzyme. The cell pellet was then resuspended in 0.5 mL of CPW and kept at 4°C. Cells were counted under the microscope and their viability was examined with fluorescein diacetate as described by Widholm (1972). Cells could be kept viable in culture at 4°C for about 24 h following isolation; however, reliable microinjection should be performed within the initial 4 h after isolation.

Measurements of $[Ca^{2+}]_{cyt}$

Levels of $[Ca^{2+}]_{cyt}$ were measured in cells isolated from leaves at the indicated senescence stages, from senescent leaves pretreated with 10^{-5} M BA, and from leaves preloaded with Ca^{2+}. Isolated parsley cells were immobilized in agarose by mounting a small drop of the cell solution on a small drop of 1.2% molten (approximately 40°C), low-temperature gelling agarose (gel point 26–30°C; Sigma type VII). The preparation was rapidly cooled to gel the agarose and then flooded with CPW medium. The fluorescent Ca^{2+} indicator fura-2 (fura-2 pentapotassium salt, Molecular Probes, Eugene, OR) at a 2 mg/mL needle tip concentration was loaded into a cell by iontophoretic injection (2 nA for 5 s). To examine the effect of Ca^{2+} loading, a mixture of fura-2 and the Ca^{2+}-saturated BAPTA (1,2-bis[2-aminophenoxy]ethane-*N,N,N',N'*-tetraacetic acid) buffer (1 μL of 10 mg/mL fura-2 and 4 μL of Ca^{2+}-saturated, 2.5 mM BAPTA [needle tip concentrations]) was injected into the cells.

Fluorescent ratio-imaging microscopy was applied on a modified microscope (model IM-35, Zeiss) equipped with a 40 × 1.3 numerical aperture fluorescent objective (Nikon; Callaham and Hepler, 1991; Pierson et al., 1994). The excitation light was filtered by a 340- and 360-nm interference filter (10 nm FWHM; Corion, Holliston, MA). The emission was imaged by a thermoelectrically cooled charge-coupled device camera (type AT200 [Photometrics, Tucson, AZ] with a TH7883A charge-coupled device chip [Thomson, Springfield, VA] at a 500-kHz data transfer rate) installed in an 80486DX-based, PC-compatible computer. Ratio images were computed after the subtraction of background images adjacent to the cell, which were taken immediately (Miller et al., 1992; Pierson et al., 1994). The absolute $[Ca^{2+}]_{cyt}$ was determined according to published methods (Gryn-kiewicz et al., 1985; Callaham and Hepler, 1991; Miller et al., 1992; Pierson et al., 1994). In addition, a 600-nm filter was applied to block the autofluorescence of the chloroplasts. Ratio images were further analyzed with an image-processing system (Image-I/AT, Universal Imaging, Media, PA).

RESULTS

Determination of Senescence Stages in Parsley Leaves

The rate of parsley leaf senescence (as indicated by Chl loss), accumulation of amino acids resulting from enhanced proteolysis, and accumulation of the lipid peroxidation products aldehyde and MDA were determined periodically during dark senescence of detached leaves. The data from d 4 were not collected in this specific experiment, but results from other corresponding experiments show that intermediate values between d 3 and 5 were obtained for all parameters assayed. Results demonstrate that, in general, the senescence-related changes in Chl content (Fig. 1A) were accompanied by a parallel moderate increase of aldehydes (Fig. 1C) and MDA (Fig. 1D). Therefore, levels of both Chl and lipid peroxidation products did not change significantly during the initial 3 d of senescence.

Chl levels decreased to 77 and 40% on d 5 and 6, respectively (Fig. 1A), whereas aldehydes were elevated by 280 and 490%, respectively (Fig. 1C), and MDA levels were elevated by 208 and 320%, respectively (Fig. 1D), during this period. Unlike Chl breakdown and lipid peroxidation, the

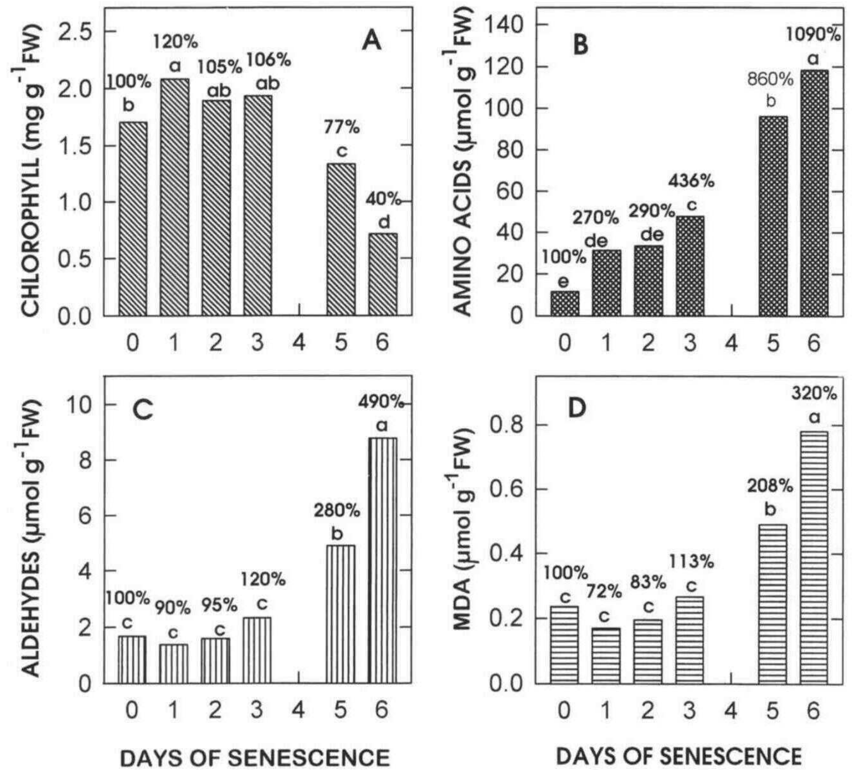

Figure 1. Changes in levels of Chl (A), amino acids (B), aldehydes (C), and MDA (D) during dark senescence of parsley leaves. Parsley bunches were senesced and leaf blades were detached periodically for measurements. Numbers above columns represent the percentages of control values at d 0 of the various parameters. Three replicates were used for each assay and different letters above the columns indicate statistical significance within senescence days at P = 5% according to analysis of variance. The results presented are from an individual representative experiment of three. FW, Fresh weight.

process of proteolysis proceeded much more rapidly, showing a 270% increase already on the 1st d of senescence and reaching an increase of 1090% after 6 d (Fig. 1B). These results indicate that Chl breakdown is closely linked to lipid peroxidation, whereas proteolysis seems to proceed independently of these two senescence-associated processes.

A similar relation between these senescence processes was found previously in parsley and other herb species (Meir et al., 1992; Philosoph-Hadas et al., 1994). Therefore, senescence rate was determined in the system of detached parsley leaves only on the basis of Chl loss and enhanced lipid peroxidation. Based on these two senescence-associated activities, three consecutive senescence stages, varying according to duration in darkness, were defined in the periodically detached leaves. These stages included fresh, nonsenescent green leaves (d 0), 3-d-senescent leaves that were still green (just before the onset of rapid Chl loss), and 6-d-senescent leaves, which lost 60% of their initial Chl level and were partially yellow (Fig. 1A).

Effect of Leaf Senescence Stage on $[Ca^{2+}]_{cyt}$ in Isolated Mesophyll Cells

Mesophyll cells were isolated from fresh, nonsenescent leaves (Fig. 2a), 3-d-senescent leaves (Fig. 4a), and 6-d-senescent leaves (Fig. 2b). The viability of nonsenescent and 3-d-senescent cells was greater than 60%, whereas that of isolated 6-d-senescent cells was approximately 20 to 30%. The chloroplasts of the senescent cells were much smaller than those of the nonsenescent cells, but the cells of 6-d-senescent leaves were often enlarged (Fig. 2b).

Fura-2 is a small, charged molecule that is subject to sequestration into cell compartments and/or extrusion from the cell (Bush and Jones, 1990; Callaham and Hepler,

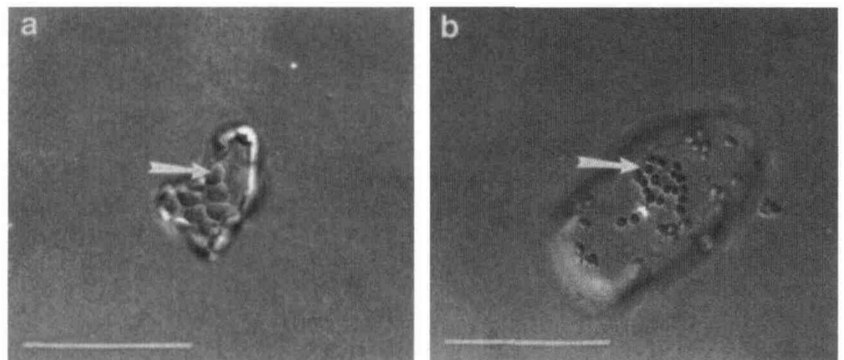

Figure 2. Light micrographs of isolated parsley mesophyll cells. a, Nonsenescent cell. b, Six-day-senescent cell. The arrows point to individual chloroplasts. Bars = 20 μm.

1991). To prevent these problems, we had originally attempted to load the cells by pressure injection with fura-2 that had been covalently linked to dextran (Miller et al., 1992), since it can remain in the cells for a much longer time because of its larger size. However, we were unable to find the conditions that allowed us to successfully apply pressure injection to the isolated parsley cells, particularly those undergoing senescence, and thus could not load fura-2-dextran. We therefore introduced free fura-2 by iontophoresis, wherein sharp needles could be used under conditions in which no volume changes were caused. With this method cell viability could be maintained; however, dye loss became a serious issue, forcing us to obtain useful ratio images within a few minutes of the time of injection.

The process of microinjection could cause misleading results, since $[Ca^{2+}]_{cyt}$ has been observed in other cell systems to usually undergo a brief elevation following impalement (Callaham and Hepler, 1991). To be certain that the images taken immediately after injection indicated an accurate depiction of $[Ca^{2+}]_{cyt}$, fura-2 was injected into cells that were still connected after mounting on the slides. Figures 3b and 4, a, and b, demonstrate such pairs of connected cells. Although only one adjacent cell was given the injection, the dye was translocated into the cytoplasm of the neighboring cell through plasmodesmata. Consequently, $[Ca^{2+}]_{cyt}$ in both cells was measured at the same time. The cells that were not treated with microinjection showed the same basal $[Ca^{2+}]_{cyt}$ as that detected in the adjacent cells that were treated.

These results indicate that the $[Ca^{2+}]_{cyt}$ in the treated cells was not appreciably altered as a result of possible wounding caused by impalement. However, similar experiments could not be performed with the 6-d-senescent cells, since such adjacent cells were rarely found because of their poor viability. Experiments performed under strictly aseptic conditions to examine the in vitro aging process of cells

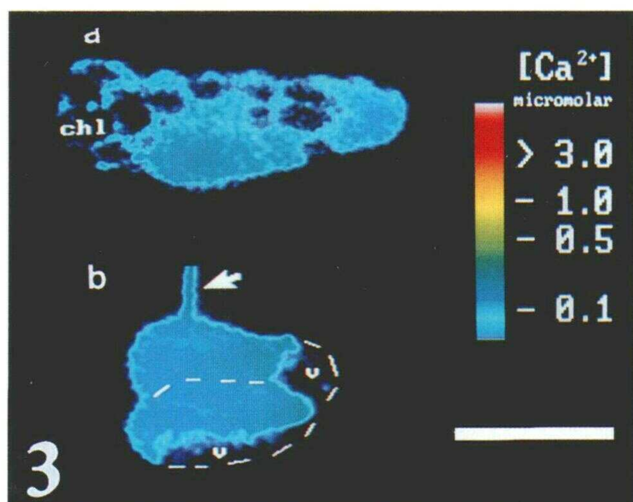

Figure 3. Pseudocolor images of mesophyll cells isolated from nonsenescent parsley leaves. a, Single cell. b, Two connected cells; the upper cell was injected where indicated by the arrow. The dashed line indicates the actual shape of the cells. chl, Chloroplasts; v, vacuole. Bar = 20 μm.

isolated from nonsenescent leaves showed a similar pattern of senescence in the isolated cells as in leaves in terms of Chl and protein loss during 4 d at 22°C (data not shown). This suggests that the isolation procedure did not damage the cells.

To determine the changes in $[Ca^{2+}]_{cyt}$ during senescence, fura-2 was injected into cells isolated from leaves at different senescence stages. The ratio images of the various cells are illustrated in Figures 3 through 7. The dark areas observed in these figures correspond to the location of either vacuoles or chloroplasts, in which the signal was below the threshold and thus was set to black. Since the cytoplasm is a very thin layer near the edge of the cells, the fura-2 signal could not always be detected; therefore, the dashed lines indicate the actual cell shape.

The $[Ca^{2+}]_{cyt}$ of nonsenescent cells was at a basal level (Fig. 3), and no significant changes in this basal level could be observed in cells isolated from 1- or 2-d-senescent leaves (Table I). However, the ratio images of cells isolated from 3-d-senescent leaves showed two different $Ca^{2+}{}_{cyt}$ levels: a basal level (0.1–0.2 μM; Fig. 4, b and c), similar to that obtained in fresh, nonsenescent cells (Fig. 3), and a higher level at approximately 0.5 to 1.0 μM (Fig. 4d). Most of the images of cells isolated from 6-d-senescent leaves indicated elevated $[Ca^{2+}]_{cyt}$, at approximately 0.5 to 1.0 μM (Fig. 5a) or higher (Fig. 5b). The distribution of cytosolic Ca^{2+} within the fura-2 injected cell population is summarized in Table I and shows that, eventually, significant and repeatable changes in $[Ca^{2+}]_{cyt}$ could be detected only in cells obtained from 3-d-senescent leaves.

Response of Cells at Different Senescent Stages to Manipulation of $[Ca^{2+}]_{cyt}$

The results of the ratiometric imaging at different senescence stages indicated that the $[Ca^{2+}]_{cyt}$ increased during parsley leaf senescence. To determine whether this increase was due to loss of the ability of senescent cells to extrude excess Ca^{2+}, we injected a mixture of fura-2 and BAPTA saturated with Ca^{2+} into nonsenescent and 6-d-senescent cells. Figure 6 illustrates a set of four consecutive images taken at 10-s intervals and depicts the response of nonsenescent cells to the additional Ca^{2+} level. The nonsenescent cell rapidly pumped out or sequestered the additional Ca^{2+}; consequently, the initial high $[Ca^{2+}]_{cyt}$ (Fig. 6a) decreased to nearly basal levels (Fig. 6d) within 40 s of injection. Meanwhile, the dye also diffused out of the cytoplasm quickly, and the images were not detectable 50 s later.

Eight nonsenescent cells were treated by injection, and five showed a very rapid efflux of the injected Ca^{2+}, as represented in Figure 6. When 6-d-senescent cells were loaded with Ca^{2+}, 15 of 17 treated cells quickly died, and the 2 senescent cells that survived showed a high level of Ca^{2+} and the inability to pump this Ca^{2+} out (Fig. 7). Thus, the initial high $[Ca^{2+}]_{cyt}$ (Fig. 7a) remained at the same level 80 s after the injection (Fig. 7h), and the dye did not disappear as rapidly as it did from nonsenescent cells (Fig. 6).

The $[Ca^{2+}]_{cyt}$ in cells of detached parsley leaves was manipulated by the application of plant growth regulators

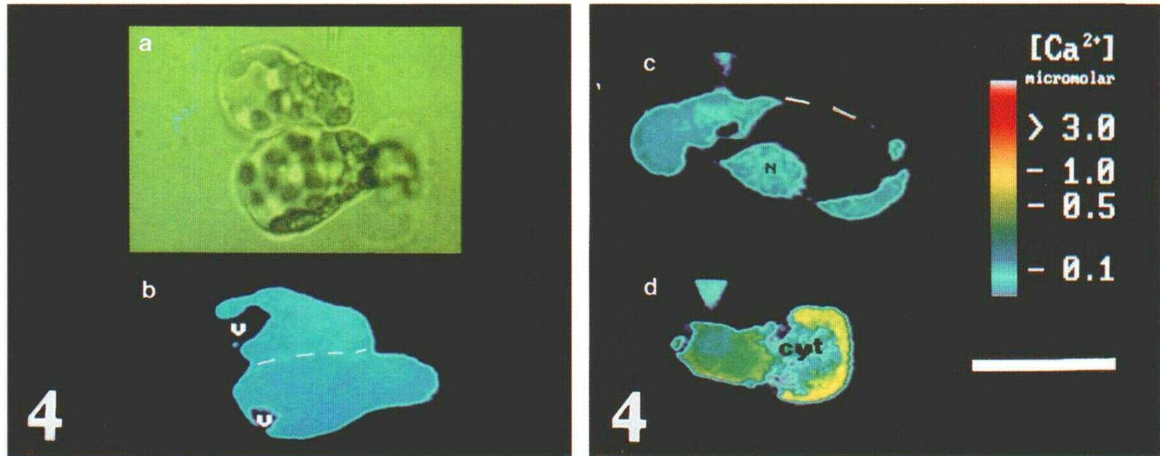

Figure 4. Pseudocolor images of mesophyll cells isolated from 3-d-senescent parsley leaves. a, Transmission image of two adjacent cells, taken after injection. b, Ratio image of the same cells demonstrated in a. c, Ratio image of a cell showing basal $[Ca^{2+}]_{cyt}$. d, Ratio image of a cell showing higher $[Ca^{2+}]_{cyt}$. cyt, Cytoplasm; N, nuclei; v, vacuole. Bar = 20 μm.

known as senescence retardants (Thimann, 1985; Nooden, 1988). The effects of various concentrations of GA_3 and the cytokinin BA on Chl degradation during senescence was examined. Results depicted in Figure 8 show that detached leaves treated with three concentrations of these growth regulators retained about 65 to 79% of their initial Chl level after 6 d of dark senescence, whereas control leaves retained only 46% of their Chl level during this period. Therefore, the treatment with 10^{-5} M BA, which retained the highest Chl level (79%), was chosen for the rest of the experiments. This concentration, which successfully retarded Chl breakdown during 6 d of senescence (Fig. 9A), also significantly retarded the process of proteolysis, resulting in the reduced accumulation of amino acids (Fig. 9B).

These results indicate that BA can retard several senescence-associated processes in detached parsley leaves. All cells (14 successful injections) isolated from BA-treated detached parsley leaves following 3 d of senescence, in which both Chl loss and proteolysis were inhibited (Fig. 9), showed a normal basal $[Ca^{2+}]_{cyt}$ (Fig. 10), similar to that previously observed in nonsenescent cells (Fig. 3), and quite unlike cells isolated from untreated 3-d-senescent leaves, of which 40% showed elevated $[Ca^{2+}]_{cyt}$ (Table I; Fig. 4). Therefore, the senescence-retarding effect of BA may be associated with its ability to maintain the cell's capability to regulate $[Ca^{2+}]_{cyt}$ at homeostatic levels.

DISCUSSION

Our results demonstrate that elevated $[Ca^{2+}]_{cyt}$ in parsley mesophyll cells is positively correlated with the progress of typical senescence-associated events occurring in whole parsley leaves, from which these cells were derived. Increases in $[Ca^{2+}]_{cyt}$ above resting levels, which

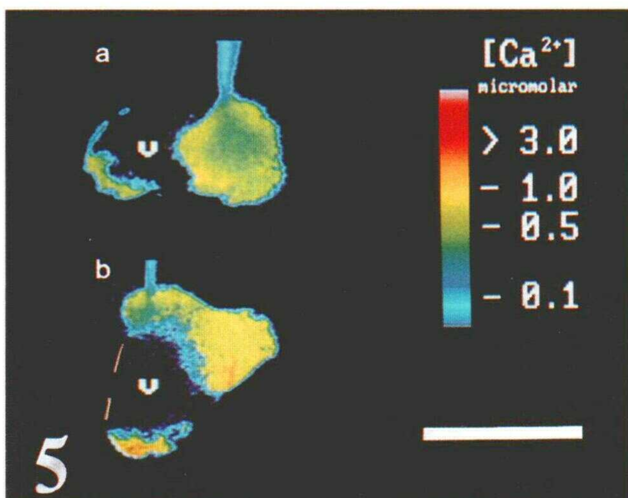

Figure 5. Pseudocolor images of two mesophyll cells isolated from 6-d-senescent parsley leaves. a, Cell with 0.5 to 1.0 μM $[Ca^{2+}]_{cyt}$. b, Cell with 0.5 to 2.0 μM $[Ca^{2+}]_{cyt}$. v, Vacuole. Bar = 20 μm.

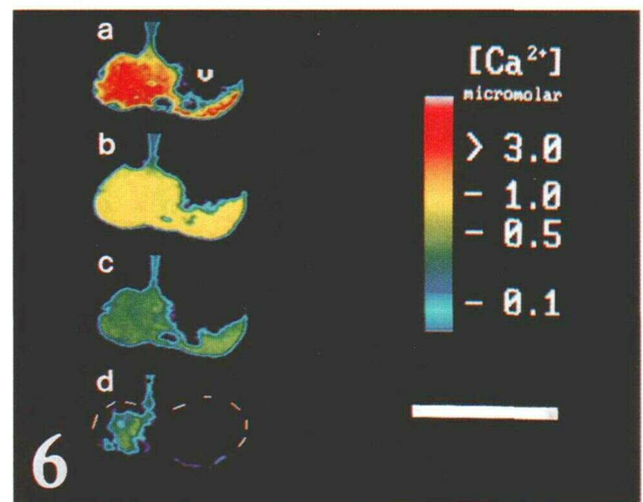

Figure 6. The capability of mesophyll cells isolated from nonsenescent parsley leaves to extrude $[Ca^{2+}]_{cyt}$. Pseudocolor images of the same cell after coinjection of saturated Ca^{2+} and fura-2 were taken sequentially at 10-s intervals. v, Vacuole. Bar = 20 μm.

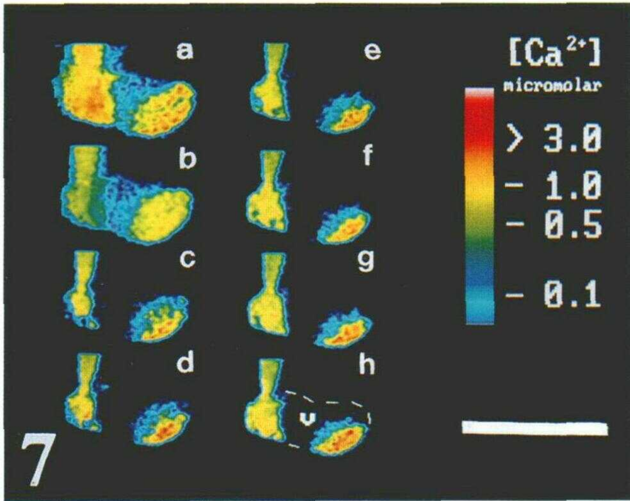

Figure 7. The capability of mesophyll cells isolated from 6-d-senescent parsley leaves to extrude $[Ca^{2+}]_{cyt}$. Pseudocolor images of the same cell after coinjection of saturated Ca^{2+} and fura-2 were taken sequentially at 10-s intervals. v, Vacuole. Bar = 40 μm.

could not be detected in nonsenescent cells or in cells isolated from 1- and 2-d-senescent leaves (Table I; Fig. 3), were first observed in 40% of the injected cells isolated from 3-d-senescent leaves (Table I; Fig. 4). At this time none of the leaves showed significant changes in the levels of Chl (Fig. 1A) or lipid peroxidation products (Fig. 1, C and D); however, proteolysis, as evidenced by an accumulation of amino acids, had already begun (Fig. 1B).

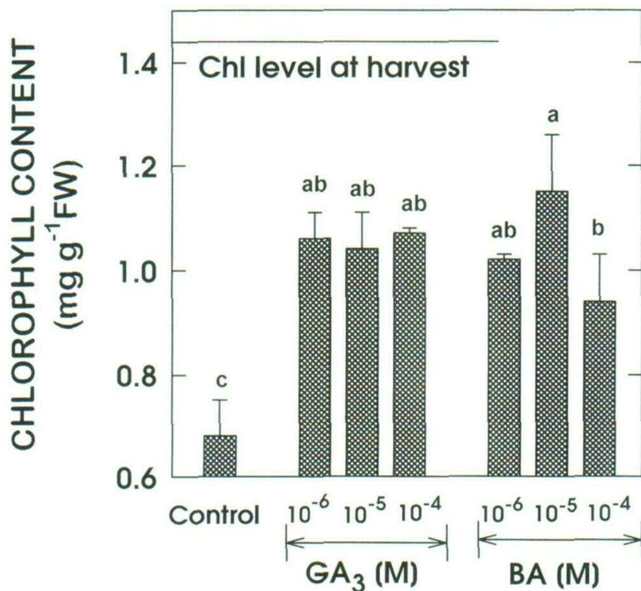

Figure 8. Effect of various concentrations of plant growth regulators on Chl levels of parsley leaves following 6 d of senescence. Detached parsley leaf blades were floated for 24 h on solutions containing the indicated concentrations of GA_3 or BA, and then leaves were incubated on water for another 5 d in darkness. Control leaves were treated with water only. The vertical bars represent SDs of three replicates, and the different letters above columns indicate the statistical significance within treatments at P = 5% according to analysis of variance. The results presented are from an individual representative experiment of three. FW, Fresh weight.

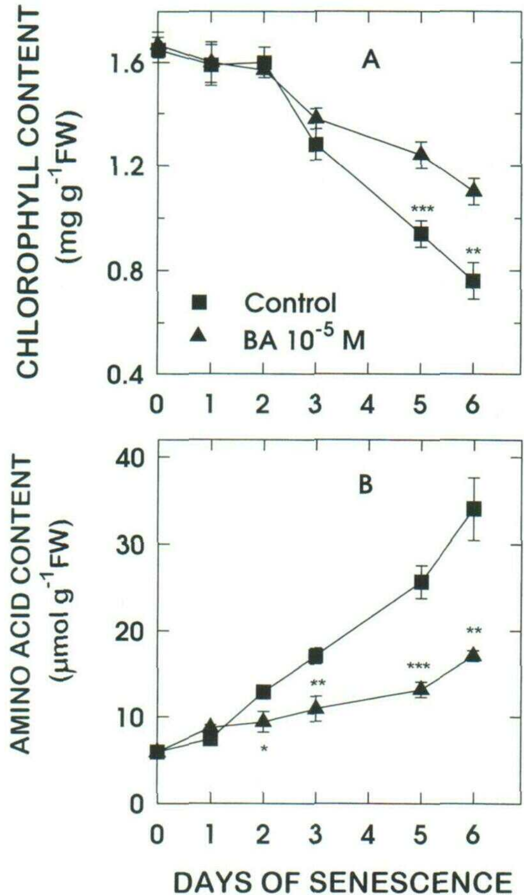

Figure 9. Effect of BA 10^{-5} M on Chl (A) and amino acid (B) content of parsley leaves during 6 d of senescence in darkness. Control leaves were treated with water only. Conditions were as detailed in Figure 8. The vertical bars represent SDs of three replicates. Mean separation by Student's *t* test between control and treated leaves for each sampling period is indicated by asterisks: *, **, and *** represent significance at P = 0.05, 0.01, and 0.001, respectively. The results presented are from an individual representative experiment of three. FW, Fresh weight.

Elevated $[Ca^{2+}]_{cyt}$ was observed in 90% of the injected cells isolated from leaves detached from 6-d-senescent bunches (Table I; Fig. 5), which lost 60% of their Chl content (Fig. 1A) and showed a remarkable increase in proteolysis (Fig. 1B) and lipid peroxidation (Fig. 1, C and D). It should be noted that when parsley bunches were senesced for 3 d the leaves still retained their initial levels of Chl (Fig. 1A) and lipid peroxidation products (Fig. 1, C and D), whereas in detached leaf blades undergoing senescence (for hormone application) the onset of Chl loss had already started on d 3 (Fig. 9A). This suggests that leaf blade detachment accelerated the senescence rate, as was previously reported (Philosoph-Hadas et al., 1991, 1994). Therefore, it should be concluded that the initial increase in $[Ca^{2+}]_{cyt}$ preceded or coincided with different senescence-associated processes such as Chl breakdown and lipid peroxidation but followed the onset of protein degradation.

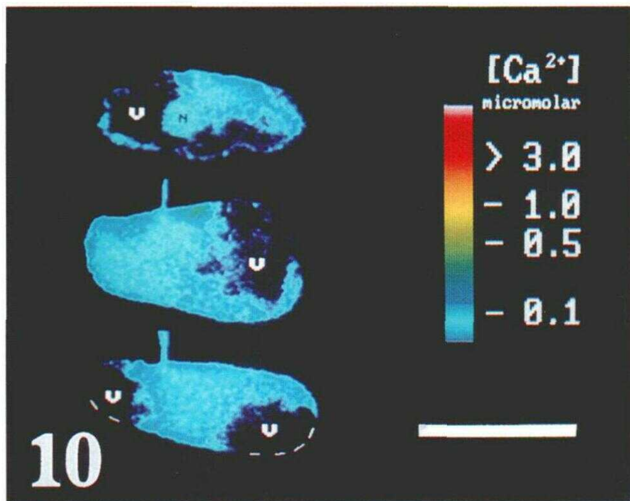

Figure 10. Pseudocolor images showing basal $[Ca^{2+}]_{cyt}$ of three mesophyll cells isolated from 3-d-senescent parsley leaves pretreated with 10^{-5} M BA. N, Nucleus; v, vacuole. Bar = 20 μm. The images presented are representative of 14 successful injections.

Previous studies in parsley and other herb leaves demonstrated that proteolysis proceeded immediately after detachment and independently of the other two closely linked senescence-associated processes (Meir et al., 1992; Philosoph-Hadas et al., 1994). Taken together, our results suggest the following sequence of events: darkness and leaf detachment trigger enhanced senescence processes that are first (d 1 and 2) manifested in increased proteolysis and followed (d 3) by the long-lasting increase of $[Ca^{2+}]_{cyt}$ and enhanced degradation of Chl and lipids.

The elevated levels of $[Ca^{2+}]_{cyt}$ observed mainly in 6-d-senescent cells (Fig. 5) are likely to result from an inability of senescent cells to regulate their $[Ca^{2+}]_{cyt}$, as suggested previously for senescing carnation petals (Paliyath and Thompson, 1988). Indeed, a striking difference in the way nonsenescent and senescent cells cope with the excess Ca^{2+} could be observed. Whereas most of the analyzed nonsenescent cells rapidly sequestered or extruded both the injected dye and excess Ca^{2+} (Fig. 6), all of the analyzed 6-d-senescent cells failed to remove the excess Ca^{2+} and also retained the injected dye (Fig. 7). Consequently, a long-lasting elevated $[Ca^{2+}]_{cyt}$ occurred in the few senescent cells that survived, indicating that one property of senescence is a loss of ability to extrude excess Ca^{2+} from the cytoplasm. Quite likely, these impairments are related to the loss of the Ca^{2+}-transport activity observed at advanced stages of parsley leaf senescence (S. Philosoph-Hadas, R. Sabato, R. Ronen, S. Lurie, and S. Meir, unpublished data). Elevated $[Ca^{2+}]_{cyt}$ may further facilitate senescence, as suggested by Leshem (1987), by triggering the activities of phospholipases A and D, which would release fatty acids from membrane lipids, resulting in membrane breakdown and cell death.

Senescence of leaves is controlled by different factors, one of which, the cytokinins, are well known for their ability to markedly delay or reverse leaf yellowing in various species (Thimann, 1985; Nooden, 1988). This effect is probably due to a slowing or inhibition of Chl breakdown and protein degradation (Thimann, 1985) or to the promotion of Chl biosynthesis and accumulation (Lechowski and Bialczyk, 1993). In the studies reported here, it is evident that the cytokinin BA also significantly retards senescence in parsley leaves, as indicated by a delay in Chl breakdown from d 3 (Fig. 9A) and in proteolysis from d 2 of senescence (Fig. 9B).

Ratiometric ion imaging also revealed that all of the cells isolated from 3-d-senescent leaves pretreated with BA retained basal levels of $[Ca^{2+}]_{cyt}$ (Fig. 10), similar to the result obtained in cells isolated from nonsenescent leaves (Fig. 3). These results suggest that in parsley leaves BA may partially retard Chl degradation (which occurs after the increase in $[Ca^{2+}]_{cyt}$), by blocking the changes in $[Ca^{2+}]_{cyt}$. This may result from the promotion in Ca^{2+}-ATPase activity by cytokinin, which has already been reported in maturing zones of soybean hypocotyls treated with zeatin (Kubowicz et al., 1982). However, since the parallel senescence-associated process of protein degradation precedes the increase in $[Ca^{2+}]_{cyt}$, its significant inhibition by BA on d 2 (Fig. 9B) cannot be attributed to the BA-blocking effect on the $[Ca^{2+}]_{cyt}$ increase.

The possibility that Ca^{2+} might participate in mediating the responses of cytokinins has been suggested previously, and a cytokinin-Ca^{2+} interaction has been noted in several systems (Hepler and Wayne, 1985). These include delayed senescence of maize leaf discs (Poovaiah and Leopold, 1973), regulation of bud formation in the moss *Funaria hygrometrica*. (Saunders and Hepler, 1983), and enhanced formation and maintenance of Chl levels in Scots pine embryos by BA (Lechowski and Bialczyk, 1993). Hahm and Saunders (1991) found that intracellular Ca^{2+} was elevated in response to cytokinin treatment in *F. hygrometrica*.

These studies, in contrast to those of senescing parsley leaves, were mostly performed in systems undergoing cell division. Moreover, the application of cytokinin caused an increase in intracellular Ca^{2+} levels (Kubowicz et al., 1982; Saunders and Hepler, 1983; Hepler and Wayne, 1985; Hahm and Saunders, 1991; Lechowski and Bialczyk, 1993), presumably through alteration of the activity of Ca^{2+} transport pumps (Kubowicz et al., 1982) or by increasing the activity of Ca^{2+} channels (Hepler and Wayne, 1985; Hahm and Saunders, 1991).

One might expect cytokinin-induced increases in $[Ca^{2+}]_{cyt}$ to be typical for meristematic zones (Saunders and Hepler, 1983; Hepler and Wayne, 1985), since cytokinins are cell-division hormones, and Ca^{2+} plays a part in mediating cell division. However, in maturing zones like those in parsley leaves, the opposite effect, maintaining basal $[Ca^{2+}]_{cyt}$ (Kubowicz et al., 1982; Fig. 10), appears to hold. It may therefore be concluded, that, unlike their effect on cell division in meristematic zones, the senescence-retarding effect of cytokinins in mature tissues is manifested in the maintenance of basal $[Ca^{2+}]_{cyt}$, which results in the retention of high Chl levels. Therefore, the BA experiments in this study, showing that basal levels of $[Ca^{2+}]_{cyt}$ are associated with retardation of leaf senescence, strengthen the correlation found between elevated $[Ca^{2+}]_{cyt}$ and advanced leaf senescence.

There is no doubt that cells isolated from senescent leaves are more fragile, more sensitive to dye injection (either free or dextranated) and Ca^{2+} loading, and show less viability after isolation. In our view, these features reflect the progress of the senescence syndrome and emphasize the extreme sensitivity of the senescent state. Nevertheless, we cannot rule out the possibility that this increased sensitivity of senescent cells to experimental manipulation might be the basis for their modified behavior in relation to $[Ca^{2+}]_{cyt}$. However, considering all of these limiting and possibly misleading factors, we still believe that by careful performance and repeating of experiments, our results are reproducible and reliable enough to ascribe the differences between senescent and nonsenescent cells to their physiological behavior rather than to experimental artifact.

This conclusion is based on the following observations. (a) Dye sequestration was virtually always observed in the vacuole; however, both Figures 6 and 7 show that the vacuole did not acquire dye. Therefore, excess dye sequestration in the older cells does not seem to account for the lingering signal observed. (b) The ratio images clearly show that, although the Ca^{2+} is elevated, it is nevertheless within the range of 1 to 2 μmol and is thus easily 100-fold less than would be found in the lumen of the ER. Therefore, the possible dye sequestration into the ER of older cells does not seem to occur. (c) Wounding caused the $[Ca^{2+}]_{cyt}$ to elevate to high levels. However, because the $[Ca^{2+}]_{cyt}$ was at basal levels (100–200 nM) in coupled cells (Figs. 3b and 4b), we assert that the reported Ca^{2+} kinetics were not due to wounding. (d) Regarding the representative quality of the 15 of 17 cells killed after Ca^{2+} injection, since this study is literally on the edge of technical feasibility, and the minority of senescent cells repeatedly survived dye injection and Ca^{2+} loading, it is reasonable to conclude that this actually reflects a physiological behavior in relation to $[Ca^{2+}]_{cyt}$. (e) The fact that cells isolated from 3-d-senescent leaves showed full recovery of their $[Ca^{2+}]_{cyt}$ in response to treatment with the senescence retardant BA further strengthens the interpretation of the results as reflecting a real physiological process. Since injection of the whole leaf tissue (which would have reduced artificial influence) was not possible, our $[Ca^{2+}]_{cyt}$ measuring technique could be applied to isolated cells only, with all of the limitations that this system imposes.

In conclusion, our results reveal that the competence of cells to maintain $[Ca^{2+}]_{cyt}$ at homeostatic levels is impaired at an early stage of leaf senescence, possibly due to the senescence-associated reduction in Ca^{2+} extrusion activity (S. Philosoph-Hadas, R. Sabato, R. Rohen, S. Lurie, and S. Meir, unpublished data). Accordingly, we demonstrated by Ca^{2+}-ratio-imaging both the elevation of $[Ca^{2+}]_{cyt}$ in mesophyll cells following senescence induction by detachment and darkness and the reduced ability of senescent cells to cope with this increased $[Ca^{2+}]_{cyt}$. The elevated $[Ca^{2+}]_{cyt}$ may serve as a signal that can further facilitate senescence through triggering a cascade of deteriorative events. Indeed, indirect evidence suggests that cytosolic Ca^{2+} might have a regulatory role in parsley leaf senescence, since various Ca^{2+} antagonists (EGTA, $LaCl_3$, and nifedipine) delayed senescence characteristics in detached leaves, whereas Ca^{2+} agonists (A23187 and Bay K-8544) enhanced the process (S. Philosoph-Hadas, R. Sabato, and S. Meir, unpublished data). Additional studies are needed to directly characterize this putative regulatory role for increases in $[Ca^{2+}]_{cyt}$ in leaf senescence.

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