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The importance of transmembrane flux of Ca^{2+} in regulating dark-induced senescence of detached corn leaves

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Abstract. Effects of compounds that influenced Ca^{2+} transport on dark-induced senescence of detached corn leaves were examined. Exogenous Ca^{2+} and ionophore A23187 did not affect dark-induced senescence. Specific Ca^{2+} chelators, 1, 2-*bis* (o-aminophenoxy)-ethane-N, N, N′, N′-tetraacetic acid and ethyleneglycol-*bis*-(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, markedly retarded dark-induced senescence. Verapamil, a Ca^{2+} channel blocker, and lanthanum chloride, a Ca^{2+} antagonist, were also found to retard dark-induced senescence. Ruthenium red, the Ca^{2+} transport inhibitor, also retarded the senescence of detached corn leaves in the dark. Collectively, our data seem to suggest that failure to maintain normal transmembrane flux of Ca^{2+} is a key cause of dark-induced senescence.

Key words: Calcium; Leaf senescence; *Zea mays*.

Introduction

Several lines of evidence show that Ca^{2+} plays an important role in regulating leaf senescence. The senescence, measured as chlorophyll loss and protein degradation, of corn and *Rumex* leaf discs was found to be retarded by added Ca^{2+} (Poovaiah and Leopold, 1973). In detached cucumber cotyledons, Ca^{2+} reduced the rate of chlorophyll degradation, ethylene production and peroxide accumulation (Ferguson *et al.*, 1983). These effects were interpreted as consequence of the Ca^{2+} acting on the plasma membrane.

In the present study, we examined the effects of compounds that influenced Ca^{2+} transport on the senescence of detached corn leaves under dark condition. We show here that the inability of the cell to maintain low cytosolic Ca^{2+} level may be a key cause of the senescence syndrome of detached corn leaves.

Materials and Methods

Corn seeds (*Zea mays* L., cv. Tainung 351) were grown in vermiculite in a greenhouse with natural light at 30°C day/25°C night for 7 days by which time the primary leaves were fully expanded. The apical 2.5-cm segments excised from primary leaves were used. Leaf segments were placed vertically in test tubes with the cut end submerging in 2 ml of test solution and incubated at 27°C for 4 days under dark condition. All test solutions were adjusted to pH 5.5.

Total chlorophyll was extracted and determined at 4 days after incubation in the dark by the method of Arnon (1949). Total chlorophyll content is expressed as mg/gFW.

All chemicals used in this study were purchased from Sigme Company, USA.

Results

The senescence of corn leaves was followed by

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[2] **Abbreviations**: BAPTA, 1, 2-*bis*(o-aminophenoxy)-ethane-N, N, N′, N′-tetraacetic acid; EGTA, ethyleneglycol-*bis*-(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid.

measuring the decrease of chlorophyll. Since exogenous Ca^{2+} has been shown to retard senescence of corn leaf discs (Poovaiah and Leopold, 1973), preliminary experiments were performed to determine concentration range over which Ca^{2+} retarded senescence of detached corn leaves in darkness. Surprisingly, we were unable to find any effect on senescence of Ca^{2+} in the range of 0.1 to 5 mM (data not shown).

Figure 1 shows the effect of EGTA and BAPTA, Ca^{2+} specific chelators (Campbell, 1983; Tsien, 1980), on the chlorophyll content of detached corn leaves in the dark. It is clear that both EGTA and BAPTA were effective in retarding dark-induced senescence. The effect of EGTA on the senescence of detached corn leaves was further investigated to see whether its effect could be reversed by the addition of Ca^{2+}. As indicated in Table 1, the retardation effect of EGTA could, indeed, be reversed by the addition of Ca^{2+}, though Ca^{2+} itself at 1 mM had no effect on senescence.

Experiments were carried out with calcium ionophore A23187 (Pressman, 1976) to further characterize the role of Ca^{2+} in dark-induced senescence of detached corn leaves . The results in Table 1 indicated that A23187 in the range of 5-50 μM had no effect on dark-induced senescence of detached corn leaves.

To see whether there is a requirement for calcium channels when dark induces senescence of detached leaves, verapamil, a calcium channel blocker (Janis *et al.*, 1985), or lanthanum chloride, a calcium antagonist (Fineran and Gilbertson, 1978; Thomson *et al.*, 1973), was applied to detached corn leaves under dark codition. It was found that verapamil significantly retarded chlorophyll loss of detached corn leaves incubated in darkness (Fig. 2). Lanthanum chloride was also found to retard chlorophyll loss, though higher concentration than verapamil was required (Fig. 2). It should be noted that toxicity of detached corn leaves treated with 5-10 mM lanthanum chloride were not visually observable.

We also investigated the effect of ruthenium red, the Ca^{2+} transport inhibitor (Bygrave, 1977), on the chlorophyll content in detached corn leaves under dark condition. In the presence of the Ca^{2+} transport inhibitor, dark-induced senescence of detached corn leaves was markedly retarded (Fig. 3).

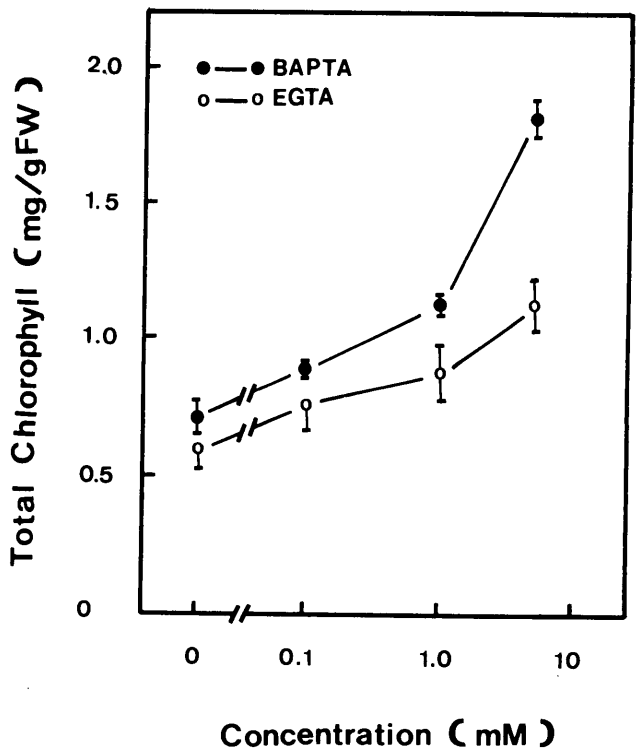

Fig. 1. Effects of EGTA and BAPTA on chlorophyll content of detached corn leaves in the dark. EGTA and BAPTA were prepared by dissolving in 10 drops of 1 N NaOH and then adding an appropriate amount of distilled water. Vertical bars represent standard errors (n=4)

Table 1. *Effects of $CaCl_2$, EGTA, EGTA+$CaCl_2$, and A23187 on chlorophyll content in detached corn leaves after 4 days in darkness. A23187 was prepared by dissolving in 10 drops of ethanol (95%), adding an appropriate amount of distilled water and heating to 80°C to remove ethanol. Values are averages with standard errors (n=4)*

Treatment	Total chlorophyll (mg/gFW)
Experiment I	
Control	0.74±0.05
$CaCl_2$, 1 mM	0.81±0.06
EGTA, 1 mM	1.09±0.05
EGTA, 1 mM+$CaCl_2$, 1 mM	0.89±0.06
Experiment II	
Control	1.24±0.07
A23187, 5 μM	1.24±0.05
A23187, 10 μM	1.25±0.03
A23187, 50 μM	1.27±0.10

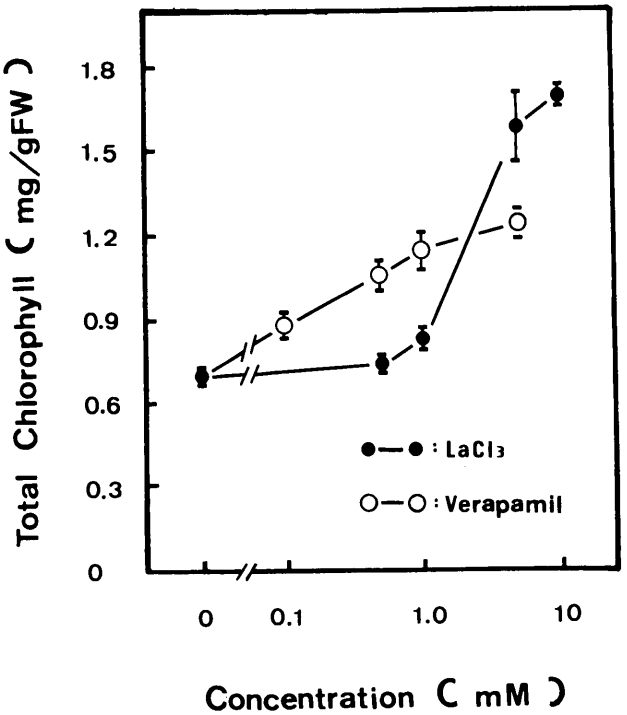

Fig. 2. Effects of verapamil and $LaCl_3$ on chlorophyll content of detached corn leaves in the dark. Verapamil was prepared by dissolving in 10 drops of ethanol (95%), then adding a appropriate amount of distilled water and heating to 80°C to remove ethanol. Vertical bars represent standard errors (n=4).

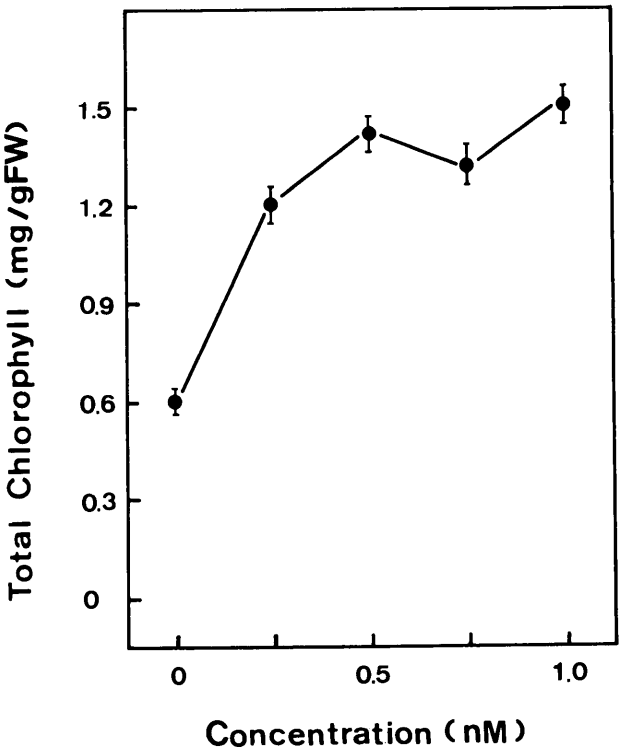

Fig. 3. Effects of ruthenium red on chlorophyll content of detached corn leaves in the dark. Verticals bars represent standard errors (n=4).

Discussion

Exogenous Ca^{2+} has been shown to delay senescence of detached cucumber cotyledons and corn and *Rumex* leaf discs (Ferguson *et al.*, 1983; Poovaiah and Leopold, 1973). The present investigation, however, demonstrated that added Ca^{2+} had no effect on dark-induced senescence of detached corn leaves. Failure to unequivocally demonstrate retardation of dark-induced senescence of detached corn leaves by exogenous Ca^{2+} suggests that corn leaves may contain a high level of Ca^{2+} in darkness.

Using the ionophore A23187 (a compound that transports Ca^{2+} across membranes), it is possible to chemically induce an increase in the intracellular Ca^{2+}. Using pea leaf system, Leshem *et al.* (1984) found that A23187 treatment resulted in the promotion of senescence. In contrast to their results, we found that A23187 did not affect dark-induced senescence of detached corn leaves, indicating that detached corn leaves incubated in the dark may already contain a high level of cytosolic Ca^{2+}.

In both mammals and plants, Ca^{2+} concentration in the cytosol is several order of magnitude less than that on cell's exterior (Ferguson, 1984). The basis for the regulatory activity of Ca^{2+} in mammalian cells is the maintenance of very low concentration of Ca^{2+} in the cytosol (Krestsinger, 1976). Ferguson (1984) suggested that an important part of the senescence process was a breakdown in Ca^{2+} regulation to the extent that increased cytosolic Ca^{2+} level resulted in some of the characteristics of senescence symptoms. The fact that exogenous Ca^{2+} and A23187 had no effect on dark-induced senescence of detached corn leaves suggested that corn leaves incubated in darkness were unable to maintain normally low level of cytosolic Ca^{2+} or the steep Ca^{2+} gradient across the plasma membrane. It seems that the influx of Ca^{2+} into cytosol is enhanced once detached corn leaves are incubated in the dark.

EGTA, BAPTA and ruthenium red have been shown to remain in the outside of the plasma membrane in animal tissues (Jones and Moll, 1983). EGTA does not enter intact plant cells (Moll and Jones, 1981), and the dye quin-2, a close relative of BAPTA (Tsien, 1980), is also difficult to introduce into plant cells (Gilroy *et al.*, 1986). If EGTA, BAPTA and ruthenium red behave similarly in cells of corn leaves, then the effect of them in retarding dark-induced senescence is more likely mediated by inhibiting the influx of Ca^{2+} into the cytosol. The importance of the influx of Ca^{2+} in inducing senescence is further supported by the observations that verapamil and lanthanum chloride markedly retard dark-induced senescence of corn leaves.

Since maintenance of normally low cytosolic Ca^{2+} concentration requires continuous pumping of Ca^{2+} from cytosol to either apoplast or cell organells (Poovaiah and Reddy, 1987), the possibility that the operation of Ca^{2+} pumping in corn leaf cells is blocked under dark condition can not be ruled out.

It is interesting to note that an elevated cytosolic Ca^{2+} retards dark-induced senescence in detached rice leaves (Huang and Kao, 1990). The diverse roles of cytosolic Ca^{2+} on leaf senescence seem to be complex. At present, it is impossible to propose a general explanation why increase in cytosolic Ca^{2+} resulted in the retardation of dark-induced senescence of detached rice leaves but is the prime factor causing senescence of detached corn leaves in the dark. Since increase in cytosolic level of Ca^{2+} in pea leaves resulted in the promotion of senescence (Leshem *et al.*, 1984), an elevated cytosolic Ca^{2+} as a key cause of senescence is unlikely limited to the leaves of C_4 or upland plants.

The present work seems to support the view that the transmembrane flux of Ca^{2+} is important in regulating senescence of detached corn leaves. We admit that all evidences described in this paper are indirect, and conclusive proof should come from direct study of Ca^{2+} transport and/or direct measurement of cytosolic Ca^{2+} level in corn leaves in response to darkness.

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Literature Cited

Arnon, D. I. 1949. Copper enzymes in chloroplast polyphenol-oxidases in *Beta vulgaris*. Plant Physiol. **24**: 1-15.

Bygrave, F. L. 1977. Mitochondrial calcium transport. Curr. Top. Bioenerg. **6**: 259-318.

Campbell, A. K. 1983. Intracellular Calcium: Its Role as Regulator. Wiley, New York.

Ferguson, I. B. 1984. Calcium in plant senescence and fruit ripening. Plant Cell Environ. **7**: 477-489.

Ferguson, I. B., C. B. Watkins, and J. E. Harman. 1983. Inhibition by calcium of senescence of detached cucumber cotyledons: effect on ethylene and hydroperoxide production. Plant Physiol. **71**: 182-186.

Fineran, B. A. and J. M. Gilbertson. 1978. Application of lanthanum uranyl salts as tracers to demonstrate apoplastic pathway for transport in the glands of the carnvorous plant *Utricularia monanthos*. Eur. J. Cell Biol. **23**: 66-72.

Gilroy, S., W. A. Hughes, and A. J. Trewavas. 1986. The measurement of intracellular calcium levels in protoplasts from higher plant cell. FEBS Lett. **199**: 217-223.

Huang, Y. and C. H. Kao. 1990. Senescence of rice leaves XXIV. Involvement of calcium and calmodulin in the regulation of senescence. Plant Cell Physiol. **31**: 1015-1024.

Janis, R. R., D. Rampe, C. M. Su, and D. L. Triggle. 1985. Ca^{2+} channel: Ligand-induced antagonism and activation. *In* T. Godfrainn (ed.), Calcium Entry Blockers and Tissue Protection. Raven Press, New York, pp. 21-30.

Jones, R. L. and C. Moll. 1983. Gibberellin-induced growth in excised lettuce hypocotyls. *In* A. Crozier (ed.), The Biochemistry and Physiology of Gibberellins. Praeger, New York, pp. 95-128.

Krestsinger, R. H. 1976. Evolution and function of calcium binding protein. Int. Rev. Cytol. **46**: 323-393.

Leshem, Y. Y., S. Sirdhara, and J. E. Thompson. 1984. Involvement of calcium and calmodulin in membrane deterioation during senescence of pea foliage. Plant Physiol. **75**: 329-335.

Moll, C. and R. L. Jones. 1981. Calcium and gibberellin-induced elongation of lettuce hypocotyl sections. Planta **152**: 450-456.

Poovaiah, B. W. and R. W. Reddy. 1987. Calcium messenger systems in plants. CRC Crit. Rev. Plant Sci. **6**: 47-103.

Poovaiah, B. W. and A. C. Leopold. 1973. Deferral of leaf senescence with calcium. Plant Physiol. **52**: 236-239.

Pressman, B. C. 1976. Biological application of ionophore. Annu. Rev. Biochem. **45**: 501-530.

Thomson, W. W., K. A. Platt, and N. Campbell. 1973. The use of lanthanum to delineate the apoplastic continuum in plants. Cytobios 8: 57-62.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry **19**: 2396-2406.

鈣離子與玉米切離葉片老化之關係

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本文主要是利用一些能影響鈣離子運移的藥劑處理玉米切離葉片，以時解鈣離子對切離葉片在暗中老化所扮演的角色。外加鈣離子或 A23187(鈣離子的 ionophore，可使 Ca^{2+}進入細胞質中)對切離葉片老化沒有明顯作用。而鈣離子的螯合物(EGTA 或 BAPTA)顯著的延緩切離葉片於暗中的老化。處理 verapamil(鈣通路抑制劑)或 La^{3+}(鈣離子的拮抗劑)，切離葉片的老化亦明顯的被延緩。鈣離子運移的抑制劑亦可延緩切離葉片老化。整體而言，我們的資料顯示，玉米切離葉片在暗中無法保持正常的鈣離子運移可能是導致老化的主因之一。