

Senescence of Rice Leaves XXIV. Involvement of Calcium and Calmodulin in the Regulation of Senescence

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Effects of compounds that influenced calcium uptake and calmodulin inhibitors on the senescence of detached rice leaves were examined. Chelators, ethyleneglycol-bis-(β -aminoethyl ether)-*N,N,N,N*-tetraacetic acid (EGTA) and 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid (BAPTA), significantly promoted senescence of detached rice leaves in the dark and light. The effect of EGTA can be reversed by treating detached rice leaves with calcium. Verapamil, a calcium channel blocker, and lanthanum chloride, a calcium antagonist, promoted dark-induced, and suppressed BA- and light-retarded senescence of detached rice leaves. Calcium ionophore A23187 and ruthenium red, believed to raise cytosolic level of Ca^{2+} , were quite effective in retarding dark-induced and ABA-promoted senescence of detached rice leaves. Calmodulin inhibitors, W-7, compound 48/80, chlorpromazine and trifluoperazine, significantly promoted dark-induced, and suppressed BA- and light-retarded senescence of detached rice leaves. It is concluded that cytosolic level of Ca^{2+} may regulate senescence of detached rice leaves through a calmodulin-dependent mechanism.

Key words: Calcium — Calmodulin — Leaf senescence — *Oryza sativa*.

Calcium is known to retard ripening process of fruit. Specifically, maintenance of relatively high calcium concentrations in fruit tissue results in a slower rate of ripening, as seen in lower respiration, reduced ethylene production, and slower softening of fruit flesh (Ferguson 1984). Leaf senescence has also been shown to be retarded by the application of calcium. Chl loss and protein degradation were both reduced in corn and *Rumex* leaf discs by calcium (Poovaiah and Leopold 1973). In whole, excised cucumber cotyledons, calcium treatment reduced the rate of Chl degradation and peroxide accumulation (Ferguson et al. 1983). They related the calcium effect to maintain cellular membranes (Ferguson et al. 1983, Poovaiah and Leopold 1973). It appears that the senescence-effect of calcium is an effect of external calcium. Using pea leaf system, Leshem et al. (1982, 1984, 1986) were able to show that senescence was promoted by increasing intracellular calcium. They concluded that elevated intracellular calcium promoted senescence through a calmodulin-mediated effect.

Abbreviations: BA, benzyladenine; BAPTA, 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid; CPZ, chlorpromazine; EGTA, ethyleneglycol-bis-(β -aminoethyl ether)-*N,N,N,N*-tetraacetic acid; TFPZ, trifluoperazine.

The present investigation was undertaken to examine the effects of calcium chloride, calcium chelators (EGTA and BAPTA), a calcium antagonist (lanthanum chloride), a calcium channel blocker (verapamil), a calcium ionophore (A23187), and anti-calmodulin drugs (W-7, compound 48/80, CPZ and TFPZ) on the senescence of detached rice leaves. Results indicate that elevated cytosolic calcium retards senescence of detached rice leaves through a calmodulin-dependent mechanism.

Materials and Methods

Rice (*Oryza sativa* cv. Taichung Native 1) seedlings were cultured as previously described (Kao 1980). The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments were floated in a Petri dish containing 10 ml of test solutions. All chemicals used in this investigation were purchased from Sigma. Incubation was carried out at 27°C in darkness or the light ($16.7 \text{ W} \cdot \text{m}^{-2}$) provided by fluorescent lamps.

Chl was extracted and quantitated as described previously (Kao 1980). All experiments were repeated at least twice. Data are presented as the results of a single experi-

ment typical of the trends seen in the repeated experiments.

Results

The effectiveness of calcium in retarding Chl degradation of detached rice leaves was tested as shown in Figure 1. Calcium was effective in retarding the decrease of Chl under both light and dark conditions. The effect of calcium in the dark was more effective than that in the light. The effect of EGTA on Chl content of detached rice leaves in the dark is presented in Figure 2. The amount of Chl was decreased by EGTA treatment. This effect was reversed by treating detached rice leaves with calcium (data not shown). Ca^{2+} chelator BAPTA was found to be more effective than EGTA in decreasing Chl content (Fig. 2).

Experiments were carried out with putative calcium channel blocker to further characterize the role of calcium in the regulation of senescence of detached rice leaves in the dark. Verapamil, a calcium channel blocker, or lanthanum chloride, a calcium antagonist, was applied to detached rice leaves in the dark. The results in Figure 3 indicate that verapamil was very effective in causing the decrease of Chl content. Lanthanum chloride also decreased Chl content in the dark (Fig. 3). These experiments suggest that Chl loss of detached rice leaves in the dark is regulated by a block of transport of calcium ions into the cytosol. Further evidence of this role for calcium was obtained using the calcium ionophore A23187 (Campbell 1983). Treatment with the ionophore at 0.01 mM resulted in higher Chl content than the control (Table 1). Ruthenium red is known to block calcium ion transport (Hinds et al. 1981, Watson et al. 1971) and is believed to

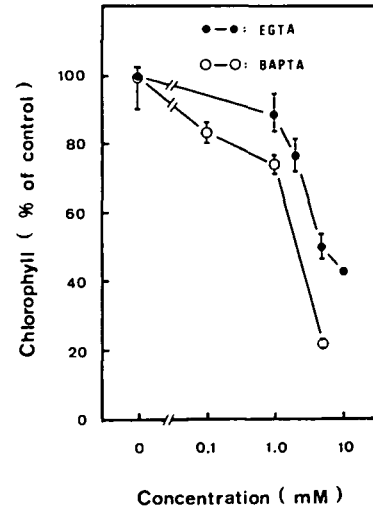


Fig. 2 Effects of EGTA and BAPTA on the Chl content of detached rice leaves in the dark. Chl was determined after 4 days in the dark. Values are averages with standard errors ($n=4$).

raise the intracellular calcium level (Bednarska 1989, Moll and Jones 1982, Vashington et al. 1972). We investigated the effect of this compound on the Chl content of detached rice leaves in the dark. Ruthenium red was found very effective in retarding Chl loss (Table 1).

We also investigated the Chl content of detached rice leaves in the dark in the presence of calmodulin antagonists. Results shown in Table 1 and Figure 4 indicate that calmodulin antagonists, W-7, compound 48/80, CPZ and TFPZ, significantly accelerated the decrease of Chl con-

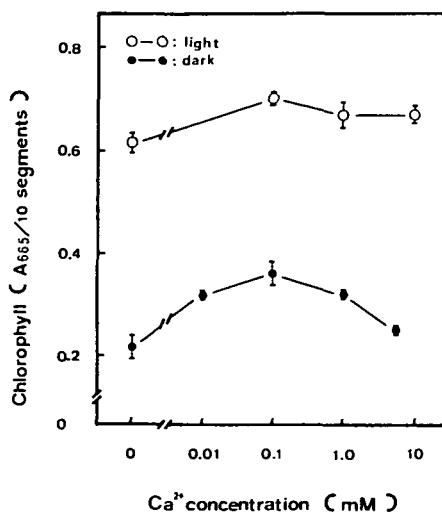


Fig. 1 Effect of calcium chloride on the Chl content of detached rice leaves. Chl was determined after 4 days in the dark or light. Values are averages with standard errors ($n=4$).

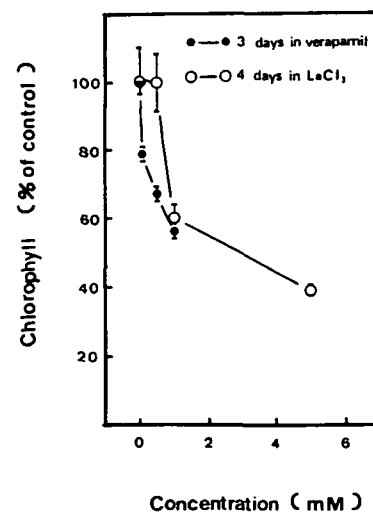


Fig. 3 Effects of verapamil and lanthanum chloride on the Chl content of detached rice leaves in the dark. Values are averages with standard errors ($n=4$).

Table 1 Effect of A23187, ruthenium red, W-7 and compound 48/80 on the Chl content of detached rice leaves in the dark

Treatment	Chl ($A_{665}/10$ segments)
A23187 (mM)	
0	0.44 ± 0.01
0.01	0.67 ± 0.02
Ruthenium red (mM)	
0	0.40 ± 0.02
0.5	0.49 ± 0.04
1.0	0.61 ± 0.02
2.0	0.71 ± 0.03
W-7 (mM)	
0	0.72 ± 0.03
0.1	0.46 ± 0.02
0.5	0.34 ± 0.03
Compound 48/80 ($\mu\text{g}/\text{ml}$)	
0	0.55 ± 0.03
100	0.43 ± 0.01

Chl was determined after 4 days in the dark. Values are averages with standard errors (n=4).

tent.

BA, a synthetic cytokinin, is known to retard Chl degradation of detached rice leaves in the dark (Kao 1980). To see whether there is a requirement for calcium channels when BA retards Chl degradation, agents known to block such channels were incorporated in the incubation media. Lanthanum chloride and verapamil significantly reduced the retardation effect of Chl loss by BA (Table 2). We also

Table 2 Effects of BA alone and BA in the presence lanthanum chloride, verapamil, CPZ, TFPZ, W-7 and compound 48/80 on the Chl content of detached rice leaves in the dark

Treatment	Chl ($A_{665}/10$ segments)
Control	
Control	0.23 ± 0.02
BA	0.78 ± 0.03
BA + LaCl ₃	0.55 ± 0.02
BA + Verapamil	0.29 ± 0.03
BA + CPZ	0.45 ± 0.02
BA + TFPZ	0.39 ± 0.02
Control	
Control	0.72 ± 0.03
BA	0.95 ± 0.02
BA + W-7	0.84 ± 0.03
Control	
Control	0.55 ± 0.03
BA	0.96 ± 0.01
BA + Compound 48/80	0.82 ± 0.01

The concentrations for BA, lanthanum chloride, verapamil, and CPZ are 1 mM, whereas those for TFPZ, W-7 and compound 48/80 are 0.5 mM, 0.1 mM and 100 $\mu\text{g}/\text{ml}$, respectively. Chl was determined after 4 days in the dark. Values are averages with standard errors (n=4).

investigated the ability of BA to retard Chl loss in the presence of calmodulin antagonists in the dark. In the presence of W-7, compound 48/80, CPZ and TFPZ, the effect of BA was significantly suppressed (Table 2), indicating there is a requirement of calmodulin for BA to retard Chl loss of detached rice leaves in the dark.

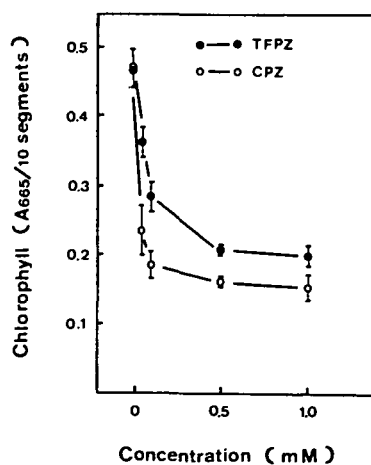
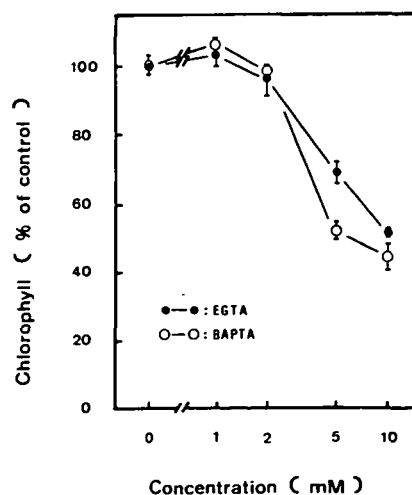
**Fig. 4** Effects of TFPZ and CPZ on the Chl content of detached rice leaves in the dark. Chl was determined after 4 days in the dark. Values are averages with standard errors (n=4).**Fig. 5** Effects of EGTA and BAPTA on the Chl content of detached rice leaves in the light. Chl was determined after 4 days in the light. Values are averages with standard errors (n=4).

Table 3 Effects of lanthanum chloride, verapamil, CPZ, TFPZ, W-7 and compound 48/80 on the Chl content of detached rice leaves in the light

Treatment	Chl ($A_{665}/10$ segments)
Control	0.56 ± 0.02
LaCl ₃	0.34 ± 0.02
Verapamil	0.21 ± 0.12
CPZ	0.46 ± 0.01
TFPZ	0.45 ± 0.01
Control	0.80 ± 0.01
W-7	0.59 ± 0.01
Control	0.79 ± 0.01
Compound 48/80	0.64 ± 0.02

The concentrations for lanthanum chloride, verapamil and CPZ are 1 mM, whereas those for TFPZ, W-7 and compound 48/80 are 0.5 mM, 0.1 mM and 100 µg/ml, respectively. Chl was determined after 4 days in the light. Values are averages with standard errors (n=4).

Light has been shown to retard Chl loss of detached rice leaves (Hurng et al. 1986). Similar to the effect of BA in the dark, calcium and calmodulin appear to be required for the effect of light. This conclusion is supported by the observations that calcium chelators, calcium channel blockers and calmodulin antagonists significantly reduced the effect of light (Fig. 5 and Table 3).

ABA significantly promoted Chl loss of detached rice leaves in the light (Table 4). ABA effect was totally abolished by ruthenium red and significantly reduced by A23187 (Table 4). These experiments seem to suggest that ABA promoted Chl loss may mediate through blocking calcium ions into the cytosol.

Table 4 Effects of ABA alone and ABA in the presence of ruthenium red or A23187 on the Chl content of detached rice leaves in the light

Treatment	Chl ($A_{665}/10$ segments)
Control	0.88 ± 0.01
ABA	0.26 ± 0.02
ABA + Ruthenium red	0.87 ± 0.03
Control	0.58 ± 0.03
ABA	0.36 ± 0.01
ABA + A23187	0.43 ± 0.02

The concentrations of ABA, ruthenium red and A23187 are 0.1, 2.0 and 0.01 mM, respectively. Chl was determined after 4 days in the light. Values are averages with standard errors (n=4).

Discussion

Several compounds expected to increase or decrease cytosolic levels of calcium were used to examine the requirement of calcium in regulating the senescence of detached rice leaves, measured as Chl loss. We also investigated effects of four inhibitors known to inhibit action of Calmodulin in animals.

EGTA is a specific Ca²⁺ chelator (Campbell 1983) used in many systems to affect endogenous Ca²⁺ concentrations (Daye 1984, Karege et al. 1982, Lehtonen 1984) by causing a depletion of cytosolic Ca²⁺ (Campbell 1983, Gilroy et al. 1986, Poovaiah and Reedy 1987). The chelator BAPTA is much more selective for Ca²⁺ than EGTA (Tsien 1980). In the present study, we found that both EGTA and BAPTA were effective in promoting Chl loss. The effect of EGTA could be reversed by the addition of Ca.

Of particular interest is the finding that ruthenium red, a hexavalent dye, significantly retarded dark-induced and ABA-promoted senescence of detached rice leaves. Ruthenium red has been shown to be a potent inhibitor of Ca²⁺ uptake and efflux by animal cells and mitochondria (Hinds et al. 1981, Watson et al. 1971). This compound also interferes with Ca²⁺ efflux from aleurone layers of barley (Moll and Jones 1983). Recently, Bednarska (1989) reported that ruthenium red raised the amount of ⁴⁵Ca²⁺ taken up by germinating pollen grains relative to the control. Based on the known mechanism of ruthenium red action in animal and plant cells, the most likely explanation of its effect on Chl loss involves its role in the regulation of cytosolic Ca²⁺ content.

The importance of the cytosolic Ca²⁺ in the regulation of rice leaf senescence is further supported by the observations that (a) lanthanum chloride, a calcium antagonist, and verapamil, a calcium channel blocker, promoted dark-induced, and reduced light- and BA-retarded senescence of detached rice leaves, and (b) calcium ionophore A23187, a compound that induces an increase in the cytosolic Ca²⁺, retarded dark-induced and ABA-promoted senescence of detached rice leaves.

Our data thus strongly suggest that an elevated level of cytosolic level of Ca²⁺ is required for retarding senescence of detached rice leaves. However, our results are inconsistent with those reported by Leshem et al. (1982, 1984, 1986), who found that senescence of pea leaves was promoted by increasing cytosolic Ca²⁺. The use of different plant species may have led this discrepancy. Recently, Leshem (1987) proposed that elevated cytosolic Ca²⁺ might promote senescence through a mechanism that triggered the catabolic process by binding to calmodulin and activating phospholipase A₂.

The use of calmodulin inhibitors on intact cells can lead to complex responses, and it cannot be assumed that

the primary target reaction or system is the only one affected. It was for this reason that we used four different calmodulin inhibitors, and it is important that we obtained essentially similar results with all four. The concentrations of calmodulin inhibitors used in this investigation are comparable with those used by others who concluded that Ca-calmodulin is probably essential for physiological processes (Elliott 1983, Elliott et al. 1983, Friedman et al. 1989, Muto and Hirose 1987). Furthermore, no toxicity (rolling or loss of turgor) was visually observable at the inhibitory concentrations.

Since calmodulin inhibitors were found to promote dark-induced and reduce BA- and light-retarded senescence, the cytosolic Ca^{2+} seems to regulate senescence through a calmodulin-mediated effect. Calcium- and calmodulin-stimulated phosphorylation of membrane proteins has been found to decrease during senescence of apples (Paliyath and Poovaiah 1984, 1985). In a recent study, we demonstrated that proton secretion activity of detached rice leaves, which was found to be originated from ATP-driven H^+ pump located in the plasma membrane, played an important role in regulating senescence of detached rice leaves (Chen et al. 1990). Proton secretion activity decreased during senescence of detached rice leaves. Phosphorylation of the plasma-membrane H^+ -ATPase has been shown to be regulated by calcium-stimulated protein kinase (Bidwai and Takemoto 1987, Schaller and Sussman 1988). Hanson and Trewavas (1982) suggested that calcium and calmodulin might regulate the activity of H^+ -ATPase of the plasma membrane. It seems that the promotion of Ca^{2+} and calmodulin-dependent protein kinase or phosphorylation by Ca^{2+} and calmodulin provides a mechanism by which intracellular Ca^{2+} can regulate senescence of detached rice leaves. The possible changes of protein kinase activity or protein phosphorylation during senescence of detached rice leaves are now the subject of further research.

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