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The Role of Ca^{2+} in Retardation Effects of Benzyladenine on the Senescence of Wheat (*Triticum aestivum* L.) Leaves

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The role of Ca^{2+} on benzyladenine (BA)-induced senescence retardation in mature wheat (*Triticum aestivum* L.) primary leaves was investigated. When an extracellular calcium chelator, ethylene glycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) together with BA, was applied to senescing leaves for 4 days of dark incubation, the content of chlorophyll and soluble protein decreased rapidly. And, the content of malondialdehyde (MDA), known to be a degradation product of membrane lipids, increased compared with the BA alone control. The BA-EGTA combination also caused the stimulation of protease and RNase activity and a rapid loss of catalase activity owing to the decline of BA effects. In the case of treatment with only intracellular calcium antagonist 3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester (TMB-8) without the BA addition, the chlorophyll content at day 4 after dark incubation decreased in parallel with the increasing concentration of the antagonist. In addition, the chlorophyll content at 10^{-5} M calcium ionophore A23187 treatment in the absence of BA was similar to that of the BA alone treatment. These results suggest that calcium may mediate the retardation effect of BA on leaf senescence by acting as a second messenger and that the calcium input from cell organelles, as well as the calcium inflow from intercellular spaces and cell walls, may be involved in modulating cytosolic calcium levels related to BA action.

Keywords: wheat, senescence, benzyladenine, calcium, second messenger

Since the function of cytokinins has been shown to be effective in inhibiting plant senescence (Richmond and Lang, 1957), further cytokinin effects have been demonstrated by the following studies: detached bean leaf growth (Leopold and Kawase, 1964), changes of lipoxygenase activity in pea leaves (Grossman and Leshem, 1978) and the RNA degradation of barley leaves (Stoddart and Thomas, 1980; Legoka and Szweykovska, 1981). It was also reported that these effects of cytokinins were transmitted to cells through the mediation of a second messenger such as other hormones (Poovaiah, 1989). The mechanisms of action of hormones were generally claimed to occur through two different ways: one is direct action on cells, and the other is modulation of the ion flux of the cells (Leopold and Nooden, 1984; Sexton and Woolhouse, 1984).

The calcium ion is regarded as the more important regulator among the second messengers of H^+, K^+, IP_3, Ca^{2+}, and the differential membrane potential, which are known to mediate hormonal actions and various physiological stimuli in plants (Cheung, 1980; Campbell, 1983; Roux, 1986; Marme, 1989). Saunders (1986) suggested that the stimulus of cytokinins was transmitted through the regulation of a cellular Ca^{2+} pool. For example, benzyladenine (BA) treatment enhanced bud formation in the algae *Funaria*, however the ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid (EGTA) treatment which was known to block the Ca^{2-} input from the outside to the inside of a cell inhibited the BA effect. In addition, he observed that a calcium ionophore A23187 mimicked the BA-enhanced effect on bud formation. It was also reported that EGTA hindered the BA effect in retarding the degradation of chlorophyll and protein during senescence (Rhee and Poovaiah, 1985; Poovaiah, 1987). Other reports, however, have emphasized an alternate role of calcium (Leshem *et al.*, 1984). In these

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studies, calcium ions enhanced the activity of phospholipase A_2 participating in the degradation of cell membrane lipids and thus accelerated the processes of senescence. In relation to the role of calcium ions, it is known that there are various sources of calcium supply in plant cells, such as cell membranes, cell walls and intercellular spaces, and organelles such as the endoplasmic reticulumn (ER), Golgi's bodies and vacuoles (Poovaiah *et al.*, 1987). Especially, the interactions of cytokinin and calcium are suggested to induce a change of cell membrane permeability during senescence (Poovaiah *et al.*, 1987).

This present study, therefore, aimed to clarify the interactions of BA and calcium as well as to correlate between intra- and extra- cellular calcium pools during senescence. To do so, we investigated the changes in the levels of characteristic metabolites and the development of activities of some enzymes in BA-treated wheat leaves with and without: the extracellular Ca^{2+} chelater, EGTA; an intracellular Ca^{2+} antagonist, 3,4,5-trimethoxybenzoicacid 8-(diethylamino) octyl ester (TMB-8); and a Ca^{2+} ionophore, calcymycin A23187.

MATERIALS AND METHODS

Plant material and growth conditions

Wheat seeds (*Triticum aestivum* L.) were surface-sterilized by soaking 30 min in a solution of 1%(v/v) sodium hypochlorite and then immersed for 24h in distilled water with aeration. These seeds were sown and allowed to germinate in glass-covered polypropylene containers ($105\times105\times100$ mm) which contained 3 layers of filter paper (Toyo No. 2) moistened with distilled water. The wheat seedlings in each growth chamber were grown for 5 days without nutrient supply at 25 ± 1°C under continuous daylight fluorescent tubes giving an approximate intensity of 20 Wm^{-2}. On day 6 the first leaves of the seedlings were excised and prepared as segments 30 mm in length. The leaf segments were floated in petri dishes (5 cm diameter) containing 10 mL distilled water, or the treatment solutions, and then incubated to senescence for up to 4 days in the same growth chamber except for those under darkness.

Preparation of treatment solutions

34 mM calcimycin A23187 was dissolved in DMSO, and 40 mM TMB-8 in 100% MtOH were prepared as stock solutions, respectively. Each stock was then diluted with DW to achieve its final concentration as a treatment solution. The concentration of the used benzyladenine solution (pH 5.6) was 1×10^{-6} M and that of EGTA in 1×10^{-6} M BA solution was 1×10^{-3} M, respectively.

Biochemical analysis

Leaf segments were placed in 100% DMSO solution and chlorophyll was leached into the fluid without grinding at 65°C by incubating for 1 h. The absorbance of the chlorophyll extract was read at 645 and 663 nm and the content of chlorophyll was calculated from the equation used by Holden (1965). In order to determine soluble protein content, leaf tissues (10 segments) were homogenized in 5 mL of cold 50 mM potassium phosphate buffer (pH 7.5) and centrifuged at 18,000 g at 4°C for 30 min. The supernatant was mixed with the same volume of chloroform and recentrifuged at 1,800 g for 5 min to remove pigments. From this supernatant, the soluble protein was precipitated with 20%(v/v) trichloroacetic acid (TCA) for 24h and the precipitate redissolved in 1 N NaOH and the protein content estimated according to Lowry *et al.* (1951). Malondialdehyde (MDA) content was determined by thiobarbituric acid (TBA) reaction (Dhindsa *et al.*, 1981) with a minor modification of the method of Heath and Packer (1968). 24 leaf segments were homogenized in 4 mL of 0.1% TCA. The homogenate was centrifuged at 10,000 g for 5 min. To 1 mL aliquots of the supernatant, 4 mL of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. After centrifuging at 10,000 g for 10 min the absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted. The content of MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

Enzyme extraction and assay

Leaf tissue (10 segments) was homogenized on ice with 0.3 g quartz sand in 3 mL of 50 mM potassium phosphate buffer (pH 7.5) using a pre-cooled mortar. The homogenate was centrifuged at 18,000 g for 30 min and the supernatant obtained was used as enzyme extract. In the case of protease, the extraction buffer additionally contained 1 mM EDTA, 4 mM DTT and 0.1 g/mL polyvinylpolypyrrolidone (PVPP). Catalase activity was assayed

according to Chance and Maehly (1955). The 3 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.05 mL enzyme extract. Enzyme activity was estimated from the decrease of absorbance at 240 nm using an extinction coefficient of 0.44×10^2 M^{-1} cm^{-1} (Chance and Maehly, 1955). Lipoxygenase activity was measured polarographically in a 3 mL reaction vessel fitted with a Clark-type oxygen electrode (Douillard and Bergeron, 1981) with a minor modification of the method of Surrey (1963). In a total volume of 3 mL, the reaction solution containing 2 mL of 50 mM potassium phosphate buffer (pH 6.9) and 0.5 mL of 15 mM linoleic acid emulsified in Tween 20 was aerated fully by magnetic stirrer at 20°C and the reaction was started by adding 0.5 mL enzyme extract. Lipoxygenase activity was estimated from the O_2 consumption in the reaction solution. RNase activity was assayed by the method of Altman *et al.* (1977). 0.1 mL of enzyme extract was incubated with 0.4 mL of RNA substrate (containing 15 mg purified *Torula* yeast RNA/16 mL of 0.1 M acetate buffer at pH 5.5) for 1h at 37°C, and the reaction was terminated by adding 0.5 mL of 2.5% (w/v) TCA and 0.3% (w/v) $La(NO_3)_3$. The reaction was left for 2h at 4°C and the clear supernatant was collected after centrifugation (1,000 g, 10 min), then the absorbance at 260 nm was read. The enzyme activity was expressed in units where one unit of RNase indicates a 0.1 increase of absorbance at 260 nm per 1h. Protease activity was assayed according to the method of Wittenbach (1978) with slight modification. 0.4 mL of enzyme extract was incubated with 0.2 mL of azocazein substrate (10 mg/mL DW) and 0.4 mL of 0.1 M citrate buffer (pH 4.8) for 2 h at 37°C, and the reaction was stopped by adding 1 mL of 20% (w/v) TCA. The reaction was left for 30 min at 4°C and the supernatant was obtained after centrifugation (1,000 g, 5 min), and then the absorbance at 340 nm was measured. One unit of protease activity is the amount of enzyme which results in an increased absorbancy of 0.01 at 340 nm per 1 h.

RESULTS AND DISCUSSION

Changes in the level of cellular components during senescence

The primary feature of the biochemical changes generated in senescing leaf cells is a dramatic decline in the levels of cellular components such as chlorophyll, protein, nucleic acid and the major cell membrane lipid (Thimann, 1980). It is well known that the degradation of these cellular components is retarded by the action of BA which is one of the synthetic cytokinins. If the calcium ion mediates the action of BA, blocking the calcium sources would be expected to decrease BA effects in retarding senescence. Therefore, we investigated the changes in the contents of chlorophyll, protein and malondialdehyde (MDA: a degradation product of membrane lipids) as senescence markers in senescing wheat leaves for 4 days dark incubation in the presence of 10^{-6} M BA with the extracellular Ca^{2+} chelator (10^{-3} M EGTA). As shown in Figure 1 and Figure 2, the decrease in the contents of chlorophyll and protein in BA only treated leaves was noticeably retarded as compared to the DW control. In the BA-treated leaves with EGTA, which caused the blocking of calcium input from the intercellular spaces to the inside of cells, the BA effect in retarding senescence began to decrease at day 1 after treatment. Thus the chlorophyll and protein content in leaves with the BA-EGTA combination treatment decreased 33% and 25% at day 4, respectively, as compared to those of BA only treated leaves. This means that calcium is active in BA's action in retarding senescence. This result, however, was not coincident with the fact that the Ca^{2+}-calmodulin complex promoted senescence processes through the activation of cell membrane-localized phospholipase A_2 (Leshem *et al.*, 1984). The destruction of cell membrane structures has been well demonstrated the key symptom of senescence. It also has been assumed that the stability and permeability of the cell membrane might be regulated by the stimuli of light and hormones. Especially, the degradation of cell membrane lipids and proteins is accompanied by the onset of membrane oxidation reactions due to free radicals and activated hydrolytic enzymes, which cause the loss of membrane integrity and thus ultimately accelerates the senescence processes (Dhindsa *et al.*, 1981). The peroxidation of a membrane lipid is largely assumed to induce such membrane destruction (Fridovich, 1976; Stewart and Bewlay, 1980; Kar and Feierabend, 1984), thus the degree of senescence can be deduced by measuring the content of MDA. In senescing wheat leaves of 4 days incubation, the BA alone treatment conspicuously reduced MDA production as compared to the DW control, but the BA-EGTA combination treatment stimulated the production of MDA up to almost the same level of the DW control (Fig. 3). Therefore, the inhibitive effect of BA on MDA pro-

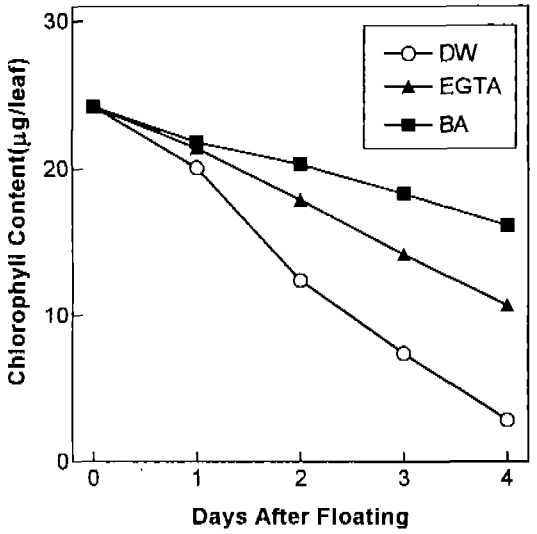

Fig. 1. Changes of total chlorophyll content in detached wheat leaves under the treatments of DW, 10^{-6} M BA and 10^{-3} M EGTA for 4 days dark incubation. In the case of EGTA treatment, 10^{-6} M BA was supplemented.

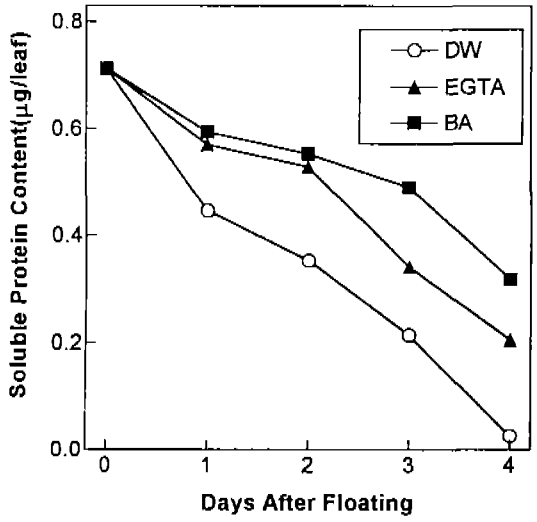

Fig. 2. Changes of soluble protein content in detached wheat leaves. Each treatments on leaves are same with those in figure 1.

duction in senescing wheat leaves indicates that BA may have a regulatory ability over senescence by a modulation of the free radical scavenging metabolism together with an associated enzyme system. It has also been reported that the changes of cytosolic calcium concentration can regulate lipoxygenase activity (Grossman and Leshem, 1978; Lynch and Thompson, 1984; Moor *et al.*, 1986). So, the antagonistic effect of EGTA on BA action in MDA production suggests that BA also may regulate the activity of enzymes related to membrane peroxidation through modulation of the calcium pool.

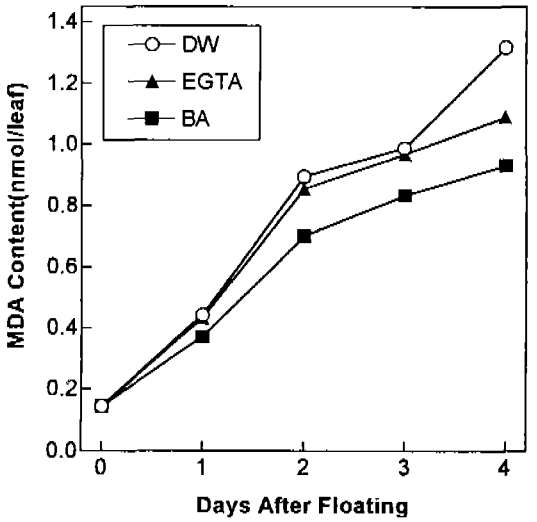

Fig. 3. Changes of malondialdehyde(MDA) content in detached wheat leaves. Each treatments on leaves are same with those in figure 1.

Changes in enzyme activities related to senescence

The processes of senescence are known to be accompanied by the increase of several enzymes activities related to the degradation of cell constituents. Namely, it has been observed that the roles of catalase, peroxidase and superoxide dismutase (SOD) are involved in the scavenging of free radicals, and the lipoxygenase (LOX) in the lipid breakdown and the protease and RNase in the degradation of proteins and ribonucleic acids are also well documented (Makovetzki and Goldschmidt, 1976; Nooden and Leopold, 1989). Calcium is well known to affect the activity of LOX and SOD (Allen and Trewavas, 1987). Martin and Thimann (1972) has suggested that the enhancement of protease and RNase synthesis in accordance with the progress of senescence could be explained in terms of calcium effects on the transcription and translation levels of their enzymes. Therefore, the development of the activities of catalase, LOX, RNase and protease were investigated in senescing wheat leaves during 4 days dark incubation. It has been speculated that H_2O_2 may leak away from abnormal cellular metabolism and give rise to even more oxygen radicals that readily attack the cell membrane leading to acceleration of senescence processes (Fridovich, 1976).

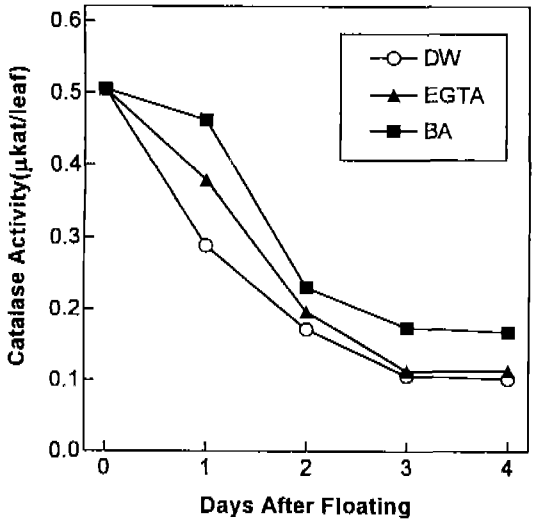

Fig. 4. Response of catalase activity in detached wheat leaves. Each treatments on leaves are same with those in figure 1.

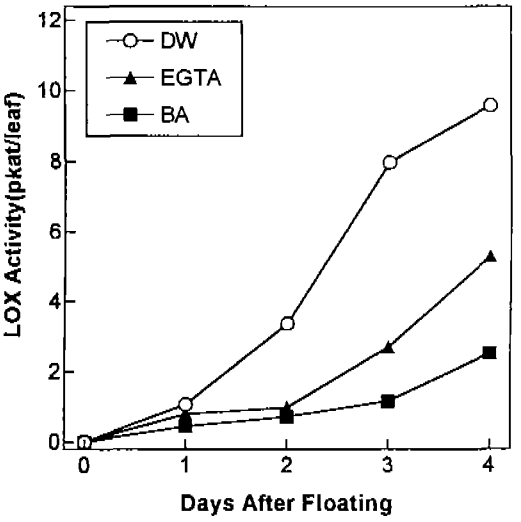

Fig. 5. Time course of lipoxygenase (LOX) activity development in detached wheat leaves. Treatments are same with those in figure 1.

The rapid removal of H_2O_2 is assumed to be associated with the maintenance of high catalase activity via a BA action, therefore, the suppression in oxidative degradation of cell constituents can be expected (Servettaz *et al.*, 1976). In the catalase activity profile during 4 days dark incubation (Fig. 4), the overall development patterns were similar to each other in all treatments including a DW control. However, the decrease of catalase activity of BA only treated leaves was retarded as compared to the DW control. The similar level of catalase activity in both the BA-EGTA combination treatment and the DW control after day 1 suggests the possibility that the amplified cellular Ca^{2+}-pool by the BA stimulus may suppress the decrease of catalase activity and thereby reduce the generation of free radicals. It has been suggested that LOX-mediated lipid peroxidation may be associated with the membrane damage and lead to an acceleration of free radical production (Kar and Feierabend, 1984; Lynch and Thompson, 1984). As presented in Figure 5, the BA treatment significantly delayed the increase of LOX activity compared to the DW control in senescing wheat leaves. Such a BA effect in delaying LOX activity development was interrupted by EGTA in the case of catalase. The LOX activity has been suggested to be regulated by the activated Ca^{2+}-calmodulin complex due to the increase of cytosolic calcium concentration (Cheung, 1980; Allan and Trewavas, 1987; Poovaiah, 1989). In the present experiment interrupting the entry of calcium into the cell by EGTA treatment, we observed a decrease of BA effects by delaying LOX activity. This result indicates the fact that the function of BA in retarding senescence may appear through a mediation of calcium. The increment of *de novo* synthesis of protease and RNase during leaf senescence promotes a degradation of basic cell components such as protein and nucleic acid (Martin and Thimann, 1972; Wittenbach, 1978; Yu and Kao, 1981). In addition these enzymes inhibit the translation processes and the synthesis of several enzymes for cellular metabolism, and thus cause a loss of cell integrity (Thomas and Stoddat, 1980; Miller and Huffaker, 1982, 1985). Stoddat and Thomas (1980) reported that mechanical wounding and unfavorable environmental stress could induce biosynthesis of these hydrolytic enzymes and thus accelerate the progress of senescence. The results shown in Figure 6 and Figure 7 indicate that BA noticeably delayed the development of activities of RNase and protease in senescing wheat leaves. However, the BA-EGTA combination treatment reduced the BA effect in delaying the development of these enzyme activities. In the time course of RNase activity development, all treatments including control gradually declined after day 3 of dark incubation. Isola and Franzoni (1981) observed that the differential appearance with time in RNase isozymes occurred in senescing leaves. It is also known that treatment by trifluoperazine (an inhibitor of calcium-calmodulin complex) and diltiazem (a calcium channel blocker) increased some RNase and

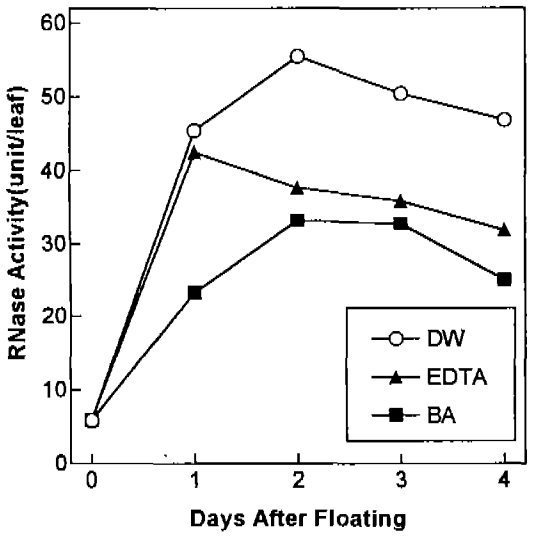

Fig. 6. Time course of RNase activity development in detached wheat leaves. Treatments are same with those in figure 1.

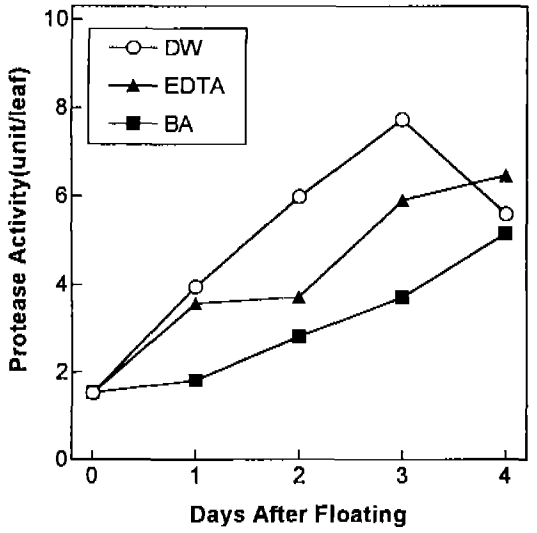

Fig. 7. Time course of protease activity development in detached wheat leaves. Treatments are same with those in figure 1.

protease isozyme activities, and thereby reduced the BA effect in retarding senescence in early senescing leaves. The results obtained in the present experiments together with other reports suggest that the decrease of RNase activity after day 3 in senescing wheat leaves is attributable to the decline of RNA substrate content and the reduction of a substrate-inducible enzyme activity. Stoddart and Thomas (1980) demonstrated that there is a close correlation between the decrease of protein content and the increase of protease activity. As presented in Figure 7, the protease activity in a DW control decreased conspicuously at day 4. It is likely that the reduced protease activity is attributable to the loss of RuBPcase content, known to be a good intracellular substrate for a thiol-protease, and/or the degradation of protease itself due to autoproteolysis. Actually, the soluble protein content in a DW control at day 4 remained at zero level (Fig. 2). The antagonistic effects of EGTA against the BA action in chlorophyll and protein content (Figs. 1, 2), RNase activity profile (Fig. 6) and protease activity profile (Fig. 7) were all apparent between day 1 and day 2 during 4 days dark incubation. This suggests that the relation between BA and calcium seems to be determined in the early stage of senescence.

Effects of the intracellular Ca^{2+} antagonist TMB-8 and the Ca^{2+} ionophore A23187 on chlorophyll content

Pretch *et al.* (1980) showed that the calcium ionophore A23187 takes a cyclic form by binding itself to a cell membrane, and thereby allows the zwitterion to enter the cell according to the passive concentration gradient. In particular the ionophore A23187 shows an almost absolute specific affinity to calcium. The intracellular calcium antagonist TMB-8 interrupts the calcium supply from cell organelles into the cytosol and reduces the efficiency of cytosolic free calcium (Brummel and Maclachlan, 1989). Using an ionophore, some evidence has implicated calcium mediation in the growth inhibition response owing to mechanical stress (Jones and Mitchell, 1989) and in overcrowding by ethylene (Faber and Kandeler, 1989). It has also been demonstrated that TMB-8 noticeably decreased the regulation activity of calcium in promoting growth response via stimulation of cell wall synthesis. And the decline in the regulation activity of calcium was attributed to the decrease of cytosolic free calcium concentration which resulted from the calcium antagonistic action of TMB-8 (Saunders and Jones, 1988; Brummel and Maclachlan, 1989). The present study was, therefore, designed to investigate calcium mediation in BA actions on senescence and an affection of BA to calcium supply sources in using calcium ionophore A23187 and an intracellular calcium antagonist TMB-8. Figure 8 showed the effect of various concentrations of TMB-8 on the chlorophyll contents in dark incubating wheat leaves in the presence and absence of 10^{-6} M BA.

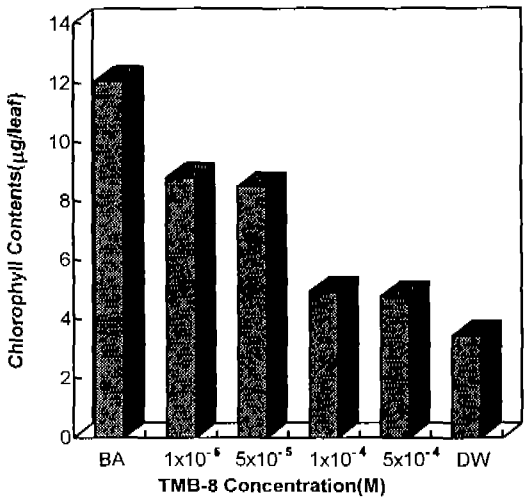

Fig. 8. Effect of TMB-8 concentrations on chlorophyll content in senescing wheat leaves after 4 days. Each treatment contained 0.05% DMSO. TMB-8 treatment was performed with 1×10^{-6} M BA.

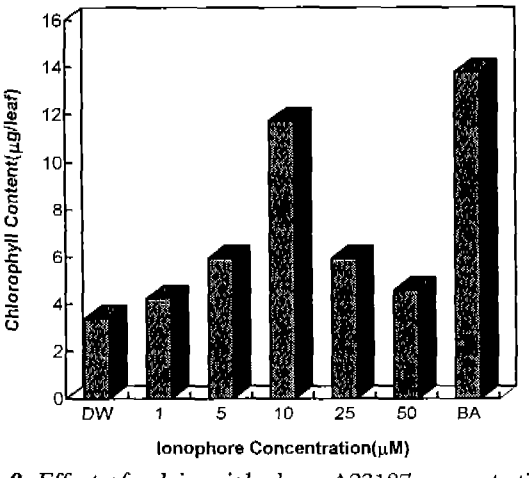

Fig. 9. Effect of calcium iolophore A23187 concentrations on chlorophyll content in senescing wheat leaves after 4 days. Each treatment contained 0.05% DMSO.

At the concentrations above 10^{-4} M, TMB-8 caused a strong inhibition on BA actions in retarding chlorophyll degradation. Indeed this result indicated that the calcium supply from the intracellular calcium sources could be involved in BA actions, like calcium input from the outside of cells. In order to confirm the presence of calcium mediation in the BA effects on senescence retardation, we investigated chlorophyll contents in senescing leaves in the presence of the ionophore A23187 without BA supplement. As shown in Figure 9, the chlorophyll content at 10^{-5} M A23187 treatment was similar to that of the BA only treated leaves. This result in-

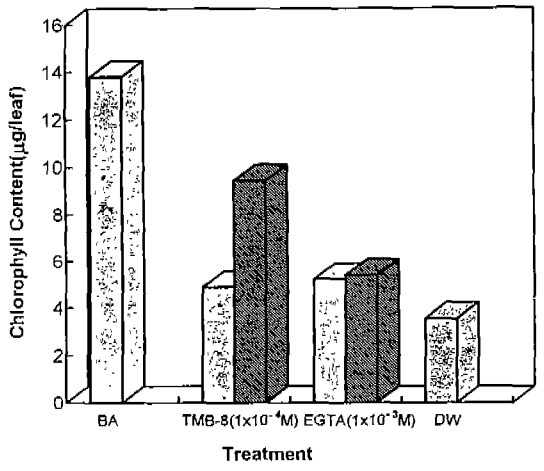

Fig. 10. Response of total chlorophyll content in senescing wheat leaves on day 4 after each treatments. Shaded bars represent transferring of the TMB-8 and the EGTA-pretreated (1 day) leaves to the calcium inophore A23187. Each treatment contained 0.05% DMSO.

dicates that the ionophore A23187 mimicked the action of BA and also strongly supports the proposition that the BA-induced retardation of senescence is mediated by calcium.

It has been reported that the range of cytosolic calcium concentration routinely lies between 10^{-7} M and 10^{-6} M and increases from 10 to 100 fold by hormonal stimulus (Moor *et al.*, 1986; Roux *et al.*, 1986; Allan and Trewavas, 1987; Marme, 1986, 1989). In addition, Allan and Trewavas (1987) observed that concentrations of calcium greater than 10^{-4} M causes a toxic effect on cells.

In senescing leaves treated with EGTA and TMB-8, changes of chlorophyll content after transfer to ionophore A23187 were measured in order to find the correlation between the intracellular calcium source and the extracellular calcium source. After transferring the EGTA-treated leaves to the ionophore, we observed no changes in chlorophyll content, however, the transfer of TMB-8 treated leaves to the same ionophore caused some recovery of BA effects reduced by TMB-8 (Fig. 10). Such a result implies that the concentration of cytosolic free calcium is controlled by the calcium supply from intra and extracellular calcium sources and that the reduced calcium supply due to the blocking the cell organelles could also be compensated for by calcium input from outside the cells. This seems likely because the extracellular space is suggested to have a 1000 times higher concentration of calcium ions than the intracellular space and because the con-

centration of cytosolic free calcium ions can be increased more or less only by calcium input from the extracellular space (Roux, 1986; Allan and Trewavas, 1987). In conclusion, our present data suggests that the BA actions in retarding senescence could be mediated by a change of the cytosolic calcium concentrations supplied from the intra and extracellular calcium sources and that the calcium inflow from intercellular spaces and cell walls may participate in the regulation of cellular calcium levels.

ACKNOWLEDGEMENTS

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