Hydrogen Peroxide, Calcium, and Leaf Senescence in Rice

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ABSTRACT

The possible involvement of calcium in the regulation of H_2O_2-, abscisic acid (ABA)- or methyl jasmonate (MJ)-induced H_2O_2 generation, protein loss, and lipid peroxidation in detached rice (*Oryza sativa* L.) leaves was investigated. Calcium chloride and calcium ionophore A23187 effectively reduced H_2O_2-promoted ABA- or MJ-induced H_2O_2 generation, protein loss, and lipid peroxidation in detached rice leaves. It appears that ABA- or MJ-induced H_2O_2 generation, protein loss, and lipid peroxidation in rice leaves is mediated through blocking the entrance of calcium ions into the cytosol.

Key words: Abscisic acid, Ca^+, H_2O_2, Leaf senescence, *Oryza sativa*.

過氧化氫、鈣與水稻葉片老化

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摘要

本研究探討鈣參與調控過氧化氫、脫落酸與甲基茉莉酸鹽所控制之過氧化氫產生、蛋白質喪失與脂質過氧化作用。氯化鈣與鈣離子載體 (calcium ionophore) A23187 均可有效的降低過氧化氫產生、蛋白質喪失與脂質過氧化作用。本研究結果顯示，脫落酸與甲基茉莉酸言之作用是由於其阻止鈣離子進入細胞質。

關鍵詞： 脫落酸、鈣離子、過氧化氫、葉片老化、甲基茉莉酸鹽、水稻。

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投稿日期：2007 年 3 月 21 日
接受日期：2007 年 4 月 23 日
作物、環境與生物資訊 4:145-150 (2007)
Crop, Environment & Bioinformatics 4:145-150 (2007)
189 Chung-Cheng Rd., Wufeng, Taichung Hsien 41301, Taiwan ROC

INTRODUCTION

Several lines of evidence indicate that Ca^{2+} plays an important role in regulating leaf senescence. The senescence, measured as chlorophyll and protein degradation, of corn and *Rumex* leaf discs was found to be retarded by the addition of 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 a consequence of the maintenance of cellular membranes by Ca^{2+} (Poovaiah and Leopold 1973, Ferguson *et al*. 1983). It appears that senescence effect of calcium is an effect of external calcium. Using pea leaf system, Leshem *et al.* (1984) and Leshem (1987) were able to show that senescence was promoted by increasing $[Ca^{2+}]_{cyt}$. They concluded that elevated intracellular calcium promoted senescence through a calmodulin-mediated effect. Further evidence for the involvement of $[Ca^{2+}]_{cyt}$ in the process of 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 (Huang *et al.* 1997).

H_2O_2 is a constituent of oxidative metabolism and itself a reactive oxygen species (ROS). It has been shown that H_2O_2 promotes leaf senescence (Parida *et al.* 1978, Mondal and Chondhuri 1981, Lin and Kao 1998, Hung and Kao 2005). Recently we reported that H_2O_2 is involved in

abscisic acid (ABA)- and methyl jasmonate (MJ)-induced senescence of rice leaves (Hung and Kao 2004, Hung *et al.* 2006). In rice leaves, it has been demonstrated that calcium ionophore A23187, believed to raise $[Ca^{2+}]_{cyt}$ (Pressman 1976), is quite effective in reducing the promotive effect of ABA and MJ on senescence of detached rice leaves (Huang *et al.* 1990, Chou and Kao 1992). It seems that calcium may also interact with H_2O_2 in the regulation of senescence of detached rice leaves. In this study the possible involvement of calcium in the regulation of H_2O_2-promoted senescence and ABA- or MJ-induced H_2O_2 accumulation in detached rice leaves was investigated.

MATERIALS AND METHODS

Rice (*Oryza sativa* L. cv. Taichung Native 1) was sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with filter paper at 37℃ under dark conditions. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 500 ml beaker containing half-strength Kimura B solution as described previously (Hsu and Kao 2005). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron with natural sunlight at 30℃ (day)/25℃ (night) and 90% relative humidity. The apical 3 cm of the third leaf was used in all experiments. A group of ten segments was floated in a Petri dish containing 10 ml of test solution. Incubation was carried out at 27℃ in the dark.

The senescence of detached rice leaves was followed by measuring the decrease in protein content. For protein extraction, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 x *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976). Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). The H_2O_2 content was measured colormetrically as described by Jana and Chundhuri (1982). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6,000 x *g* for 25 min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium sulphate in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6,000 x *g* for 25 min. The absorbance was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient 0.28 $\mu mol^{-1}\ cm^{-1}$.

The results presented were the mean of four replicates. Means were compared by either Student's *t*-test or Duncan's multiple range test at 5% level of significance.

RESULTS AND DISCUSSION

The most obvious character of leaf senescence is yellowing. Chlorophyll lost has long been considered the principal criterion of senescence. The protein breakdown that occurs during leaf senescence has been recognized since the earliest studies performed. We have shown that protein breakdown precedes chlorophyll loss during rice senescence (Kao 1980). Leaf senescence is also known to be mediated through lipid peroxidation (Thompson *et al.* 1987). Thus, senescence of rice leaves in the present study was followed by measuring the decrease in protein and MDA, an indicator of lipid peroxidation.

The effectiveness of calcium in reducing H_2O_2-promoted protein loss and lipid peroxidation of rice leaves was tested as shown in Table 1. $CaCl_2$ was effective in reducing H_2O_2-promoted senescence of rice leaves (Table 1). The endogenous H_2O_2 content in H_2O_2-treated rice leaves was also observed to be reduced by $CaCl_2$ (Table 1). The optimal concentration of $CaCl_2$ in retarding protein loss, reducing lipid peroxidation, and inhibiting the increase in H_2O_2 content caused by H_2O_2 seems to be 0.1 mM (Table 1).

To characterize further the role of calcium in H_2O_2-promoted senescence and H_2O_2-increased H_2O_2 content of rice leaves, the calcium ionophore A23187 was applied to detached rice leaves. The results shown in Table 1 indicate that the effect of H_2O_2 on senescence was significantly reduced by A23718 or A23187 + $CaCl_2$. These results provide evidence to support our earlier conclusion that the cytosolic calcium ions are important in the regulation of senescence of detached rice leaves (Huang *et al.* 1990, Chou and Kao 1992). It

Table 1 Effect of $CaCl_2$ and A23187 on the contents of protein, MDA, and H_2O_2 in rice leaves treated with H_2O_2. All measurements were made after 2 days in the dark. Values are means ± SE (n = 4). Values with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

Treatment	Protein (mg g^{-1} FW)	MDA (nmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)
H_2O	43.8 ± 0.89^{a}	25.2 ± 0.22^{d}	33.1 ± 0.72^{d}
H_2O_2 (10 mmol l^{-1})	30.0 ± 0.67^{d}	36.9 ± 0.62^{a}	43.9 ± 0.59^{a}
H_2O_2 + $CaCl_2$ (0.1 mmol l^{-1})	38.3 ± 0.59^{b}	27.1 ± 0.23^{c}	35.4 ± 0.54^{c}
H_2O_2 + $CaCl_2$ (1 mmol l^{-1})	36.9 ± 0.16^{c}	29.5 ± 0.73^{b}	37.3 ± 0.57^{b}
H_2O_2 + $CaCl_2$ (10 mmol l^{-1})	36.7 ± 0.42^{c}	29.6 ± 0.63^{b}	36.7 ± 0.60^{bc}
H_2O_2 + A23187 (10 μmol l^{-1})	38.6 ± 1.0^{b}	28.8 ± 0.89^{b}	36.6 ± 0.96^{bc}
H_2O_2 + A23187 (10 μmol l^{-1}) + $CaCl_2$ (10 mmol l^{-1})	39.8 ± 0.72^{b}	28.8 ± 0.65^{b}	36.1 ± 0.36^{bc}

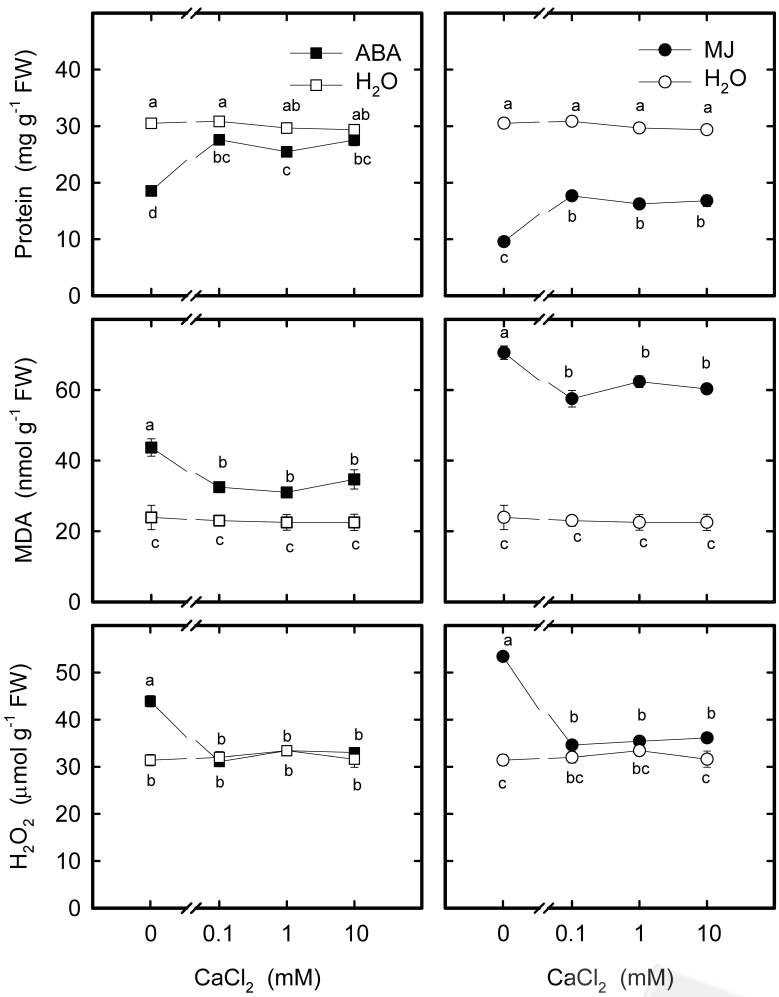

Fig. 1. Effect of $CaCl_2$ on the contents of protein, MDA, and H_2O_2 in rice leaves treated with ABA or MJ. The concentrations of ABA and MJ were 45 μM, respectively. All measurements were made after 3 days in the dark. Values are means ± SE (n = 4). Values with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

appears that H_2O_2 can be scavenged under the condition of elevated $[Ca^{2+}]_{cyt}$.

H_2O_2 treatment resulted in a marked increase in MDA content, indicating that H_2O_2 treatment caused an oxidative stress. Recently, we demonstrated that H_2O_2 is involved in ABA- and MJ-induced senescence of rice leaves (Hung and Kao 2005, Hung *et al.* 2006). Thus, it is of great interest to know whether $CaCl_2$ and A23187 also affect ABA- and MJ-induced H_2O_2 production and senescence. As indicated in Figs. 1 and 2, both $CaCl_2$ and A23187 indeed reduced ABA- and MJ-induced H_2O_2 production and senescence in detached rice leaves. Our experiments suggest that the effect caused by ABA or MJ in rice leaves leaves may be mediated through blocking the entrance of calcium ions into cytosol.

It has been shown that ABA brings about stomatal closure by an elevation of $[Ca^{2+}]_{cyt}$ (MacRobbie 1998, Assmann and Shimazaki 1999). There are also reports indicating that H_2O_2 is involved in ABA-induced stomatal closure (McAinsh *et al.* 1996, Pei *et al.* 2000, Zhang *et al.* 2001). The work by Pei *et al.* (2000) has led the discovery of H_2O_2-activated Ca^{2+} channels as an important part of the mechanism for ABA-induced stomatal closure. Leaf senescence is characterized by the orderly, progressive disassembly of mesophyll cells. It seems that the effect of ABA on guard cells is quite different from that of mesophyll cells. In mesophyll cells of rice leaves, ABA blocks the entrance of calcium ions into the cytosol, which in turn results in an accumulation of H_2O_2.

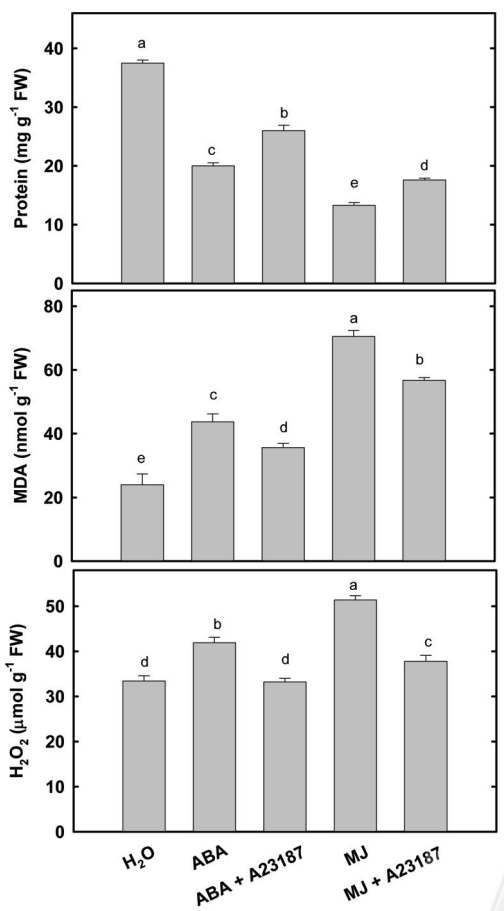

Fig. 2. Effect of A23187 on the contents of protein, MDA, and H_2O_2 in rice leaves treated with ABA and MJ. The concentrations of ABA, MJ, and A23187 were 45 μM, 45 μM, and 10 μM, respectively. H_2O_2 content was measured after 3 days in the dark. Values are means ± SE (n = 4). Values with the same letter are not significantly different at P < 0.05 level, according to Duncan's multiple range test.

ACKNOWLEDGMENTS

This work was supported by grant NSC 95-2313-13-02-007 from the National Science Council of Republic of China on Taiwan.

REFERENCES

Assmann SM, KI Shimazaki (1999) The multisensory guard cell, stomatal responses to blue light and abscisic acid. **Plant Physiol.** 119:809-815.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram-quantities of protein utilizing the principle of protein-dye binding. **Anal. Biochem.** 72:248-254.

Chou CM, CH Kao (1992) Methyl jasmonate, calcium, and leaf senescence in rice. **Plant Physiol.** 99:1693-1694.

Dreier W (1990) Senescence inhibition and promotion in isolated primary leaves from oat by the calcium-calmodulin-complex. **Biochem. Physiol. Pflanz.** 186:31-36.

Ferguson IB, CB Watkins, JE Harman (1983) Inhibition by calcium of senescence detached cucumber cotyledons: effect on ethylene and hydroxide production. **Plant Physiol.** 71:182-186.

Foyer CH, H Lopez-Delgado, JF Dat, IM Scott (1997) Hydrogen peroxide and glutathione-associated mechanism of acclimatory stress tolerance and signaling. **Physiol. Plant.** 100:241-254.

Heath RL, L Packer (1968) Phytoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxiadtion. **Arch. Biochem. Biophys.** 125:189-198.

Hsu YT, CH Kao (2005) Abscisic acid accumulation and cadmium tolerance in rice seedlings. **Physiol. Plant.** 124:71-80.

Huang F-Y, S Philosoph-Hadas, S Meir, DA Callaham, R Sebato, A Zelcer, PK Hepler (1997) Increase in cytosolic Ca^{2+} in parsley mesophyll cells correlate with leaf senescence. **Plant Physiol.** 115:51-60.

Huang Y, CH Kao (1992a) The importance of transmembrance flux of Ca^{2+} in regulating derk-induced senescence of detached corn leaves. **Bot. Bull. Acad. Sin.** 33:17-21.

Huang Y, CH Kao (1992b) Cadmium in the regulation of corn leaf senescence by light. **Bot. Bull. Acad. Sin.** 33:161-165.

Huang Y, CT Chen, CH Kao (1990) Senescence of rice leaves. XXIV. Involvement of calcium and calmodulin in the regulation of senescence. **Plant Cell Physiol.** 31:1015-1020.

Hung KT, CH Kao (2004) Hydrogen peroxide is necessary for abscisic acid-induced senescence of rice leaves. **J. Plant Physio**l. 161:3347-3357

Hung KT, CH Kao (2005) Nitric oxide counteracts the senescence of rice leaves induced by hydrogen peroxide.. **Bot. Bull. Acad. Sin.** 46:21-28.

Hung KT, YT Hsu, CH Kao (2006) Hydrogen peroxide is involved in methyl jasmonate-induced senescence of rice leaves. **Physiol. Plant.** 127:293-303.

Jana S, MA Chundhuri (1982) Glycolate metabolism of three submerged aquatic angiosperms during aging. **Aquat. Bot.** 12:345-354.

Kao CH (1980) Senescence of rice leaves IV. Influence of benzyladmine on chlorophyll degradation. **Plant Cell Physiol.** 21:255-262.

Leshem YY (1987) Membrane phospholipid catabolism and Ca^{2+} activity in control of senescence. **Physiol. Plant.** 69:551-559.

Leshem YY, S Sridhara, JE Thompson (1984) Involvement of calcium and calmodulin in membrane deterioation during senescence of pea foliage. **Plant Physiol.** 75:329-335.

Lin JN, CH Kao (1998) Effect of oxidative stress by hydrogen peroxide on senescence of rice leaves. **Bot. Bull. Acad. Sin.** 39:161-165.

MacRobbie EAC (1998) Signal transduction and ion channels in guard cells. **Phil. Trans. R. Soc. Lond. B** 353:1475-1488.

McAinsh MR, H Clayton, TA Mansfield, AM Hetherington (1996) Changes in stomatal behavior and cytosolic free calcium in response

to oxidative stress. **Plant Physiol.** 111:1031-1042.

Mondal R, MA Choudhuri (1981) Role of hydrogen peroxide in senescence of excised leaves of rice and maize. **Biochem. Physiol. Pflanz.** 176:700-709.

Parida RK, M Kat, D Mishra (1978) Enhancement of senescence in excised rice leaves by hydrogen peroxide. **Can. J. Bot.** 56: 2937-2941.

Pei ZM, Y Murata, G Benning, S Thomine, B Klusener, GJ Allen, E Grill, JL Schroeder (2000) Calcium channel activated by hydrogen peroxide mediated abscisic acid signaling in guard cells. **Nature** 406:731-734.

Poovaiah BW, AC Leopold (1973) Deferral of leaf senescence with calcium. **Plant Physiol.** 52:230-239.

Pressman BC (1976) Biological application of ionophore. **Annu. Rev. Biochem.** 45:501-530.

Savithramma N, PM Swamy (1989) Influence of $Ca(NO_3)_2$ on the changes in the lipid composition, peroxidation and superoxide dismutase activity in intact primary leaves of cowpea during senescence. **Proc. Indian Acad. Sci. (Plant Sci.)** 99:139-145.

Thompson JE, RL Legge, RF Barber (1987) The role of free radical in senescence and wounding. **New Phytol.** 105:317-344.

Zhang X, L Zhuang, F Dong, J Gao, DW Galbraith, C-P Song (2001) Hydrogen-peroxide is involved in abscisic acid induced stomatal closure in *Vicia faba*. **Plant Physiol.** 126:1438-1448.

－編輯：謝兆樞