Calcium is involved in the abscisic acid-induced ascorbate peroxidase, superoxide dismutase and chilling resistance in *Stylosanthes guianensis*

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Abstract

The objective of this work was to test whether Ca^{2+}, a second messenger in stress response, is involved in ABA-induced antioxidant enzyme activities in *Stylosanthes guianensis*. Plants were sprayed with abscisic acid (ABA), calcium channel blocker, $LaCl_3$, calcium chelator, ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetid acid (EGTA), and ABA in combination with $LaCl_3$ or EGTA. Their effects on superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities and chilling resistance were compared. The results showed that ABA decreased electrolyte leakage and lipid peroxidation but increased maximum photochemical efficiency measured as variable to maximum fluorescence ratio (F_v/F_m) under chilling stress. Treatment with $LaCl_3$ or EGTA alone and in combination with ABA increased electrolyte leakage and lipid peroxidation, decreased F_v/F_m, suggesting that the block in Ca^{2+} signalling decreased chilling resistance of *S. guianensis* and the ABA-enhanced chilling resistance. ABA-induced SOD and APX activities were suppressed by $LaCl_3$ or EGTA. The results suggested that Ca^{2+} is involved in the ABA-enhanced chilling resistance and the ABA-induced SOD and APX activities in *S. guianensis*.

Additional key words: chlorophyll fluorescence, electrolyte leakage, EGTA, $LaCl_3$, lipid peroxidation.

Introduction

Abiotic stresses are major limiting factors in crops production. Much of the injury to plants caused by abiotic stresses is associated with oxidative damage at cellular level (Bowler *et al*. 1992). Oxidative stress may be a significant factor in relation to chilling induced injury (Fadzillah *et al*. 1996, Prasad *et al*. 1994). Higher plants have active oxygen-scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT), and some low molecules of non-enzyme antioxidants, such as ascorbic acid (AsA) and reduced glutathione (GSH) (Bowler *et al*. 1992). These systems protect membranes from the deleterious effects of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide (H_2O_2), hydroxyl radicals and singlet oxygen, which are produced at elevated rates when plants are exposed to abiotic stress conditions, including chilling (Guo *et al*. 2006). Chilling leads to unfavourable changes in cell membranes resulting in cell damage which can be measured as a level of electrolyte leakage (Huang and Guo 2005). One of the most susceptible cellular organelles is the chloroplasts and chilling leads to the decrease in photosystem 2 maximum photochemical efficiency, which is generally expressed as variable to maximum fluorescence ratio (F_v/F_m), in *Oryza sativa* and *Vitis vinifera* (Guo *et al*. 2007, Bertamini *et al*. 2007). The level of lipid peroxidation can be monitored using the peroxidation product, malondialdehyde (MDA), as a marker (Huang and Guo 2005).

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; EDTA - ethylene diaminetetraacetic acid; EGTA - ethylene glycol-bis (β-amino ethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetid acid; F_m - maximal fluorescence; F_0 - initial fluorescence; F_v - variable fluorescence; F_v/F_m - maximum photochemical efficiency; GR - glutathione reductase; GSH - reduced glutathione; MDA - malondiadehyde; POD - peroxidase; PPFD - photosynthetic photon flux density; PS 2 - photosystem 2; PVP - polyvinylpolypyrrolidone; SOD - superoxide dismutase; TBA - 2-thiobarbituric acid.
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Stylosanthes guianensis, originated in the tropics, is an important pasture legume with high yield and quality, acidity-tolerance, and adaptation to low fertility soil in tropical and subtropical countries (Meijer *et al*. 1981, Miles and Grof 1997, Miles and Lascano 1997). Chilling injury of *S. guianensis* is a serious problem in subtropical cultivated areas. Abscisic acid (ABA) treatment increased the chilling resistance of *S. guianensis* (Zhou *et al*. 2005a). The ABA biosynthesis inhibitor sodium tungstate decreases the content of ABA, inhibits ABA-induced antioxidant enzymes activity, and results in serious chilling injury (Zhou and Guo 2005). Exogenous ABA treatment also enhances the chilling resistance of other crops, such as *Zea mays*, *Litchi chinensis*, and *Eremochloa ophiruoides* (Anderson *et al*. 1994, Zhou *et al*. 2002, Lu *et al*. 2005).

ABA plays important roles in plant tolerance to stresses (Bravo *et al*. 1998). ABA treatments enhance activities of antioxidant enzymes in various species such as *Zea mays* (Jiang and Zhang 2001) and *Oryza sativa* (Lin *et al*. 2001). Our previous studies show that ABA increases antioxidant enzyme activity in *S. guianensis* and turfgrasses (Zhou *et al*. 2005a, Lu *et al*. 2003). Ca^{2+}, H_2O_2 and NO are signal molecules involved in the responses of plant to abiotic stress. H_2O_2 and NO have been well demonstrated to be signal molecules involved in the ABA-induced activities of antioxidant enzymes (Jiang and Zhang 2002a,b, Hu *et al*. 2005, Zhou *et al*. 2005b). Ca^{2+} concentration in cytosol is increased under environmental stress (Krol *et al*. 2006), which regulates gene expression and the relative physiological and biochemical reactions (Bush 1995, Monroy and Dhindsa 1995, Gong *et al*. 1998a). $CaCl_2$ treatment increases antioxidant enzyme activities and heat resistance in *Festuca arundinacea* and *Poa pratensis* under heat stress (Jiang and Huang 2001, Larkindale and Huang 2004, Agarwal *et al*. 2005). ABA-enhanced heat resistance in maize is demonstrated to be associated with the ABA-induced antioxidant enzyme activities, in which calcium is involved (Gong *et al*. 1998b). However, the studies on involvement of Ca^{2+} in ABA-induced antioxidant enzyme activity and chilling resistance are still limited. In the present study, *S. guianensis* seedlings were sprayed with calcium channel blocker $LaCl_3$, or calcium chelator EGTA to investigate the effects of ABA on chilling resistance and antioxidant enzymes under the block of calcium signal, and to find out whether Ca^{2+} is involved in ABA-enhanced chilling resistance and ABA-induced antioxidant enzymes in *S. guianensis*.

Materials and methods

Plants and treatments: *Stylosanthes guianensis* Schumach. & Thonn. seedlings were grown in a green-house as described before (Zhou *et al*. 2005a). The germinated seeds were sown in 15-cm diameter plastic pots containing mixture of peat and perlite (3:1, v/v). Plants were grown under natural light in a greenhouse with temperature from 25 to 30 °C, with irrigating daily and fertilizing once a week by 0.3 % of N-P-K fertilizer (15-15-15). Eight-week-old plants in similar size were divided into six groups. Each group contained six to twelve pots of plants as replicates. The group plants were then uniformly sprayed respectively with the following solutions: *1*) water (control); *2*) 37.9 μM ABA (*Lomon BioTechnology Co*, Chengdu, China); *3*) 37.9 μM ABA + 5 mM $LaCl_3$; *4*) 5 mM $LaCl_3$; *5*) 37.9 μM ABA + 5 mM ethyleneglycol-bis(β-amino ethyl ether)-*N,N,N',N'*-tetraacetid acid (EGTA); and *6*) 5 mM EGTA. All the potted plants were placed in a growth chamber at air humidity of 80 %, photosynthetic photon flux density (PPFD) of 160 μmol m^{-2} s^{-1} and 28 °C for 24 h. Half of the plants in each group were still kept in the growth chamber at 28 °C as control, the other half of plants were moved to another growth chamber with temperature at 8 °C for chilling treatment. The second and third fully expanded leaflets were selected for measurements. All data were subjected to analysis of variances according to the model for completely randomized design using a *SPSS* program. Differences among treatment means were evaluated by Duncan's multiply range test at 0.05 probability level.

Electrolyte leakage was determined according to the method used before (Zhou *et al*. 2005a). Samples of six leaflets were rinsed with distilled water and immersed in 6 cm^3 of distilled water for 12 h. The conductivity of the solution (R1) was measured using a conductivity meter (*Model DDS-11A*, *Shanghai Leici Instrument*, Shanghai, China). Samples were then heated in a boiling water bath for 20 min, and then cooled to room temperature. The conductivity of killed tissues (R2) was again measured. Electrolyte leakage was calculated as the ratio of R1 to R2.

Maximal photochemical efficiency of PS 2: A pulse-modulated fluorometer (*Model FMS-1*, *Hansatech Instruments*, Norfolk, UK) was used to measure F_v/F_m. For dark-adaptation, leaves were covered for 20 min. Initial fluorescence (F_0) was measured at PPFD < 0.05 μmol m^{-2} s^{-1}, following by a saturating pulse (3000 μmol m^{-2} s^{-1}) to determine maximum fluorescence (F_m). Maximum photochemical efficiency of PS 2 was estimated by the equation: $F_v/F_m = (F_m - F_0)/ F_m$.

MDA and superoxide dismutase activity assay: Fresh leaves (0.5 g) were ground in a mortar with a pestle in 5 cm^3 of 50 mM phosphate buffer (pH 7.8), containing 2 % (m/v) PVP. The homogenates were centrifuged at 13 000 *g* for 20 min at 4 °C. The supernatants were used for assays of MDA concentration and SOD activity (Huang and Guo 2005, Zhou *et al*. 2005b). The mixture of 1.5 cm^3 of the supernatants and 2.5 cm^3 of 0.5 % (m/v) 2-thiobarbituric acid (TBA) dissolved in 20 % (v/v)

trichloroacetic acid solution was incubated in a boiling water bath for 30 min and then was cooled to room temperature. Absorbance at 600 nm and 532 nm was determined. MDA concentration was calculated as $(A_{532}-A_{600})/1.55 \times 10^5$.

The reaction solution for determination of SOD activity contained 13 μM methionine, 63 μM ρ-nitroblue tetrazolium chloride (NBT), 1.3 μM riboflavin, 50 mM phosphate buffer (pH 7.8), and enzyme extract. The reaction solution was incubated for 10 min under fluorescent light of 80 μmol m^{-2} s^{-1}. Absorbance was determined at 560 nm with a spectrophotometer (*Model UV-2010*, *Hitachi*, Tokyo, Japan). One unit of SOD activity was defined as the amount of enzyme required for inhibition of photochemical reduction of NBT by 50 %.

Ascorbate peroxidase activity assay: APX activity was determined spectrophotometerically according to the method of Nakano and Asada (1981). Leaves (0.3 g) were ground in a mortar and pestle in 3 cm^3 of 50 mM cool phosphate buffer (pH 7.0, containing 1 mM ascorbic acid (AsA), 1 mM EDTA). The homogenates were centrifuged at 13 000 *g* for 20 min at 4 °C. The supernatants were used for assay of enzyme activity. The 3 cm^3 reaction solution contained 50 mM phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 and 0.1 cm^3 enzyme extract. APX activity was calculated by following the decrease in absorbance of AsA (coefficient of absorbance 2.8 mM^{-1} cm^{-1}) within 1 min at 290 nm.

Protein content: Protein content in enzyme extracts was determined according to the method of Bradford (1976). 0.025 cm^3 of enzyme extract was added to 3 cm^3 of 0.01 % (m/v) Coomassie Brilliant Blue G-250 solution (4.7 % (m/v) ethanol, 8.5 % (m/v) phosphoric acid). The absorbance at 595 nm was spectrophotometerically recorded after protein-dye binding for 5 min. Protein content was calculated with comparison to a curve made by bovine serum albumin as a standard.

Results and discussion

Effects of ABA, calcium channel blocker, and calcium chelator on electrolyte leakage, F_v/F_m and MDA: Electrolyte leakage in *S. guianensis* increased with the chilling days (Fig. 1), which has been observed before (Zhou *et al*. 2005a). ABA-treated plants had lower electrolyte leakage. At 4 d and 7 d post chilling treatment, electrolyte leakage in ABA-treated plants was 35.7 and 22.7 % lower than that in water-treated plants, respectively. $LaCl_3$ is a Ca^{2+} channel blocker, and EGTA is a Ca^{2+} specific chelator (Tester 1990, Monroy and Dhindsa 1995). The plants treated with ABA in combination with either $LaCl_3$ or EGTA had higher electrolyte leakage compared to those treated with ABA alone. $LaCl_3$ or EGTA treatment alone significantly increased electrolyte leakage under chilling condition, suggesting that Ca^{2+} is necessary for plant chilling resistance. All treatments had low electrolyte leakage under control condition, indicating that these chemicals had no injury effect on plants (Fig. 1).

Maximum photochemical efficiency (F_v/F_m) is a measure of the intrinsic efficiency of PS 2 photochemistry and appears to be of primary importance in studies of stress effects on plants (Bertin *et al*. 1997). F_v/F_m in *S. guianensis* decreased under chilling (Fig. 2), which was consistent with previous observation (Zhou *et al*. 2006). ABA-treated plants had the highest level of F_v/F_m under chilling conditions. ABA in combination with either $LaCl_3$ or EGTA treated plants had lower F_v/F_m than ABA alone treated plants. Treatment with $LaCl_3$ or EGTA alone decreased F_v/F_m under chilling condition to values even lower than that in water treatment. The plants showed no significant difference in F_v/F_m among all the treatments under control condition (Fig. 2).

MDA is a product of lipid peroxidation, which presents the oxidative damage in membranes. MDA content increased in the leaves under chilling, this increase was reduced by ABA treatment (Fig. 3). MDA content was lower by 31.1% in ABA-treated plants than water-treated plants. The other treatments including $LaCl_3$, EGTA and ABA in combination with $LaCl_3$ or EGTA increased MDA content (Fig. 3).

The data indicated that ABA reduced the chilling

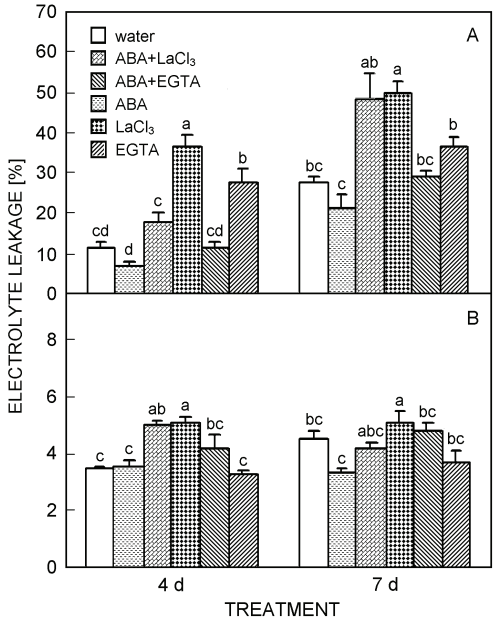

Fig. 1 Effects of ABA, $LaCl_3$ and EGTA on electrolyte leakage in leaves of *S. guianensis* at 4 d and 7 d following chilling stress (*A*) or under normal temperature (*B*). *Vertical bars* represent SE (n = 4). Different letters indicate significant difference at $P \leq 0.05$ level among a given treatment time according to Duncan's multiply range test.

injury of *S. guianensis*, which is consistent with our previous studies (Zhou *et al*. 2005a,b, Zhou *et al*. 2006). Treatments with either Ca^{2+} channel blocker or Ca^{2+} chelator increased the injury of *S. guianensis* under chilling conditions (Figs. 1-3), suggesting Ca^{2+} is involved in the chilling resistance of *S. guianensis*. The two chemicals were also shown to decrease chilling tolerance in *Oryza sativa* (Zong *et al*. 2003). Ca^{2+} was demonstrated to be involved in the thermotolerance of tobacco (Gong *et al*. 1998a) and ABA-induced thermotolerance in maize (Gong *et al*. 1998b). Our results showed that ABA-induced chilling resistance was reversed by $LaCl_3$ or EGTA. Therefore, it is suggested that Ca^{2+} is also involved in ABA-induced chilling resistance.

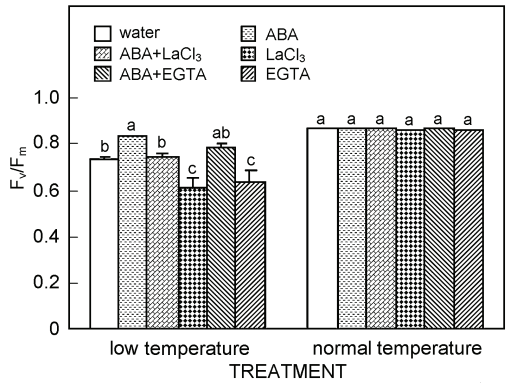

Fig. 2 Effects of ABA, $LaCl_3$ and EGTA on F_v/F_m in leaves of *Stylosanthes guianensis* at 4 d after chilling stress or under normal temperature. *Vertical bars* represent SE (n = 6). Different letters indicate significant difference at $P \leq 0.05$ level among a given treatments.

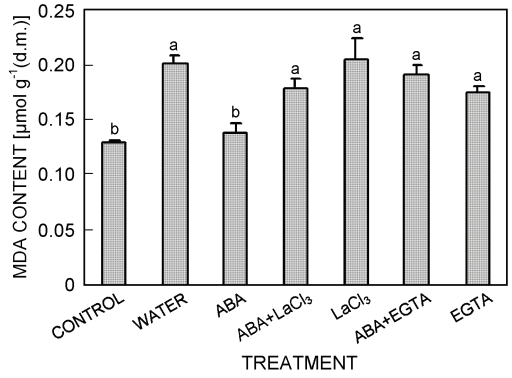

Fig. 3 Effects of ABA, $LaCl_3$ and EGTA on MDA content in leaves of *S. guianensis* at 4 d following chilling stress. Control plants were sprayed by water and grown at normal temperature. Other plants were sprayed by water, ABA, ABA + $LaCl_3$, $LaCl_3$, ABA + EGTA and EGTA, respectively, and grown at low temperature for 4 d. *Vertical bars* represent SE (n = 3). Different letters indicate significant difference at $P \leq 0.05$.

Effects of ABA, calcium channel blocker, and calcium chelator on SOD and APX: Superoxide dismutase activity was not influenced by 5-d chilling treatment. It was increased by ABA treatment under control and chilling conditions (Fig. 4), which is in accordance to previous observations (Zhou *et al*. 2005a,b). The plant treated with $LaCl_3$, EGTA, and ABA in combination with $LaCl_3$ or EGTA had the similar SOD activity to the water-treated plants under control condition, indicating Ca^{2+} is involved in ABA-induced SOD activity. SOD activity is even lower in the plants treated with $LaCl_3$, EGTA, and ABA in combination with $LaCl_3$ or EGTA than water-treated plants under chilling (Fig. 4). It is possible that these treatments caused more significant injury to plants (Fig. 1) and lead to a lower SOD activity.

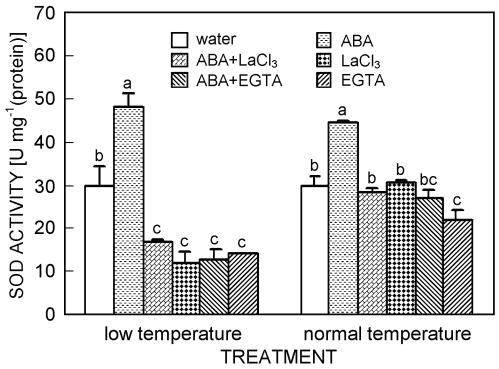

Fig. 4. Effects of ABA, $LaCl_3$ and EGTA on SOD activity in leaves of *S. guianensis* at 4 d following chilling stress or under normal temperature. *Vertical bars* represent SE (n = 3). Different letters indicate significant difference at $P \leq 0.05$ level among a given treatments.

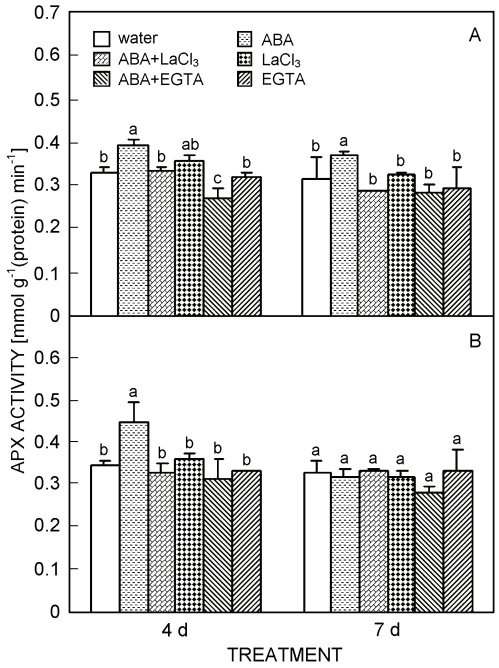

Fig. 5. Effects of ABA, $LaCl_3$ and EGTA on APX activity in leaves of *S. guianensis* at 1 d and 4 d following chilling stress (*A*) or under normal temperature (*B*). *Vertical bars* represent SE (n = 3). Different letters indicate significant difference at $P \leq 0.05$ level among a given treatment time.

ABA had no effect on APX activity at day 4 under control condition, thus we determined its effect at day 1. APX activity was induced by ABA at day 1 under control and chilling conditions, and the ABA-induced APX activity was inhibited by $LaCl_3$ and EGTA (Fig. 5). The data indicated that Ca^{2+} is involved in the ABA-induced SOD and APX activities.

It has been observed that ABA induces the expression of SOD and APX genes in maize and *Chlamydomonas reinhardtii* (Zhu and Scandalios 1994, Guan and Scandalios 2000, Yoshida *et al*. 2004). Ca^{2+} is involved in the ABA-induced closure of stomata (Cousson 2007). ABA increases the cytosolic Ca^{2+} concentration by inducing both Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular stores (Pei *et al*. 2000, Murata *et al*. 2001). ABA-induced H_2O_2 production and the H_2O_2-activated Ca^{2+} channels are important mechanisms for ABA signalling in guard cell (Pei *et al*. 2000). Ca^{2+} functions as a signal downstream of H_2O_2 in the ABA-induced antioxidant enzymes activity (Jiang and Zhang, 2003).

In summary, we have found that the application of either $LaCl_3$ or EGTA reverses the effect of ABA on chilling-treated plants, *i.e*., it leads to the decrease in plant chilling resistance (determined as the rate of electrolyte leakage, maximum photochemical efficiency of PS 2 and the level of lipid peroxidation) and to the decrease in SOD and APX activities. Thus, we can conclude that calcium is involved in transduction of ABA signal which leads to the increase in chilling resistance in *S. guianensis* and to the activation of SOD and APX during chilling.

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