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Effects of Calcium and Calmodulin Antagonists on Chilling Stress-Induced Proline Accumulation in *Jatropha curcas* L.

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Abstract Regulation of proline accumulation in plants under chilling stress remains unclear. In this paper, we treated *Jatropha curcas* seedlings under chilling stress with exogenous calcium chloride ($CaCl_2$), the plasma membrane Ca^{2+}-channel blocker lanthanum chloride ($LaCl_3$), calmodulin antagonists, chlorpromazine (CPZ), and trifluoperazine (TFP) and investigated the effects of calcium and calmodulin (CaM) on proline accumulation and chilling tolerance. The results showed that $CaCl_2$ treatment significantly enhanced chilling stress-induced proline accumulation. $CaCl_2$ also induced an almost immediate and rapid increase of Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and glutamate dehydrogenase activities, the key enzymes in the glutamate pathway of proline biosynthesis, and up-regulated *P5CS* expression, but it decreased the activity of proline dehydrogenase (ProDH), a key enzyme of proline degradation, and inhibited *ProDH* expression. Treatment with $LaCl_3$, CPZ, and TFP exhibited the opposite effects to those by $CaCl_2$ treatment. Moreover, $CaCl_2$, $LaCl_3$, CPZ, and TFP had little effect on the activities of ornithine aminotransferase and arginase, the key enzymes in the ornithine pathway of proline biosynthesis. These results indicated that Ca^{2+}-CaM might be involved in signal transduction events, leading to proline accumulation in *J. curcas* seedlings under chilling stress, and that Ca^{2+}-induced proline accumulation is a combined result of the activation of the glutamate pathways of proline biosynthesis and the simultaneous inhibition of the proline degradation pathway. In addition, $CaCl_2$ treatment increased tissue vitality, decreased the content of the lipid peroxidation product malondialdehyde (MDA), and alleviated electrolyte leakage in *J. curcas* seedlings under chilling stress, indicating that exogenous Ca^{2+} can enhance chilling tolerance, and proline might be a key factor in this increased chilling tolerance.

Keywords Calcium · Calmodulin antagonists · Proline biosynthesis and degradation · Chilling stress · Chilling tolerance · *Jatropha curcas*

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Introduction

Chilling stress (0–15 °C) is a major environmental factor that affects metabolism, growth, development, distribution, and cultivation of chilling-sensitive plants from tropical and subtropical regions (Ruelland and others 2009; Jeon and Kim 2013; Kalisz and others 2015). Chilling-sensitive plants, such as maize, tobacco, tomato, and *Jatropha curcas* L., can be irreparably damaged when the temperature drops below 10 °C, mainly due to chilling stress-induced dysfunctions at the cellular level that include damage to membranes, osmotic stress, generation of reactive oxygen species (ROS), protein denaturation, and accumulation of the lipid peroxidation product malondialdehyde (MDA); osmotic stress is considered as one of the major injuries of chilling stress (Jan and others 2009; Heidarvand and Amiri

2010; Janska and others 2010). To cope with osmotic stress caused by chilling stress, higher plants have developed mechanisms of osmotic adjustment through the accumulation of compatible solutes such as proline, betaine, and soluble sugars (Trovato and others 2008; Szabados and Savoure 2010). Among these plant-compatible osmolytes (or plant protectants), proline is considered of major importance, as it has been reported to accumulate in a large number of species in response to stresses such as excess salinity, drought, cold, nutrient deficiency, heavy metals, pathogen infection, and high acidity (Delauney and Verma 1993; Ashraf and Foolad 2007; Trovato and others 2008).

Proline fulfills diverse functions in plants. As an amino acid, it is a structural component of proteins, but it also functions as an osmoticum, a sink of energy and reducing power, a nitrogen-storage compound, a hydroxy-radical scavenger, and a compatible solute that protects enzymes (Kishor and others 2005; Lehmann and others 2010; Szabados and Savoure 2010). In higher plants, proline can be synthesized from either glutamate or ornithine. The key enzymes involved in proline biosynthesis are Δ^1-pyrroline-5-carboxylate synthetase (P5CS) and glutamate dehydrogenase (GDH) in the glutamate pathway, and arginase and ornithine aminotransferase (OAT) in the ornithine pathway (Zhao and others 2001; Zhang and others 2008; Yang and others 2009). The onset of stress-induced proline accumulation is correlated with transcriptional activation of the *P5CS* gene (Yang and others 2009; Szabados and Savoure 2010). Metabolism and accumulation of proline also depend on its degradation, which are catalyzed by the mitochondrial enzyme proline dehydrogenase (ProDH), and the *ProDH* gene plays a key role in controlling proline levels in plants (Lehmann and others 2010; Szabados and Savoure 2010; Chen and others 2014).

Various studies have indicated proline accumulates in response to cold shock in several plants, and proline contributes to the maintenance of enzymes from denaturation, interacts with membrane systems, regulates cytosolic pH, balances the ratio of NADH/NAD^+ functions as a source of energy, and helps plants to detoxify ROS (Konstantinova and others 2002; Ruiz and others 2002; Javadian and others 2010; Ao and others 2013). Although much is known about proline metabolism, information on signaling mechanisms that regulate proline synthesis and degradation in plants under chilling stress is still unclear. Several signaling molecules and ions including abscisic acid (ABA), H_2O_2, Ca^{2+}, and phospholipase D have been suggested (Knight and others 1997; Trovato and others 2008; Yang and others 2009; Szabados and Savoure 2010).

Ca^{2+} is the most versatile intracellular messenger discovered so far, because it is involved in the regulation of almost all known cellular functions and reactions (Petersen and others 2005). In plant cells, the calcium ion is a ubiquitous intracellular second messenger involved in numerous signaling pathways. Variations in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]$cyt) combine a large array of signals and responses (Lecourieux and others 2006; Kim 2013). Calmodulin (CaM), the ubiquitous calcium sensor protein, is involved in almost all intracellular events (Yang and Poovaiah 2003; Arshi and others 2010). It has been shown that CaM binds and regulates more than 300 target proteins and that its structural plasticity is crucial for enabling its interaction with diverse partners (Yang and Poovaiah 2003).

In plants, the Ca^{2+}-CaM-mediated signal network affects many aspects of plant growth, development, and responses to environmental changes (Yang and Poovaiah 2003; Kim 2013). Exogenous application of calcium conferred enhanced tolerance to salt (Guimarães and others 2011; Upadhyaya and others 2011), drought (Issam and others 2012; Xu and others 2013), and cold (Wang and others 2009; Zhou and Guo 2009; Liu and others 2015) stresses. However, the studies on calcium-induced cold resistance in plants are still limited.

In recent years, a few studies suggested that the calcium messenger system was involved in proline accumulation of plants. Exogenous $CaCl_2$ treatment led to a significant accumulation of proline in *Cichorium intybus* L. (Arshi and others 2010). Proline accumulation in the leaves of *Cassia angustifolia* Vahl. was 1.6 times higher after treatment with $CaCl_2$ and NaCl than in the controls (treatment with NaCl alone) (Arshi and others 2005). A further increase in proline concentration was observed with the addition of calcium chloride, and calcium appears to confer greater osmoprotection to plants under water deficit (Jaleel and others 2007).

Jatropha curcas is a tropical plant (a chilling-sensitive plant) that grows well on marginal land. It is considered an important drought-tolerant plant that belongs to the tribe *Jatropheae* in the *Euphorbiaceae* family (Carels 2009; Mukherjee and others 2011). In preliminary experiments, we found that exogenous $CaCl_2$ treatment promoted chilling stress-induced proline accumulation in *J. curcas* seedlings. It also increased chilling tolerance of *J. curcas* seedlings. Treatment with the calcium-channel blocker $LaCl_3$ and CaM antagonists, chlorpromazine (CPZ), and trifluoperazine (TFP), exhibited contrary results to those of the $CaCl_2$ treatment (data not shown). We hypothesize that the Ca^{2+}-CaM messenger system plays an important role in the rapid accumulation of cellular proline induced by chilling stress. However, there is little information available on the relationship among Ca^{2+}-CaM, chilling stress, and proline metabolism in plants. In the present study, the objective was to investigate the effects of calcium and CaM

on proline accumulation and chilling tolerance. In addition, the possible metabolic pathways of Ca^{2+}-CaM that promote chilling stress-induced proline accumulation were investigated.

Materials and Methods

Plant Material and Treatments

Seeds of *J. curcas*, a mix of cultivars, were collected from Yunnan Province, China. Seeds were surface sterilized in 1 % $CuSO_4$ for 30 min, and then pre-soaked for imbibition in distilled water for 24 h. The pre-soaked seeds were sowed on six layers of wetted filter papers (a 0.2–0.3-cm layer of distilled water was present above the surface of filter paper) in covered trays (200 seeds per tray) and germinated in the dark at 25 °C for 7 days. The surface water layer was maintained by adding 50 mL of water per day. Seedlings of uniform size were then selected and transferred into pots (20 × 18 × 15 cm^3, 20 seedlings per pot) containing silica sand wetted with 1/2 Hoagland's solution. The pots were placed in a climate chamber (day/night temperature: 25/20 °C, relative humidity: 75 %, photoperiod: 16 h, photon flux density: 300 μmol m^{-2} s^{-1}) and sequentially grown for 14 days. The seedlings were cultured on 1/2 Hoagland's solution (200 mL per pot), and the surface water layer was maintained by adding 100 mL of 1/2 Hoagland's solution per day.

In preliminary experiments, *J. curcas* seedlings under chilling stress were treated with 0–30 mM $CaCl_2$, 0–1500 μM $LaCl_3$, 0–1000 μM CPZ, and 0–1000 μM TFP for 96 h, and the proline content was determined. For the following experiments, 10 mM $CaCl_2$, 500 μM $LaCl_3$, 200 μM CPZ, and 200 μM TFP were chosen because these concentrations significantly induced or inhibited proline accumulation compared to the seedlings subjected to chilling treatment alone (data not presented).

Seedlings of uniform size (21 days after sowing, approximately 10.0 cm in height, and 60 seedlings per tray) were gently transferred to 1/2 Hoagland's solution and subjected to five treatments for 96 h. These included (1) 2 °C, (2) 10 mM $CaCl_2$ + 2 °C, (3) 500 μM $LaCl_3$ + 2 °C, (4) 200 μM CPZ + 2 °C, and (5) 200 μM TFP + 2 °C. The control group (60 seedlings per tray) continued to be cultured in 1/2 Hoagland's solution at 25 °C for 96 h.

Measurement of Tissue Vitality, Electrolyte Leakage, and Malondialdehyde Content

The vitality of *J. curcas* seedlings leaves was measured using triphenyl tetrazolium chloride (TTC): 0.2 g of leaf samples were cultured in 0.6 % TTC solution at 27 °C for 15 h, and then the TTC solution was drained off and leaf samples were homogenized in 95 % (v/v) ethanol. The crude homogenate was heated in a 80 °C water bath for 10 min to extract formazan. The homogenate was then diluted with 95 % ethanol to a final volume of 25 mL, and the mixture was centrifuged at 10,000×*g* for 10 min. The absorbance of the supernatant at 485 nm was determined spectrophotometrically. Electrolyte leakage was measured according to the method described by Gong and others (2001). The content of MDA was determined by the thiobarbituric acid reaction as described by Bailly and others (1996).

Measurement of Proline Content

Proline in leaves of *J. curcas* seedlings was extracted and measured according to the method described by Bates and others (1973).

Enzyme Assays

GDH, P5CS, OAT, arginase, and ProDH in leaves of *J. curcas* seedlings were extracted and assayed according to our previously described method (Yang and others 2009), and the activities of GDH, P5CS, OAT, arginase, and ProDH were expressed as nmol NADH oxidized mg^{-1} protein min^{-1}, nmol NADPH oxidized mg^{-1} protein min^{-1}, μmol NADH oxidized mg^{-1} protein min^{-1}, nmol ornithine mg^{-1} protein min^{-1}, and nmol $NADP^+$ mg^{-1} protein min^{-1}, respectively.

Assay of CaM Activity

CaM in leaves of *J. curcas* seedlings was assayed according to our previously described method (Gong and others 1997). One unit was defined as the amount of CaM that stimulates 0.01 units of 3′,5′-cyclic nucleotide phosphodiesterase to 50 % of the maximum activity of the enzyme when saturated with CaM in the presence of 100 μM Ca^{2+} in a 3-mL reaction volume at 30 °C, pH 7.5.

RNA Isolation and Quantitative Real-Time RT-PCR (RT-qPCR)

Seedlings of *J. curcas* were treated with five treatments as described above. Total RNA was isolated from leaves of *J. curcas* seedlings after 24, 48, 72, and 96 h of treatments with an RNAiso for Polysaccharide-rich Plant Tissue kit (TaKaRa Biotechnology, Dalian, China) according to manufacturer's recommendation and used to generate a cDNA pool. RT-qPCR was performed using an ABI 7500 Fast Real-Time PCR instrument (Applied Biosystems,

Foster City, CA, USA). The following gene-specific primers for RT-qPCR were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA): for *P5CS* (GenBank accession No. GU358610) (5′-GGCAGATGGACTCCTGTTAGA-3′ and 5′-TTTCATTTGACCGCTTGGC-3′, amplicon size: 164 bp), for *ProDH* (GenBank accession No. KF879446) (5′-CGAGGCTGTAAAGTGTGTAAGG-3′ and 5′-CAAGTAGGTTCAGAGGGCAAAT-3′, amplicon size: 209 bp), and for actin gene (GenBank accession No. HM044307), which was used as internal control (5′-GTGTTATGGTTGGGATGGGT-3′ and 5′-AAGCACTGGGTGTTCCTCTG-3′, amplicon size: 188 bp).

RT-qPCR was performed using One Step SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa Biotechnology, Dalian, China) according to manufacturer's recommendations. Relative expression levels were analyzed by the comparative CT method using Microsoft Excel 2010 as described by Livak and Schmittgen (2001).

Statistical Analysis

All experiments were repeated at least three times with two independent biological replicates. The results were processed statistically with SPSS 15.0 software (IBM Corp., Armonk, NY, USA). The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range test was employed to determine significance differences between treatments at $P < 0.05$ level. Figures were drawn by SigmaPlot 10.0 (Systat Software, San Jose, CA, USA), error bars represent standard error, and each point in figure represents the mean ± SE of at least three experiments.

Results

Effect of Ca^{2+} on Proline Accumulation and Metabolic Pathways in Leaves of *J. curcas* Seedlings Under Chilling Stress Conditions

During the treatment with 2 °C for 96 h, the proline content in leaves of *J. curcas* seedlings was about 2.83 times higher than that in plants not subjected to chilling stress ($P < 0.01$, Fig. 1). Interestingly, treatments with 10 mM $CaCl_2$ for 96 h remarkably enhanced the accumulation of proline in leaves of *J. curcas* seedlings under chilling stress ($P < 0.01$, Fig. 1), whereas treatment with the plasma membrane Ca^{2+}-channel blocker $LaCl_3$ exhibited the opposite effect to that observed in $CaCl_2$ treatment ($P < 0.05$, Fig. 1).

During chilling stress at 2 °C for 96 h, the activities of P5CS and GDH in leaves of *J. curcas* seedlings increased rapidly in the first 72 h and 48 h of stress conditions, respectively, and then this increase became relatively

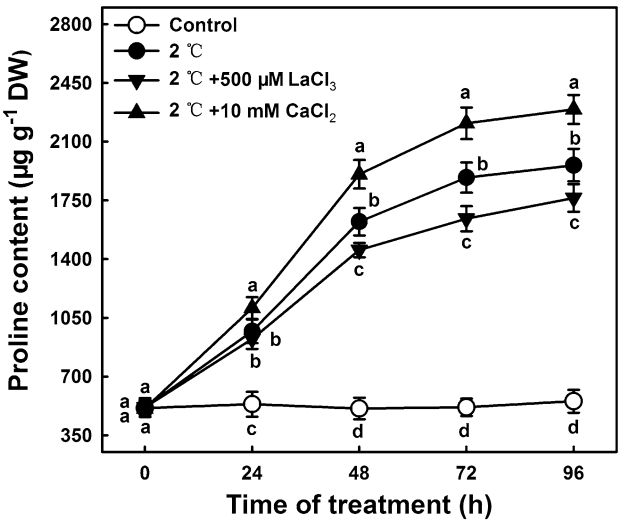

Fig. 1 Effects of $CaCl_2$ and $LaCl_3$ on proline content in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 10 mM $CaCl_2$ + 2 °C, or 500 μM $LaCl_3$ + 2 °C for 96 h and proline content was measured. The control seedlings continued to be cultured in 1/2 Hoagland's solution at 25 °C for 96 h. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

gentle in the subsequent 24 and 48 h of stress conditions, respectively (Fig. 2a, b). Treatment with 10 mM $CaCl_2$ significantly increased P5CS ($P < 0.01$) and GDH ($P < 0.01$) activities in *J. curcas* seedlings under chilling stress conditions compared to the seedlings subjected to chilling treatment alone (Fig. 2a, b). However, treatment with 500 μM $LaCl_3$ for 96 h significantly decreased the activities of P5CS and GDH ($P < 0.05$, Fig. 2a, b).

During the treatment with chilling stress at 2 °C for 96 h, arginase and OAT activities in leaves of *J. curcas* seedlings showed similar changes. In the early phase of chilling stress (0–24 h), there was a small change in the level of activities of arginase and OAT compared to the control. With prolonged chilling treatment (24–96 h), the activities of arginase and OAT increased dramatically (Fig. 2c, d), reaching levels that were 2.32 and 2.06 times higher, respectively, by the end of the treatment than those in plants not exposed to chilling stress ($P < 0.01$, Fig. 2c, d). In contrast, both $CaCl_2$ and $LaCl_3$ treatments had no significant effect on the activities of arginase and OAT ($P > 0.05$, Fig. 2c, d).

Chilling stress led to a gradual decline of ProDH activity in leaves of *J. curcas* seedlings (Fig. 3). Interestingly, compared to the seedlings exposed to chilling treatment alone, $CaCl_2$ treatment significantly decreased the activity of ProDH under chilling stress conditions ($P < 0.01$), but treatment with 500 μM $LaCl_3$ increased the ProDH activity ($P < 0.01$, Fig. 3).

To further determine the effect of Ca^{2+} on biosynthesis and degradation of proline under chilling stress, the mRNA

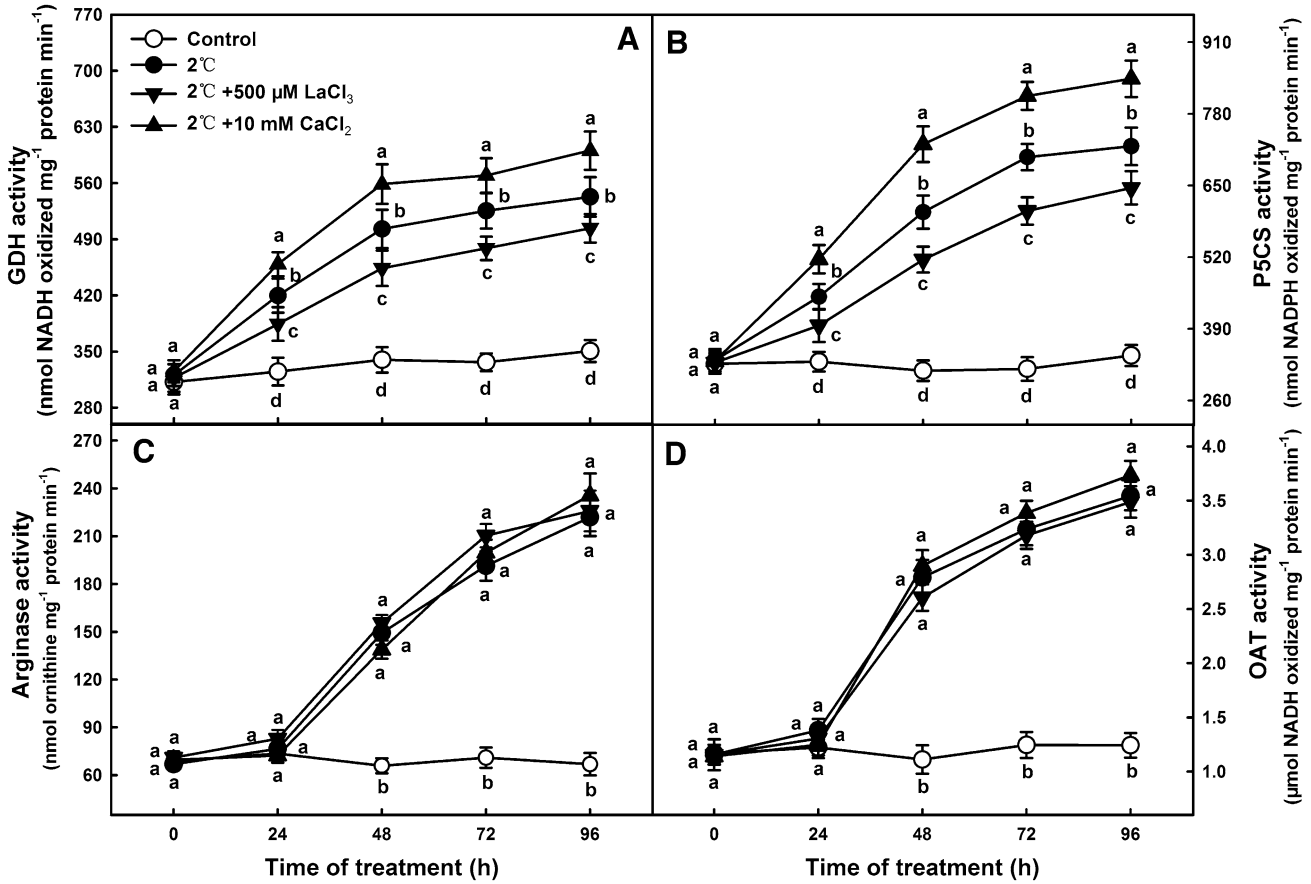

Fig. 2 Effects of $CaCl_2$ and $LaCl_3$ on activities of the key enzymes of proline biosynthesis in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 10 mM $CaCl_2$ + 2 °C, or 500 μM $LaCl_3$ + 2 °C for 96 h and the activities of four key enzymes of proline metabolism were assayed: **a** GDH, **b** P5CS, **c** arginase, **d** OAT. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

levels of *P5CS* and *ProDH* in leaves of *J. curcas* seedlings were detected. As shown in Fig. 4, during the chilling stress treatment at 2 °C for 96 h, chilling stress significantly up-regulated *P5CS* expression. Compared to the seedlings exposed to chilling treatment alone, treatment with 10 mM $CaCl_2$ remarkably up-regulated *P5CS* expression, but $LaCl_3$ treatment down-regulated *P5CS* expression under chilling stress ($P < 0.01$, Fig. 4a). In contrast, *ProDH* expression was inhibited under chilling stress conditions, and $CaCl_2$ treatment down-regulated *ProDH* expression, but $LaCl_3$ treatment up-regulated *ProDH* expression in seedlings compared to those that had undergone chilling treatment alone ($P < 0.01$, Fig. 4b).

Effect of CaM Antagonists on Proline Accumulation and Metabolic Pathways in Leaves of *J. curcas* Seedlings Under Chilling Stress Conditions

CPZ and TFP have been commonly used as CaM antagonists of choice in many studies (Martínez-Luis and others 2007). In the present work, we studied the effects of 200 μM CPZ or TFP on CaM activity in leaves of *J. curcas* seedlings under chilling stress conditions. Compared with the control, chilling stress significantly increased CaM activity ($P < 0.01$), whereas CPZ and TFP decreased CaM activity compared to the seedlings subjected to chilling treatment alone ($P < 0.05$, Fig. 5a).

Treatments with CPZ and TFP for 96 h significantly decreased the content of proline compared to that in the seedlings treated with chilling stress alone ($P < 0.05$) (Fig. 5b). They also induced a decrease in the activities of GDH and P5CS ($P < 0.05$, Fig. 6a, b), but both CPZ and TFP treatments had little effect on the activities of arginase and OAT ($P > 0.05$, Fig. 6c, d). In addition, CPZ and TFP treatments markedly increased ProDH activity in seedlings compared to that in seedlings under chilling treatment alone ($P < 0.01$, Fig. 7).

Treatments with CPZ and TFP also changed the expression level of *P5CS* and *ProDH* under chilling stress conditions. Thus, they down-regulated *P5CS* and up-

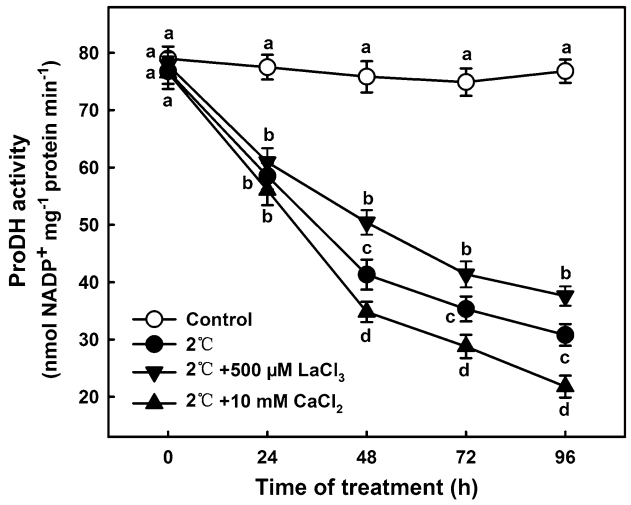

Fig. 3 Effects of $CaCl_2$ and $LaCl_3$ on ProDH activity in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 10 mM $CaCl_2$ + 2 °C, or 500 μM $LaCl_3$ + 2 °C for 96 h and the activity of ProDH was assayed. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

regulated *ProDH* expression in seedlings compared to that in seedlings subjected to chilling treatment alone ($P < 0.01$, Fig. 8).

Effect of Ca^{2+} and CaM Antagonists on Some Physiological Parameters of *J. curcas* Seedlings Under Chilling Stress Conditions

To explore the effect of Ca^{2+} and CaM on chilling tolerance of *J. curcas* seedlings, seedlings were treated with 2 °C, 10 mM $CaCl_2$ + 2 °C, 500 μM $LaCl_3$ + 2 °C, 200 μM CPZ + 2 °C, and 200 μM TFP + 2 °C for 96 h, and tissue vitality, electrolyte leakage, and MDA content in leaves of *J. curcas* seedlings were assayed daily. The results showed that chilling stress at 2 °C for 96 h decreased tissue vitality ($P < 0.01$, Fig. 9a) and increased electrolyte leakage ($P < 0.01$, Fig. 9b) and MDA content ($P < 0.01$, Fig. 9c) in seedlings. Interestingly, treatment with 10 mM $CaCl_2$ significantly increased tissue vitality (Fig. 9a) and decreased electrolyte leakage (Fig. 9b) and MDA content (Fig. 9c) compared to the seedlings subjected to chilling treatment alone. Treatments with $LaCl_3$ ($P < 0.05$, Fig. 9), CPZ, and TFP exhibited opposite effects to those with $CaCl_2$ treatment ($P < 0.05$, Fig. 10).

Discussion

Proline accumulates in many plant species in response to environmental stress. The main biological function of proline accumulation is associated with the osmotic

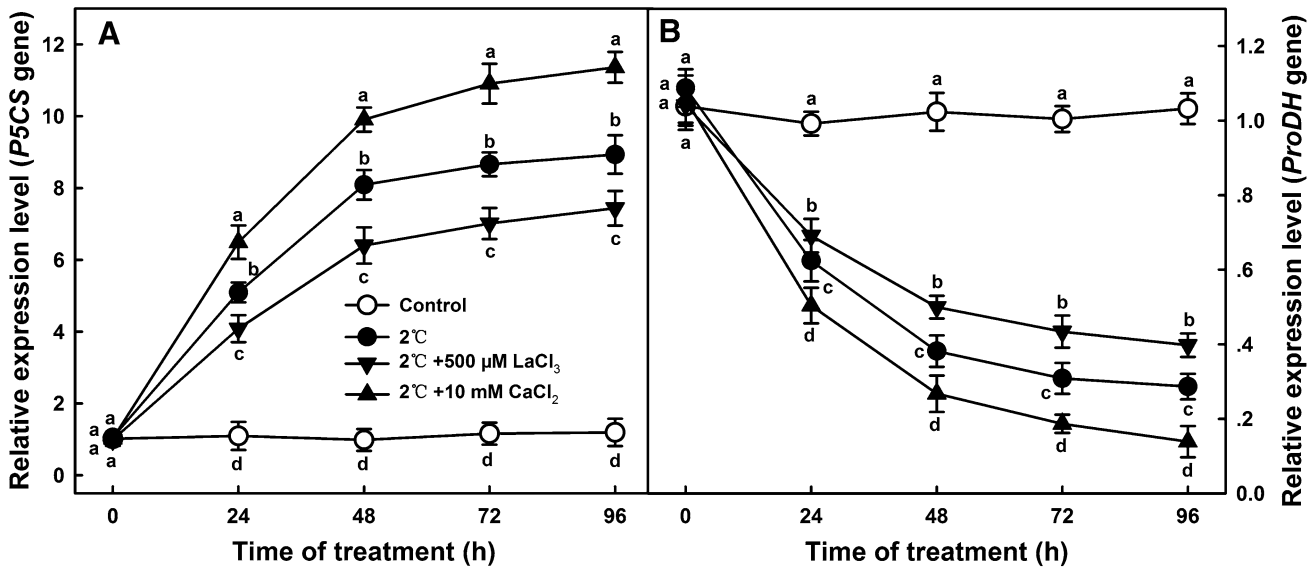

Fig. 4 Effects of $CaCl_2$ and $LaCl_3$ on the expression of *P5CS* and *ProDH* genes in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 10 mM $CaCl_2$ + 2 °C, or 500 μM $LaCl_3$ + 2 °C for 24, 48, 72 and 96 h, and *P5CS* and *ProDH* genes expression were analyzed by RT-qPCR. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

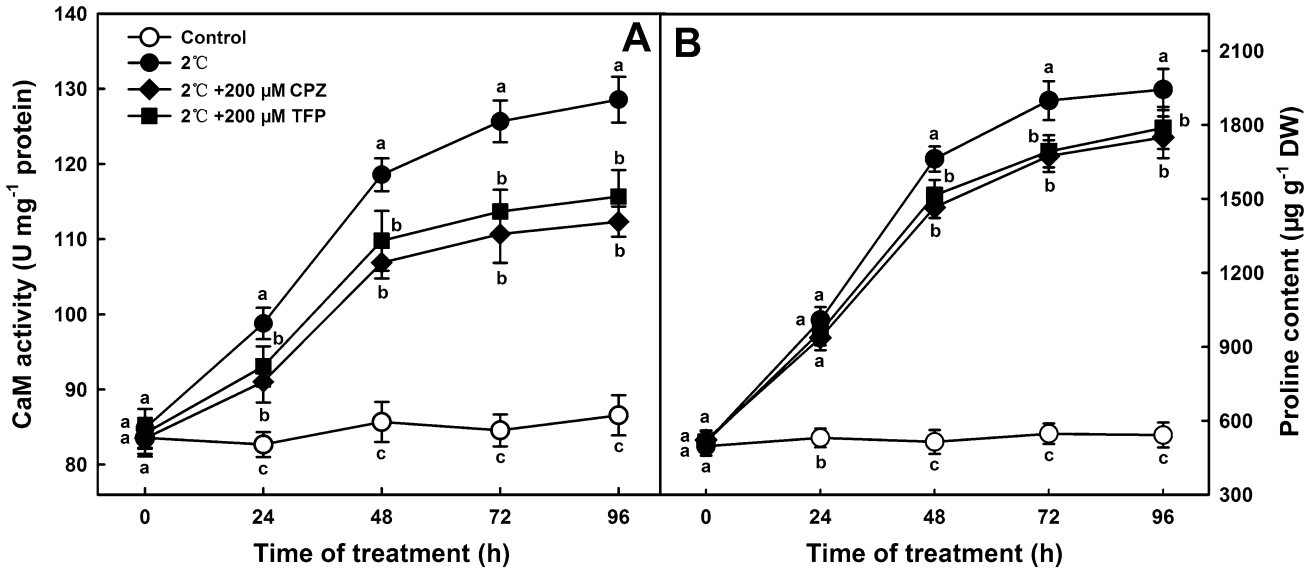

Fig. 5 Effects of CPZ and TFP on CaM activity (**a**) and proline content (**b**) in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 200 μM CPZ + 2 °C, or 200 μM TFP + 2 °C for 96 h and proline content and CaM activity were measured. The control seedlings continued to be cultured in 1/2 Hoagland's solution at 25 °C for 96 h. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

regulation of plant cells, the regulation of nitrogen and energy metabolism, and the protection of cell membrane system (Trovato and others 2008; Szabados and Savoure 2010). Studies have indicated variations in proline metabolism and accumulation in response to cold stress in several plants (Ruiz and others 2002; Javadian and others 2010; Ao and others 2013). Proline accumulation during cold acclimation showed a significant positive correlation with freezing tolerance in bermudagrass (Zhang and others 2011). Javadian and others (2010) reported that proline accumulation was greater than that of other amino acids under cold stress. They also observed higher proline accumulation in winter wheat cultivars than in spring ones, which was explained by proline's major role in coping with cold stress and maintaining favorable osmotic potential between the cell and its surrounding. Ruiz and others (2002) found that cold shock led to a significant accumulation of proline in green bean plants where OAT and ProDH appeared as determinant in this accumulation. *P5CS* was essential for cold tolerance in rice (Hur and others 2004), and overexpression of *P5CS* in *Eucalyptus saligna* Sm. accelerated proline synthesis (Dibax and others 2010). Chen and others (2014) reported that increased *P5CS* and *ProDH* expression caused proline accumulation in chrysanthemum species under cold acclimation, without the need for OAT expression changes. Our results partly agree with these authors in that the present results clearly showed that chilling stress could lead to a significant accumulation of proline (Fig. 1), an early and rapid increase of activities of P5CS and GDH, the key regulatory enzymes of the glutamate pathway (Fig. 2a, b), and an up-regulation of *P5CS* expression in leaves of *J. curcas* seedlings (Fig. 4a), indicating that the glutamate pathway of proline biosynthesis was activated in the early phase of chilling stress. Interestingly, activities of arginase and OAT, the key enzymes of the ornithine pathway, significantly increased only after 24 h of chilling stress (Fig. 2c, d), indicating that the activation of the ornithine pathway of proline biosynthesis by chilling stress was delayed compared to the activation of the glutamate pathway. In addition, chilling stress treatment decreased the activity of ProDH, the key enzyme of proline degradation, and down-regulated *ProDH* expression (Figs. 3, 4b). These results suggest that the chilling stress-induced proline accumulation is the result of the combined sequential activation of the glutamate and ornithine pathways of proline biosynthesis and simultaneous inhibition of proline degradation.

Although the importance of proline accumulation in the adaptation of plants to abiotic stress including chilling stress have been demonstrated, little is known about the regulation of proline biosynthesis and degradation. Proline accumulation is generally believed not to be a primary response to stress and was found to be dependent on de novo protein synthesis, suggesting the existence of a signaling cascade controlling proline biosynthesis (Delauney and Verma 1993; Trovato and others 2008; Szabados and Savoure 2010). Several signaling molecules and ions, including ABA, NO, Ca^{2+}, H_2O_2, phospholipase D, and

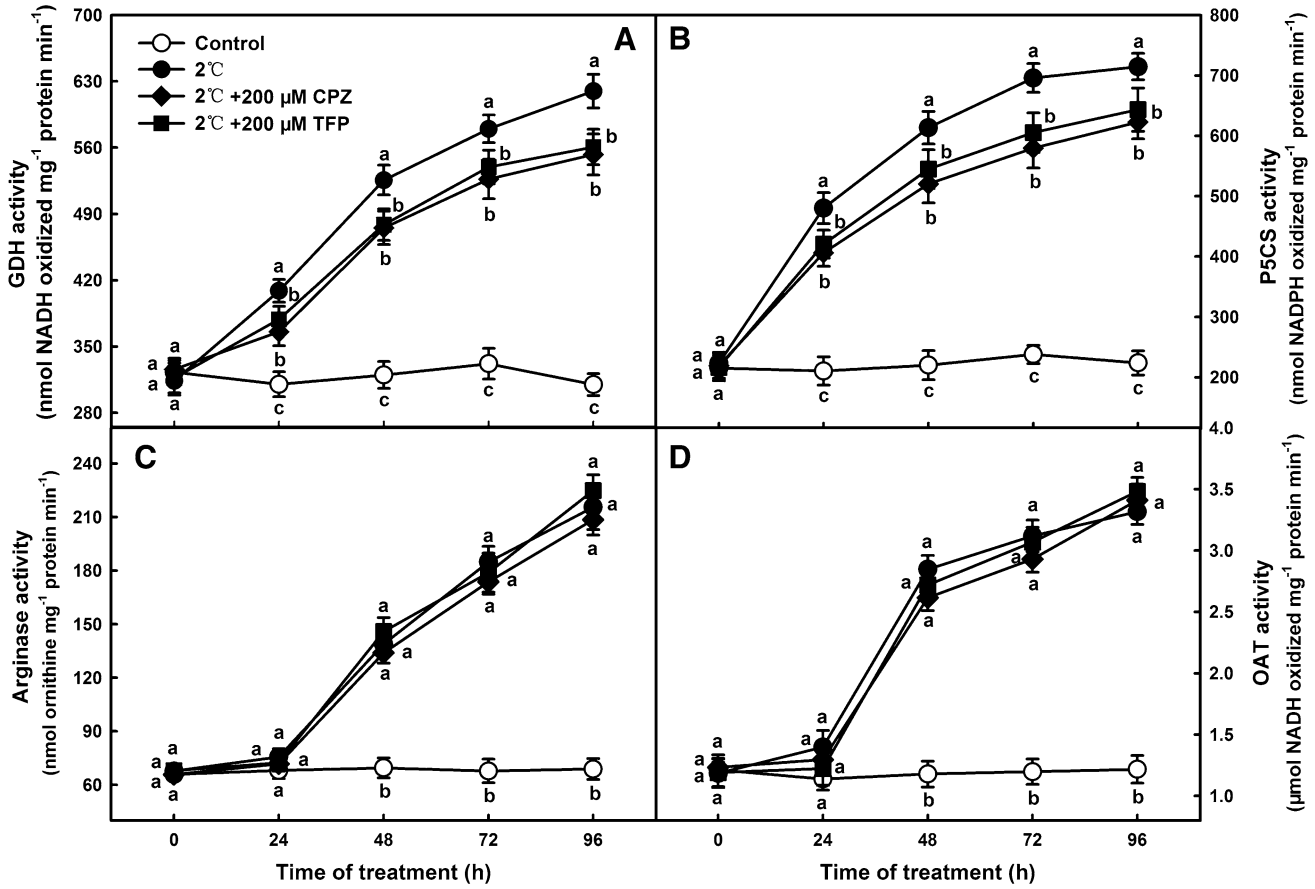

Fig. 6 Effects of CPZ and TFP on activities of the key enzymes of proline biosynthesis in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 200 μM CPZ + 2 °C, or 200 μM TFP + 2 °C for 96 h and the activities of four key enzymes of proline metabolism were assayed: **a** GDH, **b** P5CS, **c** arginase, **d** OAT. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

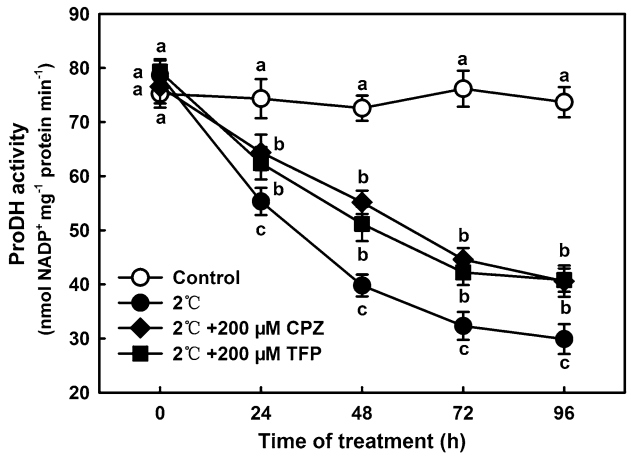

Fig. 7 Effects of CPZ and TFP on ProDH activity in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 200 μM CPZ + 2 °C, or 200 μM TFP + 2 °C for 96 h and the activity of ProDH was assayed. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

salicylic acid (SA) are involved in the regulation of proline metabolism in plants under abiotic stress (Kishor and others 2005; Trovato and others 2008; Misra and Saxena 2009; Yang and others 2009; Lehmann and others 2010). The effect of ABA on the expression of *P5CS* and *ProDH* during abiotic stress has been described and reviewed in a number of studies (Verslues and Bray 2006; Lehmann and others 2010; Sharma and Verslues 2010). In banana fruit, NO treatment significantly enhanced the accumulation of proline under chilling stress, which resulted from the increased P5CS activities and decreased ProDH activity (Wang and others 2013). Treatment with 0.5 mM SA elevated proline content in the shoots of lentil seedlings possibly by enhancing pyrroline-5-carboxylate reductase activity and decreasing the activity of ProDH (Misra and Saxena 2009). The impact of SA on proline accumulation was further increased under high salinity conditions, leading to the assumption that the stress-protective effect of SA might partially be achieved via control of proline metabolism (Misra and Saxena 2009; Lehmann and others

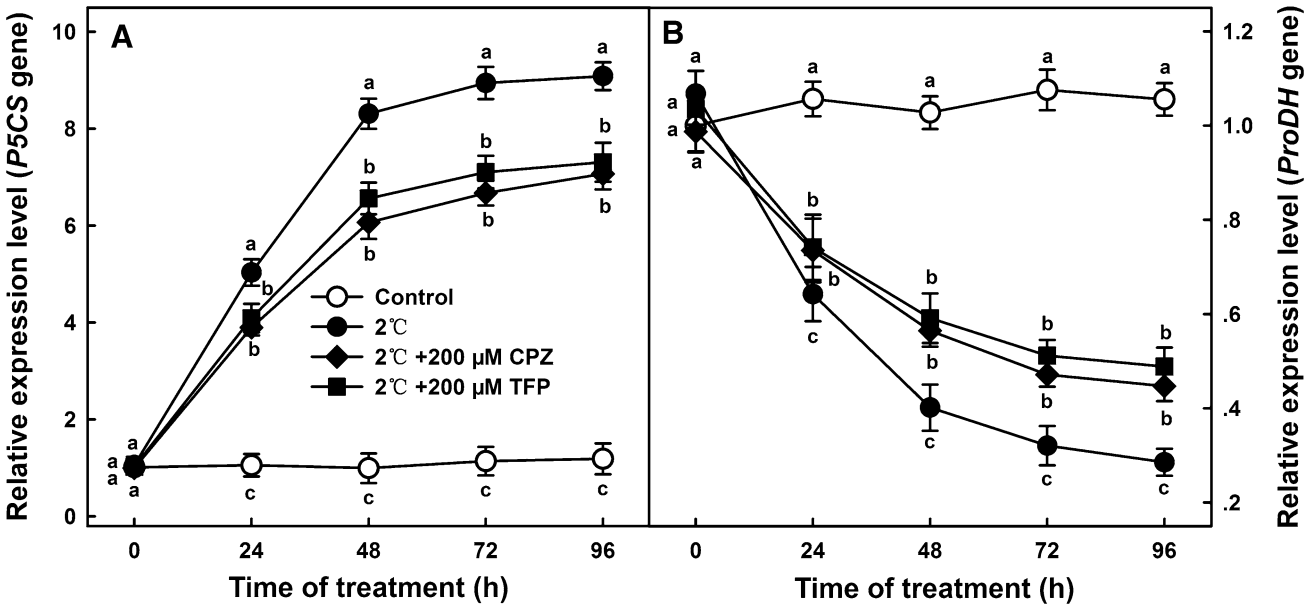

Fig. 8 Effects of CPZ and TFP on the expression of *P5CS* and *ProDH* genes in leaves of *J. curcas* seedlings under chilling stress. Seedlings were treated with 2 °C, 200 μM CPZ + 2 °C, or 200 μM TFP + 2 °C for 24, 48, 72 and 96 h, and *P5CS* and *ProDH* genes expression were analyzed by RT-qPCR. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

2010). Proline content and the activity of P5CS were enhanced with $CaCl_2$ treatment in *Centella asiatica* (L.) Urb. under salt stress (Murugan and Sathish 2005). Interestingly, the expression of *P5CS1* in *Arabidopsis* seedlings was inhibited by calcium-chelator EGTA and calcium-channel blockers lanthanum and verapamil under osmotic stress (Knight and others 1997). The addition of CaM antagonists CPZ or TFP enhanced the increase in the levels of proline, indicating that the CaM-mediated signal transduction might be negatively involved in the regulation of proline accumulation via a modification in ProDH properties (Lee and Liu 1999).

Recently, several studies confirmed a correlation between proline metabolism and Ca^{2+}-CaM messenger system under chilling stress. Nayyar and others (2005) reported that proline content in chickpea seedlings was significantly increased with $CaCl_2$ treatment under chilling stress. Ruiz and others (2002) found that Ca^{2+}-CaM-dependent NAD kinase induced the foliar accumulation of proline in response to cold shock, boosting OAT activity, and lowering ProDH activity in green bean plants. However, little is still known about the regulation of proline metabolism under chilling stress.

In the present study, $CaCl_2$ treatment increased significantly accumulation of proline in *J. curcas* seedlings under chilling stress (Fig. 1). $CaCl_2$ also induced a rapid increase of GDH and P5CS activities, the crucial enzymes of glutamate pathway (Fig. 2a, b), and up-regulated *P5CS* expression (Fig. 4a), whereas treatment with the plasma membrane Ca^{2+}-channel blocker $LaCl_3$ and CaM antagonists CPZ, and TFP exhibited the opposite effects to those of $CaCl_2$ treatment (Figs. 1, 2a,b, 4a, 5b, 6a, b, 8a). These data indicate that the glutamate pathway of proline biosynthesis is activated by Ca^{2+} treatment, and Ca^{2+}-CaM plays an important role in proline accumulation in *J. curcas* seedlings under chilling stress. However, $CaCl_2$, $LaCl_3$, CPZ, and TFP treatments had little effect on the activities of OAT and arginase, the key enzymes of the ornithine pathway (Figs. 2c, d, 6c, d), indicating that Ca^{2+}-CaM has no significant effect on the ornithine pathway of proline biosynthesis under chilling stress. In addition, $CaCl_2$ treatment decreased the activity of ProDH and inhibited the expression of *ProDH* (Figs. 3, 4b). However, $LaCl_3$, CPZ, and TFP treatments increased ProDH activity and activated the expression of *ProDH* (Figs. 3, 4b, 7, 8b), suggesting that Ca^{2+}-CaM is involved in the inhibition of proline degradation under chilling stress.

A number of studies have confirmed that Ca^{2+} plays a critical role in the regulatory response of plants to cold stress (Wang and others 2009; Zhou and Guo 2009). Schaberg and others (2011) found that Ca^{2+} modulated the stress-induced growth performance and photosynthetic efficiency of red spruce foliage during the cold season. Shi and others (2014) reported that $CaCl_2$ treatment alleviated the ROS burst and cell damage triggered by chilling stress via activating antioxidant enzymes, the non-enzymatic glutathione antioxidant pool in bermudagrass. In this study, exogenous application of $CaCl_2$ increased tissue vitality

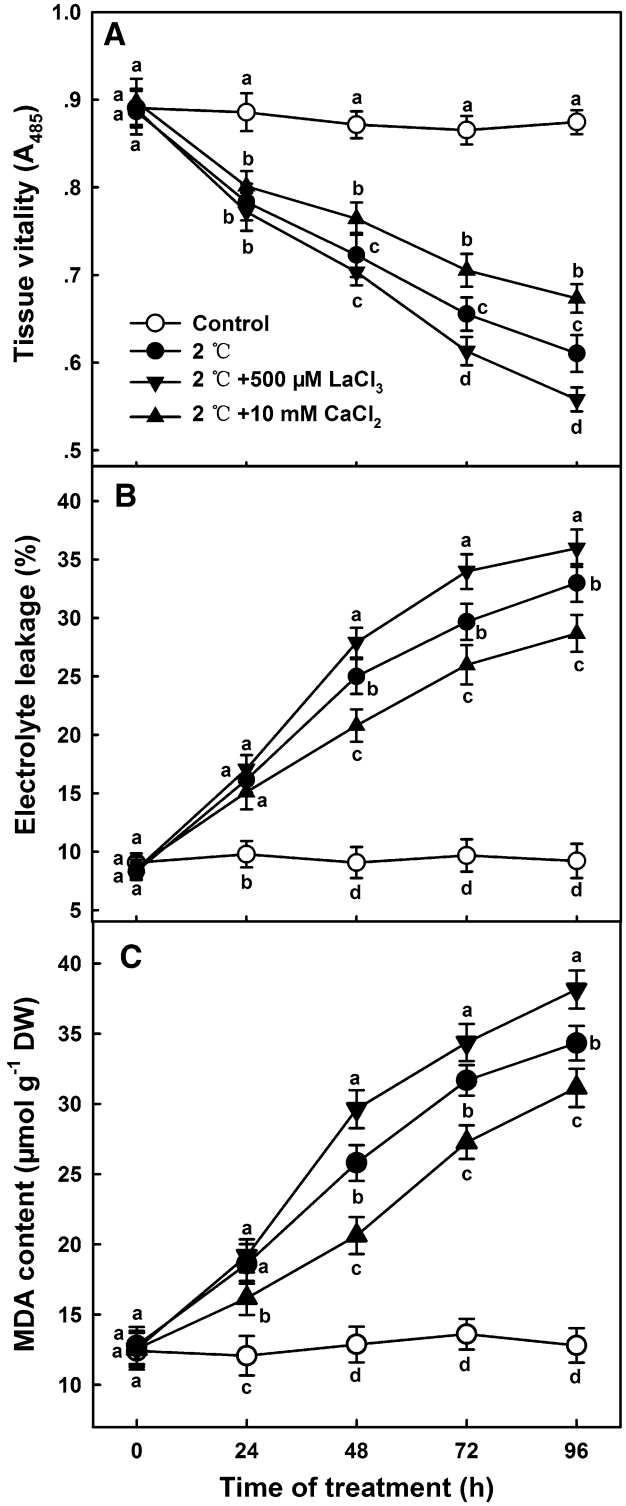

Fig. 9 Effects of $CaCl_2$ and $LaCl_3$ on tissue vitality (**a**), electrolyte leakage (**b**) and MDA content (**c**) in leaves of *J. curcas* seedlings under chilling stress. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

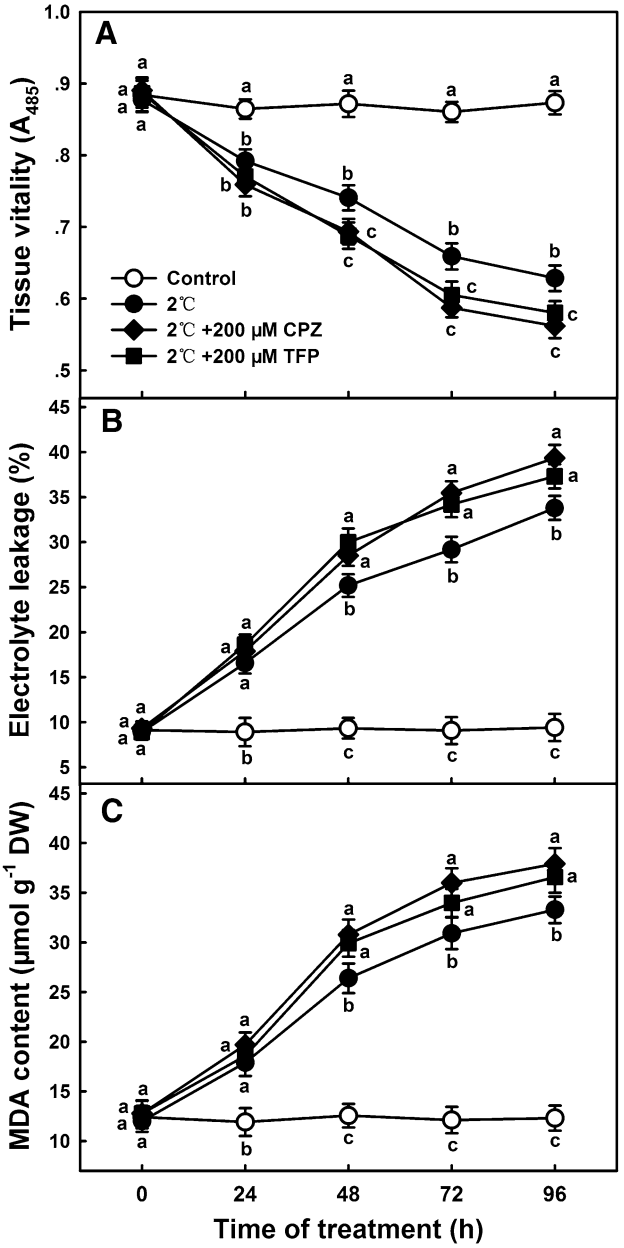

Fig. 10 Effects of CPZ and TFP on tissue vitality (**a**), electrolyte leakage (**b**) and MDA content (**c**) in leaves of *J. curcas* seedlings under chilling stress. Each *point* represents the mean ± SE of at least six replicates of three independent experiments. Treatments marked with *different letters* at a given sampling date are significantly different at $P < 0.05$

and decreased electrolyte leakage and MDA content (Fig. 9), while $LaCl_3$ (Fig. 9), and CPZ and TFP (Fig. 10) reversed $CaCl_2$ effects in *J. curcas* seedlings under chilling stress. These results showed that calcium could improve cold tolerance of *J. curcas* seedlings. However, the mechanism of calcium-induced cold stress tolerance

remains unclear. The results presented herein and in other studies (Ruiz and others 2002; Nayyar and others 2005) indicate that calcium treatment increases significantly the accumulation of proline. In addition, exogenous proline increases chilling tolerance in plants under chilling stress (Kumar and Yadav 2009; Jonytienė and others 2012). Therefore, we speculate that proline is a key factor in calcium-induced chilling tolerance. However, in plants, the acquisition of chilling tolerance is a complex event, involved in many physiological, biochemical, and molecular aspects, while the molecular mechanism of calcium-induced chilling tolerance in *J. curcas* seedlings needs to be further investigated in future studies.

Our results indicate a possible involvement of Ca^{2+}-CaM in signal transduction events leading to proline accumulation and regulation of proline biosynthesis and degradation in *J. curcas* seedlings under chilling stress. The Ca^{2+}-CaM-induced proline accumulation in *J. curcas* seedlings under chilling stress might be the result of the combined activation of the glutamate pathways of proline biosynthesis and inhibition of the proline degradation pathway. In addition, exogenous Ca^{2+} can improve chilling tolerance of *J. curcas* seedlings and the acquisition of this chilling tolerance may be involved in proline.

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