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ORIGINAL PAPER

Combined action of antioxidant defense system and osmolytes in chilling shock-induced chilling tolerance in *Jatropha curcas* seedlings

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Abstract Low non-freezing temperature is one of the major environmental factors affecting growth, development and geographical distribution of chilling-sensitive plants, *Jatropha curcas* is considered as a sustainable energy plants with great potential for biodiesel production. In this study, chilling shock at 5 °C followed by recovery at 26 °C for 4 h significantly improved survival percentage of *J. curcas* seedlings under chilling stress at 1 °C. In addition, chilling shock could obviously enhance the activities of antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), and the levels of antioxidants ascorbic acid (AsA) and glutathione (GSH), as well as the contents of osmolytes proline and betaine in leaves of seedlings of *J. curcas* compared with the control without chilling shock. During the process of recovery, GR activity, AsA, GSH, proline and betaine contents sequentially increased, whereas SOD, APX and CAT activities gradually decreased, but they markedly maintained higher activities than those of control. Under chilling stress, activities of SOD, APX, CAT, GR and GPX, and contents of AsA, GSH, proline and betaine, as well as the ratio of the reduced antioxidants to total antioxidants [AsA/(AsA + DHA) and GSH/(GSH + GSSG)] in the shocked and non-shock seedlings all dropped, but shocked seedlings sustained significantly higher antioxidant enzyme activity, antioxidant and osmolyte contents, as well as ratio of reduced antioxidants to total antioxidants from beginning to end compared with control. These results indicated that the chilling shock followed by recovery could improve chilling tolerance of seedlings in *J. curcas*, and antioxidant enzymes and osmolytes play important role in the acquisition of chilling tolerance.

Keywords Antioxidant enzymes · Antioxidants · Chilling shock · Chilling tolerance · *Jatropha curcas* L. · Osmolytes

Abbreviations

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic acid
BADH	Betaine aldehyde dehydrogenase
BSO	Buthionine sulfoximine
CAT	Catalase
DHA	Dehydroascorbate
DW	Dry weight
γEC	γ-Glutamylcysteine
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
GPX	Guaiacol peroxidase
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Due to the fact that its seed contains high oil content, the *Jatropha curcas* belongs to the tribe *Jatropheae* in the

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Euphorbiaceae family, considered as an important energy plant with great potential for biodiesel production (Carels 2009; King et al. 2009; Mukherjee et al. 2011). The seed of *J. curcas* contains 30–40 % oil with 79 % unsaturated fatty acids and 21 % saturated fatty acids, and seed oil can yield a high quality biodiesel, which has led to a surge of interest in *J. curcas* across the globe (King et al. 2009; Mukherjee et al. 2011). Compared to soybean oil, castor oil and palm oil, *J. curcas* oil has a good oxidation stability, low viscosity and a low pour point, and these indexes are close to those of fossil diesel and up to the American and European standards (Carels 2009; King et al. 2009; Mukherjee et al. 2011).

Plants are constantly exposed to a wide range of abiotic and biotic stresses such as extreme in temperature, drought, high salinity, heavy metal and mechanical stimulation due to their sessile and poikilothermic character (Ciarmiello et al. 2011; Li and Gong 2011a). Among these stresses, low temperature is the major environmental factors that limit growth, development and geographical distribution of plants, which causes significant crop losses (Jan et al. 2009; Janska et al. 2010). According to their responses to low temperature, plants can be classified into chilling-sensitive plants and chilling-tolerant plants, they differ in their tolerance to chilling (0–15 °C) and freezing (<0 °C). Chilling tolerance plants from temperate regions are chilling tolerant, although most are not very tolerant to freezing but can increase their freezing tolerance by being exposed to chilling, non-freezing temperatures, a phenomenon known as cold acclimation (Heidarvand and Amiri 2010; Ruelland et al. 2009). In contrast, plants of tropical and subtropical origins, that is, chilling-sensitive plants, including many crops such as maize, rice and tomato are sensitive to chilling stress and largely lack the capacity for cold acclimation (Ruelland et al. 2009; Survila et al. 2010). Chilling-sensitive plants can be irreparably damaged when the temperature drops below 10 °C, mainly due to (1) membrane injury, that is, loss in the integrity of membrane and intracellular organelles leads to solute leakage and the disruption of compartmentalization (Lukatkin 2003, 2012; Jan et al. 2009; Janska et al. 2010), (2) osmotic stress, namely water deficiency because of decrease in absorption of water by roots, restriction of stomata closure and reduction in water activity (Ruelland et al. 2009; Survila et al. 2010; Lukatkin et al. 2012), and (3) oxidative stress, in other words, the excess production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot-}$) leads to peroxidation of membrane lipids, protein oxidation, inactivation of enzymes, DNA damage, and so forth (Prasad et al. 1994; Lukatkin 2002a; Lukatkin et al. 2012; Suzuki et al. 2012). To cope with chilling injury, higher plants have developed several strategies, such as the antioxidation defense system including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and guaiacol peroxidase (GPX), and low molecular weight antioxidants ascorbic acid (AsA) and glutathione (GSH), as well as osmolytes, namely compatible solutes like proline and betaine (Lukatkin 2002b; Foyer and Noctor 2009, 2011; Jaleel et al. 2009; Szarka et al. 2012).

In general, chilling-sensitive plants exposed to low temperature (≥10 °C) for several days or several weeks, can improve their resistance to subsequent low temperature (0–5 °C) (Jan et al. 2009; Janska et al. 2010; Ruelland et al. 2009). Three-day-old maize seedlings did not survive chilling stress at 4 °C for 7 days unless they were pre-exposed to 14 °C for 3 days (Prasad et al. 1994). Lange and Cameron (1997) have reported that sweet basil subjected to chill hardening at 10 °C for 4 h daily for 2 days could increase average shelf life at 5 °C. Our previous results also found that in chilling-sensitive plant *J. curcas*, chill hardening at 10 or 12 °C for 1 and 2 days greatly lowered death rate and alleviated electrolyte leakage as well as accumulation of the lipid peroxidation product malondialdehyde (MDA) of *J. curcas* seedlings under severe chilling stress at 1 °C for 1–7 days (Ao et al. 2013). Surprisingly, in maize seedlings, cold-shock pretreatment at 1 °C for 4 h, followed by a 6-h recovery at 26.5 °C, significantly enhanced survival rate under severe chilling stress at 1 °C (Li et al. 2011).

On the basis of the above-mentioned results, we hypothesize that chilling-sensitive plant *J. curcas* (Liang et al. 2007; Zheng et al. 2009) may improve chilling tolerance by short-term exposure at low non-freezing temperature, that is, a 4-h chilling shock treatment at 5 °C, but detailed evidence is not clear. In this article, effect of chilling shock on chilling tolerance and involvement of antioxidant defense system and osmolytes were investigated, the objective was to examine the possible role of antioxidant defense system and osmolytes in chilling shock-induced chilling tolerance in *J. curcas* seedlings.

Materials and methods

Plant materials and treatments

Seeds of *J. curcas*, a mix of cultivars, were collected from Yuanmou, Yunnan Province, China. Seeds were surface sterilized in 1 % $CuSO_4$ for 15 min and rinsed thoroughly with sterilized distilled water according to our previous methods (Li and Gong 2011b), and then pre-soaked for imbibition in distilled water for 24 h. The soaked seeds were sowed on six layers of wetted filter papers in trays (200 seeds per tray) with covers and germinated at 26 °C in the dark for 5 days. Then, germinated seeds were selected

and transferred to pot containing sterilized soil with perlite, peat and sand (1:2:1) as well as wetted 1/2 MS (Murashige and Skoog 1962) basal salts in climate chamber with 26/20 °C (day/night), 300 μmol m^{-2} s^{-1} and 16-h photoperiod (with light from 6:00 to 22:00 h) and sequentially grown for 7 days.

To understand the effect of chilling shock on chilling tolerance, 12-day-old seedlings were transferred to a climate chamber with 5 °C and 300 μmol m^{-2} s^{-1} (the control seedlings always grown in the climate chamber with above-mentioned parameters) for 2 or 4 h to carry out chilling shock. To avoid effect of circadian clock on the following physiological and biochemical indexes, chilling shock began at 9:00 after lighting. At the end of chilling shock, shocked seedlings were transferred to another climate chamber with above cultural conditions for 2 or 4 h for recovery. Then, shocked and non-shock seedlings were subjected to chilling stress in a climate chamber with 1 °C, 300 μmol m^{-2} s^{-1} for 1, 2, 3, 4 or 5 days; survival percentage of seedlings, antioxidant enzyme activities, antioxidant and osmolyte contents in fresh leaves collected from the same plants over time was determined according to the following methods, respectively.

Determination of survival percentage

On the fifth day of chilling stress at 1 °C, seedlings were recovery growth in the climate chamber with above cultural conditions for a week, and survival percentage of seedlings was counted and expressed as %. The seedlings which could become green and re-grow during the process of recovery were considered as survival (Gong et al. 2001).

Antioxidant enzyme activities assay

During the course of chilling shock and chilling stress, antioxidant enzymes SOD, APX, CAT, GPX and GR in fresh leaves of *J. curcas* seedlings were extracted and measured according to our methods described previously (Ao et al. 2013). Fresh leaves (0.5 g) were ground in a mortar with a pestle in 5 ml of extraction buffer contained 50 mM Tris–HCl (pH 7.0), 0.1 mM EDTA, 1 mM AsA, 1 mM dithiothreitol and 5 mM $MgCl_2$. The homogenates were centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatants were used for assays of antioxidant enzymes. Detailed measurement protocols were described as below, respectively.

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The 3 mL reaction mixture contained 50 mM Tris–HCl (pH 7.8), 13.37 mM methionine, 0.1 mM NBT, 0.1 mM riboflavin, 0.1 mM EDTA and 0.1 mL enzyme extract. One unit of enzyme activity was defined as the amount of the enzyme bringing about 50 % inhibition of the photochemical reduction of NBT, and the activity of SOD was expressed as U g^{-1} DW.

APX activity was measured by monitoring the rate of AsA oxidation at 290 nm. The assay mixture contained 50 mM Tris–HCl (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.1 ml enzyme extract. APX was detected according to the reduction value of the absorbance at 290 nm per unit time, and APX activity was counted using the extinction coefficient 2.8 mM^{-1} cm^{-1} at 290 and expressed as μmol g^{-1} DW min^{-1}.

CAT activity was determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm. The 3 ml reaction mixture consisted of 50 mM Tris–HCl (pH 7.0), 0.1 mM EDTA, 12.5 mM H_2O_2 and 0.1 mL enzyme extract. CAT activity was computed using the extinction coefficient 0.04 mM^{-1} cm^{-1} at 240 and expressed as μmol H_2O_2 g^{-1} DW min^{-1}.

GR was assayed by monitoring the increase in absorbance at 412 nm. The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.2 mM NADPH and 0.1 mL enzyme extract, and distilled water to make up a volume of 1 mL. Reaction was initiated by adding 10 mM GSSG (oxidized glutathione). GR activity was calculated using the extinction coefficient 6.2 mM^{-1} cm^{-1} at 340 and expressed as μmol g^{-1} DW min^{-1}.

POD activity was estimated by measuring the increase in absorbance at 470 nm due to guaiacol oxidation. The reaction mixture contained 50 mM Tris–HCl (pH 7.0), 10 mM guaiacol, 5 mM H_2O_2 and 0.1 ml enzyme extract. GPX activity was counted using the extinction coefficient 26.6 mM^{-1} cm^{-1} at 470 nm and expressed as μmol g^{-1} DW min^{-1}.

Measurement of water soluble antioxidant content

In addition to antioxidant enzyme activities, AsA and GSH contents in fresh leaves of *J. curcas* seedlings were extracted and measured as our procedures described previously (Ao et al. 2013). Briefly, fresh leaves (0.5 g) were ground in a mortar with a pestle in 3 ml of 5 % (v/v) sulfosalicylic acid. The homogenates were centrifuged at 10,000×*g* for 15 min at 4 °C. The supernatants were used for assays of ascorbic acid and glutathione. Ascorbic acid was determined using a method based on the reduction of ferric ion to ferrous ion with reduced ascorbic acid in acid solution and then the formation of the red chelate between ferrous ion and 2,2′-dipyridyl, which absorbs at 525 nm. GSH and GSSG were determined by the 5,5-dithiobis-(2-nitrobenzoic acid)-GR recycling procedure, change in absorbance of the reaction mixtures was measured at 412 nm. The contents of AsA, DHA, GSH and GSSG as well as the ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) were expressed as μmol g^{-1} DW and %, respectively.

Determination of proline and betaine contents

Besides, during the course of chilling shock and chilling stress, the contents of proline and betaine in fresh leaves of *J. curcas* seedlings were determined according to our previous procedures (Li and Gong 2013; Xu et al. 2011). Proline was estimated colorimetrically as ninhydrin complex in toluene. Fresh leaf material (0.5 g) was homogenized in 5 mL of 3 % aqueous sulfosalicylic acid and centrifuged at 10,000×*g* for 15 min. 2 mL of the supernatants was mixed with 2 ml of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was placed in a water bath at 100 °C for 1 h. Then, the mixture was extracted with 4 mL of toluene, and the absorbance was measured at 520 nm. To determine the content of betaine in leaves, fresh leaves were ground in a mortar with a pestle in distilled water, the homogenates were transferred to conical flask in a rotary shaker (120 rpm) with 30 °C for 12 h to extract betaine and centrifuged at 10,000×*g* for 15 min, and then supernatants were mixed with Reinecke salt and generated red precipitates, which is solvable in 70 % acetone solution and the absorbance was determined at 525 nm. Proline and betaine contents were expressed as μmol g^{-1} DW, respectively.

Statistics analysis

All experiments were repeated at least three times and two replications in each time. The results were processed statistically using one-way analysis of variance (ANOVA, variance test, LSD). Figures were drawn by SigmaPlot 10.0, error bars represent standard error and each data in the figures represent the mean ± SE of at least three experiments.

Results

Effect of chilling shock on chilling tolerance of *J. curcas* seedlings

After 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 2 or 4 h, seedlings all showed visible symptoms of wilting due to the loss of turgor, a indicator of osmotic stress, in particular, 4-h chilling shock showed more obviously than 2 h, but symptoms disappeared after a 4-h recovery at 26 °C, implying that short chilling shock could trigger osmotic stress (data not shown). In addition, the shocked and non-shock seedlings were transferred to chilling stress at 1 °C, and survival percentage was counted, as shown in Fig. 1. The chilling shock alone did not improve survival percentage of seedlings under chilling stress, but the chilling shock followed by recovery did, especially in a 4-h chilling shock followed by a 4-h recovery displayed very significant difference compared to control ($P < 0.01$), suggesting that recovery after chilling shock plays an important role in the acquisition of chilling shock-induced chilling tolerance in *J. curcas* seedlings.

Effect of chilling shock and chilling stress on antioxidant enzyme activities and antioxidant levels of *J. curcas* seedlings

To better understand the possible mechanisms of the above-mentioned chilling shock-induced chilling tolerance, the activities of the antioxidant enzymes SOD, APX, CAT, GPX and GR, and the levels of the antioxidants AsA and GSH in leaves of *J. curcas* seedlings were determined. During the course of the chilling shock at 5 °C, activities of SOD, APX, CAT and GR all raised with the extension of chilling shock time, in particular, activities of SOD, APX and CAT showed more significant difference than those of other antioxidant enzymes (Figs. 2, 3, 4, 5). In contrast, GPX activity gradually dropped (Fig. 6). During the process of recovery at 26 °C, activities of GR and GPX sequentially increased with the prolongation of time, while SOD, APX and CAT activities decreased gradually (Figs. 2, 3, 4, 5, 6). Under chilling stress at 1 °C, antioxidant enzymes activities in shocked and non-shock

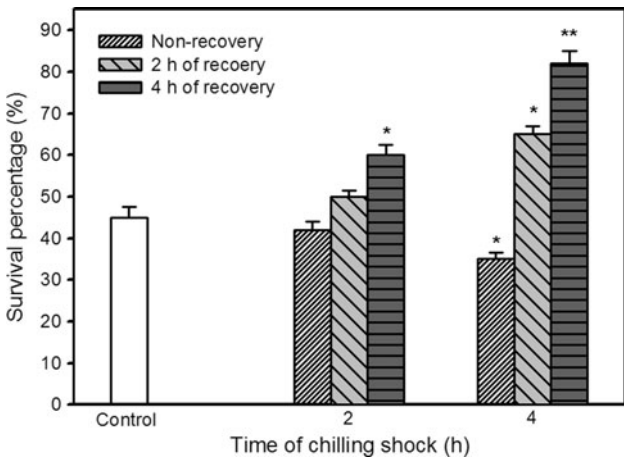


Fig. 1 Effect of chilling shock and followed by recovery on survival percentage of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 2 or 4 h, respectively, and then recovery at 26 °C for 2 or 4 h, respectively. Finally, the shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 5 days. Survival percentage (%) of the seedlings was counted after recovery under normal growth conditions for a week, and about 200 seedlings were investigated each treatment. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

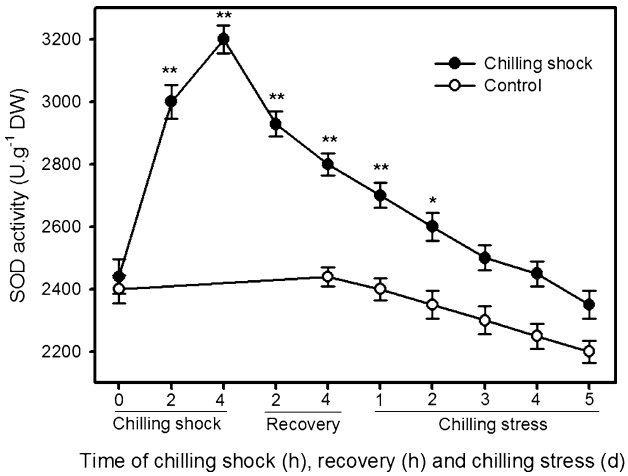


Fig. 2 Effect of chilling shock and chilling stress on SOD activity in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. SOD activity in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

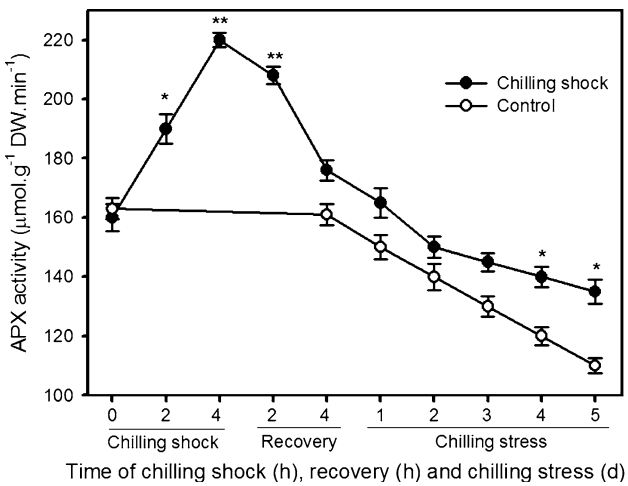


Fig. 3 Effect of chilling shock and chilling stress on APX activity in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. APX activity in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

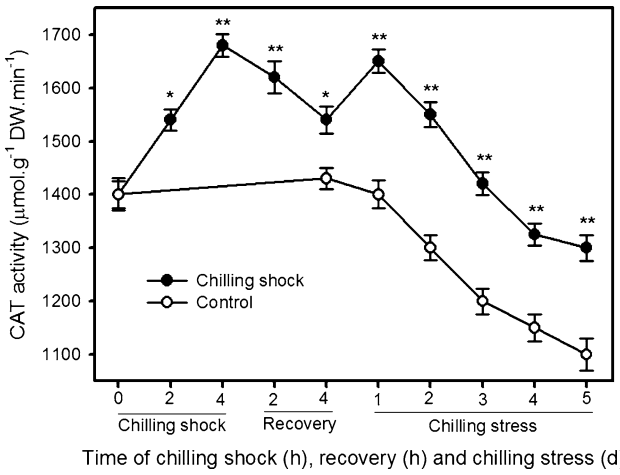


Fig. 4 Effect of chilling shock and chilling stress on CAT activity in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. CAT activity in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

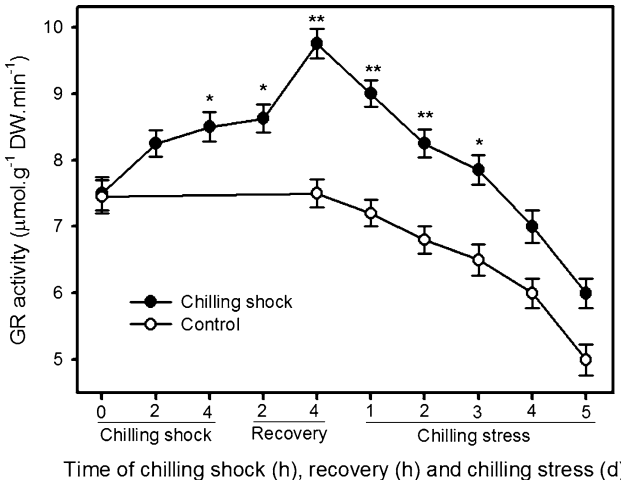


Fig. 5 Effect of chilling shock and chilling stress on GR activity in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. GR activity in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

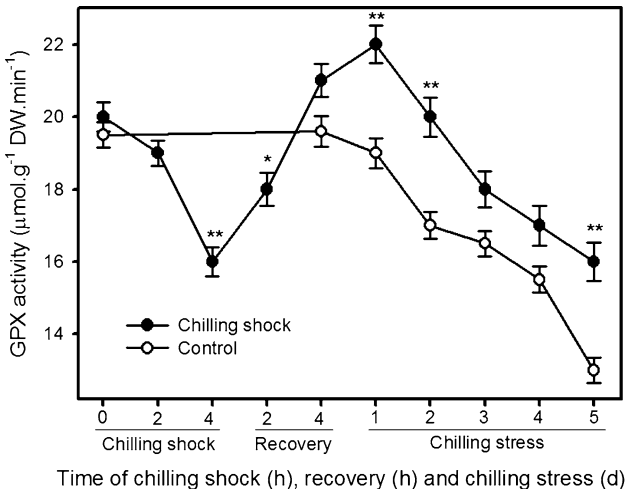


Fig. 6 Effect of chilling shock and chilling stress on GPX activity in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. GPX activity in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

seedlings all declined unceasingly except activities of CAT and GPX ascended on the first day of chilling stress, but antioxidant enzymes activities in shocked seedlings maintained higher levels from beginning to end as compared with control (Figs. 2, 3, 4, 5, 6).

In addition to antioxidant enzymes, levels of AsA, DHA and GSSG showed increase in chilling shock and decrease in recovery (Figs. 7a, b, 8b), similar to activities of SOD, APX and CAT (Figs. 2, 3, 4). GSH level and the ratio of AsA/(AsA + DHA) went up gradually during the process of chilling shock and recovery (Figs. 7c, 8a), like the trend of GR activity (Fig. 5), while the change of GSH/(GSH + GSSG) ratio was not obvious in both chilling shock and recovery (Fig. 8c). During the process of chilling stress, DHA and GSSG levels gradually ascended (Figs. 7b, 8b), whereas AsA and GSH levels as well as ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG) declined unceasingly (Figs. 7a, c, 8a, c), and the shocked seedlings sustained higher levels of reduced AsA and GSH, and ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG), as well as lower contents of oxidized antioxidants DHA and GSSG compared to the control (Figs. 7, 8).

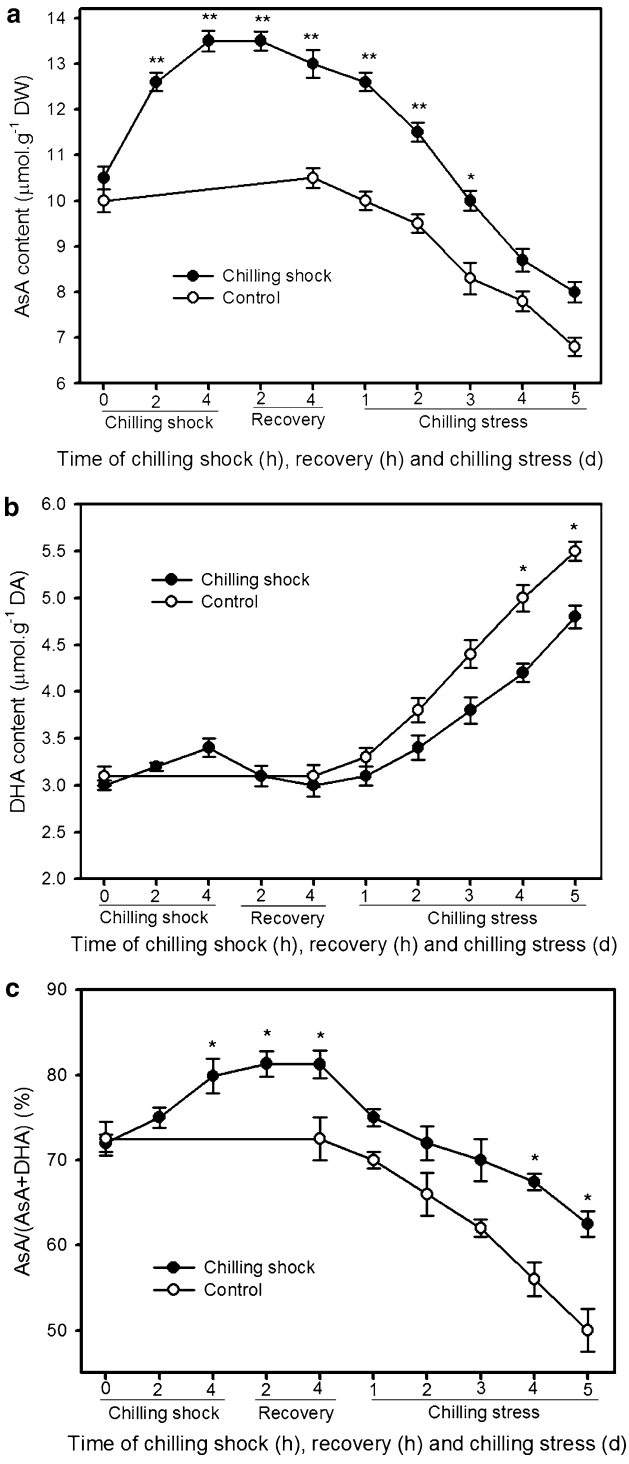
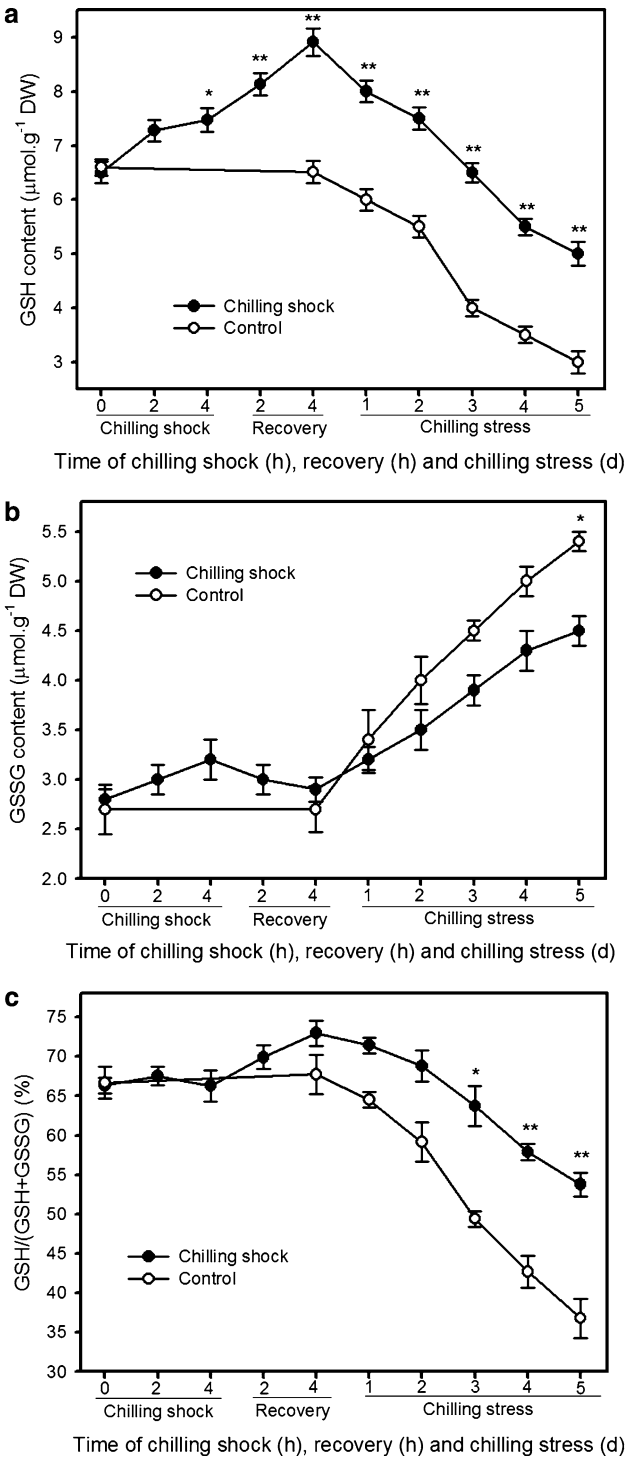


Fig. 7 Effect of chilling shock and chilling stress on levels of AsA (**a**) and DHA (**b**) as well as the ratio of AsA/(AsA + DHA) (**c**) in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. AsA and DHA contents as well as AsA/(AsA + DHA) ratio in leaves of the seedlings were measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively



◀ **Fig. 8** Effect of chilling shock and chilling stress on levels of GSH (**a**) and GSSG (**b**) as well as the ratio of GSH/(GSH + GSSG) (**c**) in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. GSH and GSSG contents as well as GSH/(GSH + GSSG) ratio in leaves of the seedlings were measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

Effect of chilling shock and chilling stress on osmolyte contents of *J. curcas* seedlings

Both proline and betaine are considered as major osmolytes against osmotic stress caused by cold, drought and salt stress in plants (Ashrak and Foolad 2007; Chen and Murata 2008). In the present work, chilling shock could gradually enhance the contents of proline and betaine in leaves of seedlings in *J. curcas* from beginning to end, and both unceasingly increased during the course of recovery and reached maximum values at 2 or 4 h of recovery, respectively (Figs. 9, 10), similar to GR activity, AsA and GSH levels as well as ratio of AsA/(AsA + DHA) (Figs. 5, 7a, c, 8a). During the process of chilling stress, contents of proline and betaine in shocked and non-shock seedlings all declined gradually, but decrease in the speed of both proline and betaine in shocked seedlings was significantly alleviated as compared with control (Figs. 9, 10), being analog to changes in antioxidant defense system (Figs. 2, 3, 4, 5, 6, 7, 8). These results indicated that chilling shock could also improve contents of endogenous proline and betaine in the leaves of *J. curcas* seedlings and remit reduction in their contents under chilling stress.

Discussion

Although chilling-sensitive plants of tropical and subtropical origins largely lack the capacity for cold acclimation, a number of studies showed that exposure of low non-freezing moderate temperature for several days or weeks can allow plants to withstand subsequent and more severe low non-freezing temperature stress (Prasad et al. 1994; Lange and Cameron 1997; Guy 1999; Chinnusamy et al. 2007; Heidarvand and Amiri 2010; Survila et al. 2010; Ciarmiello et al. 2011; Catala et al. 2012). Interestingly, maize seedlings subjected to a 4-h cold-shock pretreatment at 1 °C, followed by a 6-h recovery at 26.5 °C, significantly enhanced survival rate under severe chilling stress at 1 °C (Li et al. 2011). In the present study, 12-day-old seedlings of *J. curcas* were exposed to 5 °C for 2 or 4 h, followed by recovery at 26 °C for 2 or 4 h, could obviously enhance survival percentage under chilling stress at 1 °C, and the combination of a 4-h chilling shock and a 4-h recovery showed the most significant difference ($P < 0.01$) as compared with the control seedlings without the chilling shock (Fig. 1), implying that short-term chilling shock

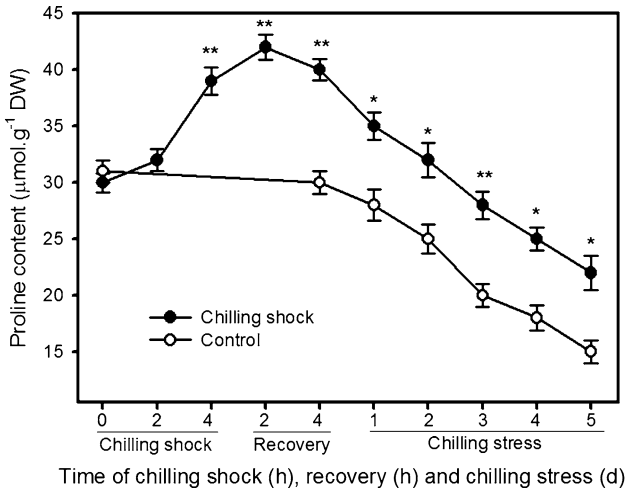


Fig. 9 Effect of chilling shock and chilling stress on content of proline in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. Proline content in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

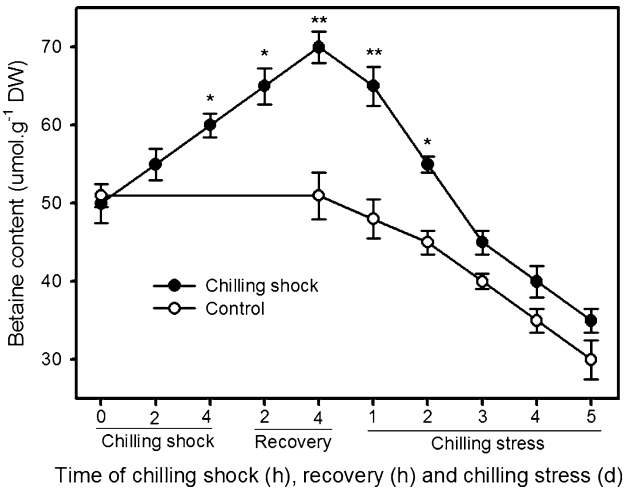


Fig. 10 Effect of chilling shock and chilling stress on content of betaine in leaves of *J. curcas* seedlings under chilling stress at 1 °C. 12-day-old seedlings of *J. curcas* were subjected to chilling shock at 5 °C for 4 h, followed by recovery at 26 °C for 4 h, and then shocked and non-shock seedlings were exposed to chilling stress at 1 °C for 1, 2, 3, 4 or 5 days. Betaine content in leaves of the seedlings was measured during the process of chilling shock, recovery and chilling stress daily. *Error bars* represent standard error and each data in the figures represent the mean ± SE of at least three experiments. *Asterisk* and *double asterisks* indicate significant difference ($P < 0.05$) and very significant difference ($P < 0.01$) from the control without chilling shock, respectively

followed by recovery could improve chilling tolerance of seedlings in *J. curcas*.

Enhancement in antioxidant defense system is one of the mechanisms of plants adapt to adverse environments including chilling stress (Guy 1999; Chinnusamy et al. 2007; Survila et al. 2010; Ciarmiello et al. 2011; Catala et al. 2012). Cellular redox homeostasis is considered to be an "integrator" of information from metabolism and the environment controlling plant growth and acclimation responses (Foyer and Noctor 2009; Jaleel et al. 2009; Scheibe and Dietz 2012). The breaking of metabolic homeostasis due to chilling stress results in a greater production of ROS, but plants can keep ROS at a physiological level that is not harmful via synergistic effect of antioxidant enzymes and antioxidants, suggesting that enhancement in antioxidant defense system under chilling stress has been correlated with tolerance to the stress (Lukatkin 2002a, b; Leipner and Stamp 2009). Chilling stress could increase GPX activity and reduce SOD activity in leaves of tobacco seedlings, and CAT activity was little affected (Xu et al. 2010; Leipner and Stamp 2009). In alfalfa, activity of SOD increased straight away under chilling stress, whereas CAT, APX and GR activities were slightly increased after chilling treatment (Wang et al. 2009). Our previous results also showed that the cold-shock pretreatment at 1 °C for 4 h enhanced the activities of antioxidant enzymes GPX, CAT, APX, GR and SOD in maize mesocotyls and remained significantly higher activities of antioxidant enzymes after chilling stress (Li et al. 2011). In the present work, chilling shock at 5 °C could trigger increase in SOD, APX, CAT and GR activities and reduced the activity of GPX, as well as maintained markedly higher antioxidant enzymes activities in leaves of *J. curcas* seedlings under chilling stress at 1 °C from beginning to end as compared with the control (Figs. 2, 3, 4, 5, 6).

Low molecular antioxidants (e.g., AsA, GSH), the heart of the redox hub, serve not only to limit the lifetime of the ROS signals, but also to participate in an extensive range of other redox signaling and regulatory functions by affecting components of the antioxidant ascorbate-glutathione cycle (Foyer and Noctor 2009, 2011; Jaleel et al. 2009). Low temperatures at 14 °C could enhance antioxidants AsA and GSH contents, followed by increase in resistance to chilling stress compared with the seedlings grown at 20 °C (Kocsy et al. 2001). Pretreatment of herbicide safeners greatly improved the GR activity and total GSH content, which increased the relative protection from chilling from 50 to 75 % in maize seedlings (Kocsy et al. 2001). On the contrary, maize seedlings treated with buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine (γEC) synthetase, significantly increased cysteine and reduced GSH content and GR activity, which, in turn,

weakened chilling tolerance (Kocsy et al. 2001). In the present work, chilling shock at 5 °C for 2 or 4 h could improve the contents of AsA and GSH, and the ratios of AsA/(AsA + DHA) and GSH/(GSH + GSSG), as well as obviously alleviated decrease in their levels under chilling stress (Figs. 7a, c, 8a, c), whereas the contents of oxidized DHA and GSSG generally sustained lower levels as compared with the control (Figs. 7b, 8b). These data demonstrated that antioxidant defense system may underlie plants chilling tolerance.

Accumulation of osmolytes such as proline and betaine is another adaptive mechanism of plant to unfavorable environments, in particular, osmotic stress caused by chilling, drought and salt stress (Ashrak and Foolad 2007; Chen and Murata 2008). Many studies found that chilling tolerance is correlated with increased osmolyte concentrations in many crops (Ruelland et al. 2009; Szabados and Savoure 2010), and accumulating osmolytes have multiple protective functions such as osmotic adjustment, protein and biomembrane stabilizing, ROS-scavenging, redox buffering, and so forth (Szabados and Savoure 2010; Theocharis et al. 2012). The *OsMYB2*-overexpressing rice seedlings not only accumulated greater amounts of proline, but also enhanced activities of antioxidant enzymes GPX, SOD and CAT, which, in turn, improved tolerant to chilling stress at 2 °C for 3 days (Yang et al. 2012). Songstad et al. (1990) found that maize suspension cultures exposed to 4 °C for 4 weeks inhibited cell growth, but this inhibition was reversed when 3–48 mM proline was present in the medium during the chilling stress. In addition, seedlings of *Arabidopsis thaliana* with the cloned *codA* gene enabled to accumulate betaine, which, in turn, did not exhibit symptoms of chlorosis when exposed to a low temperature in the light, but wild-type plants did (Hayashi et al. 1997). The overexpression of betaine aldehyde dehydrogenase (BADH) gene from *Spinacia oleracea* in the transgenic sweet potato not only increased BADH activity as well as accumulation of betaine and proline, but also improved gene expression and activities of antioxidant enzymes, which, in turn, reduced ROS accumulation, ultimately enhanced tolerance to chilling stress (Fan et al. 2012). Our previous study found that betaine treatment not only could enhance germination percentage of *J. curcas* seeds under low temperature at 18 °C, but also alleviate increase in electrolyte leakage and accumulation of MDA in *J. curcas* seedlings under chilling stress at 2 °C (Dai et al. 2012). In the present work, chilling shock could induce leaves wilting, hinting that chilling shock could trigger osmotic stress (data not shown), which, in turn, led to accumulation of osmolytes proline and betaine, and the shocked seedlings sustained significantly higher levels from beginning to end under chilling stress at 1 °C as compared with the control (Figs. 9, 10). These results suggested that osmolytes play a crucial role in the acquisition of chilling stress tolerance in plants.

In summary, it is clearly shown that 12-day-old seedlings of *J. curcas* subjected to chilling shock followed by recovery could improve survival percentage under chilling stress. In addition, chilling shock could improve activities of SOD, APX, CAT and GR, and levels of antioxidants AsA and GSH, as well as contents of osmolytes proline and betaine. Under chilling stress, the shocked seedlings maintained higher antioxidant enzyme activities, antioxidant and osmolyte contents, as well as ratio of reduced antioxidant to total antioxidant from beginning to end compared with control. These data indicated that chilling shock followed by recovery could improve chilling tolerance, and antioxidant defense system and osmolyte play important role in chilling shock-induced chilling tolerance of *J. curcas* seedlings.

Author contribution In this work, Zhong-Guang Li carried out conception, design and writing the article, Ling-Xuan Yuan, Wang Qiu-Lin, Zhi-Liu Ding and Chun-Yang Dong coordinated the study and carried out data analysis and interpretation. All authors have read and approved the final manuscript and have no conflicts of interest with regard to this research or its funding.

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References

Ao PX, Li ZG, Fan DM, Gong M (2013) Involvement of antioxidant defense system in chill hardening-induced chilling tolerance in *Jatropha curcas* seedlings. Acta Physiol Plant 35:153–160

Ashrak M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216

Carels N (2009) *Jatropha curcas*: a review. Adv Bot Res 50:39–86

Catala R, Diaz A, Salinas J (2012) Molecular responses to extreme temperatures. Plant Biotechnol Agric 5:287–307

Chen THH, Murata N (2008) Glycinebetaine: an effective protectant against abiotic stress in plants. Trends Plant Sci 13:499–505

Chinnusamy V, Zhu JH, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trend Plant Sci 12:444–451

Ciarmiello LF, Woodrow P, Fuggi A, Pontecorvo G, Carillo P (2011) Plant genes for abiotic stress. In: Shanker A, Venkateswarlu B (eds) Abiotic stress in plants—mechanisms and adaptations. Venkateswarlu Intech, Rijeka

Dai X, Li ZG, Gong M (2012) Effect of gibberellin, calcium and betaine on seed germination and resistance of *Jatropha curcas* L. seedling to low temperature and drought Stress. Plant Sci J 30:205–213

Fan WJ, Zhang M, Zhang HX, Zhang P (2012) Improved tolerance to various abiotic stresses in transgenic sweet potato (*Ipomoea batatas*) expressing spinach betaine aldehyde dehydrogenase. PLoS ONE 7:e37344

Foyer CH, Noctor G (2009) Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antioxid Redox Signal 11:861–905

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant Physiol 155:2–18

Gong M, Chen B, Li ZG, Guo LH (2001) Heat-shock-induced cross adaptation to heat, chilling, drought and salt stress in maize seedlings and involvement of H_2O_2. J Plant Physiol 158:1125–1130

Guy C (1999) Molecular responses of plants to cold shock and cold acclimation. J Mol Microbiol Biotechnol 1:231–242

Hayashi H, Alia Mustardy L, Deshnium P, Ida M, Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase; accumulation of glycine betaine and enhanced tolerance to salt and cold stress. Plant J 12:133–142

Heidarvand L, Amiri RM (2010) What happens in plant molecular responses to cold stress? Acta Physiol Plant 32:419–431

Jaleel CA, Riadh K, Gopi R, Manivannan P, Ines J, Al-Juburi HJ, Zhao CX, Shao HB, Panneerselvam R (2009) Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. Acta Physiol Plant 31:427–436

Jan N, Hussain M, Andrabi KI (2009) Cold resistance in plants: a mystery unresolved. Electr J Biotechnol 12:1–15

Janska A, Marsik P, Zelenkova S, Ovesna J (2010) Cold stress and acclimation—what is important for metabolic adjustment? Plant Biol 12:395–405

King AJ, He W, Cuevas JA, Freudenberger M, Ramiaramanana D, Graham IA (2009) Potential of *Jatropha curcas* as a source of renewable oil and animal feed. J Exp Bot 60:2897–2905

Kocsy G, Galiba G, Brunold C (2001) Role of glutathione in adaptation and signalling during chilling and cold acclimation in plants. Physiol Plant 113:158–164

Lange DL, Cameron AC (1997) Pre and postharvest temperature conditioning of greenhouse-grown sweet basil. HortScience 32:114–116

Leipner J, Stamp P (2009) Chilling stress in maize seedlings. In: Bennetzen JL, Hake SC (eds) Handbook of maize: its biology. Springer Science + Business Media, LLC, Norwell

Li ZG, Gong M (2011a) Mechanical stimulation-induced cross-adaptation in plants: an overview. J Plant Biol 54:358–364

Li ZG, Gong M (2011b) Effects of different chemical disinfectant on seed germination and seedling growth of *Jatropha curcas* L. Seed 30:4–7

Li ZG, Gong M (2013) Mechanical stimulation-induced chilling tolerance in tobacco (*Nicotiana tabacum* L.) suspension cultured cells and its relation to proline. Russ J Plant Physiol 60:149–154

Li HY, Li CG, Gong M (2011) Short-term cold-shock at 1 °C induced chilling tolerance in maize seedlings. Int Conf Biol Environ Chem 1:346–349

Liang Y, Chen H, Tang MJ, Yang PF, Shen SH (2007) Responses of *Jatropha curcas* seedlings to cold stress: photosynthesis-related proteins and chlorophyll fluorescence characteristics. Physiol Plant 131:508–517

Lukatkin AS (2002a) Contribution of oxidative stress to the development of cold-Induced damage to leaves of chilling-sensitive plants: 1. Reactive oxygen species formation during plant chilling. Russ J Plant Physiol 49:622–627

Lukatkin AS (2002b) Contribution of oxidative stress to the development of cold-Induced damage to leaves of chilling-sensitive plants: 2. The activity of antioxidant enzymes during plant chilling. Russ J Plant Physiol 49:782–788

Lukatkin AS (2003) Contribution of oxidative stress to the development of cold-induced damage to leaves of chilling-sensitive plants: 3. Injury of cell membranes by chilling temperatures. Russ J Plant Physiol 50:271–274

Lukatkin AS, Brazaityte A, Bobinas C, Duchovskis P (2012) Chilling injury in chilling-sensitive plants: a review. Agriculture 99:111–124

Mukherjee P, Varshney A, Johnson TS, Jha TB (2011) *Jatropha curcas*: a review on biotechnological status and challenges. Plant Biotechnol Rep 5:197–215

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–479

Prasad TK, Anderson MD, Martin BA, Stewart CR (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6:65–74

Ruelland E, Vaultier MN, Zachowski A, Hurry V (2009) Cold signalling and cold acclimation in plants. Adv Bot Res 49: 35–150

Scheibe R, Dietz KJ (2012) Reduction–oxidation network for flexible adjustment of cellular metabolism in photoautotrophic cells. Plant Cell Environ 35:202–216

Songstad DD, Duncan DR, Widholm JM (1990) Proline and polyamine involvement in chilling tolerance of maize suspension cultures. J Exp Bot 41:289–294

Survila M, Heino P, Palva ET (2010) Genes and gene regulation for low-temperature tolerance. In: Jenks MA, Wood AJ (eds) Genes for plant abiotic stress. Blackwell, Iowa

Suzuki N, Koussevitzky S, Mittler R, Miller G (2012) ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ 35:259–270

Szabados L, Savoure A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15:89–97

Szarka A, Tomasskovics B, Bánhegyi G (2012) The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. Int J Mol Sci 13:4458–4483

Theocharis A, Clément C, Barka EA (2012) Physiological and molecular changes in plants grown at low temperatures. Planta 235:1091–1105

Wang WB, Kim YH, Lee HS, Deng XP, Kwak SS (2009) Differential antioxidation activities in two alfalfa cultivars under chilling stress. Plant Biotechnol Rep 3:301–307

Xu SC, Li YP, Hu J, Guan YJ, Ma WG, Zheng YY, Zhu SJ (2010) Responses of antioxidant enzymes to chilling stress in tobacco seedlings. Agric Sci China 9:594–1601

Xu SL, Li ZG, Gong M (2011) Protective action of exogenous betaine on seed germination and seedlings growth of *Jatropha curcas* under PEG stress. Seed 30:29–33

Yang A, Dai XY, Zhang WH (2012) A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. J Exp Bot 63:2541–2556

Zheng YL, Feng YL, Lei YB, Yang CY (2009) Different photosynthetic responses to night chilling among twelve populations of *Jatropha curcas*. Photosynthetica 47:559–566