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ORIGINAL PAPER

Involvement of compatible solutes in chill hardening-induced chilling tolerance in *Jatropha curcas* seedlings

Ping-Xing Ao · Zhong-Guang Li · Ming Gong

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Abstract Low temperature is a major environmental factor that affects metabolism, growth, development, distribution and production of chilling-sensitive plant, and *J. curcas* L. is a sustainable energy plant with great potential for biodiesel production due to the fact that its seed contains high oil content, which has attracted much attention worldwide. Our previous work found that the chill hardening improved the chilling tolerance of *J. curcas* seedlings (Ao et al. in Acta Physiologiae Plantarum 35:153–160, 2013), but its mechanism still remains elusive. In present work, the mechanism of chill hardening-induced chilling tolerance was further investigated in *J. curcas* seedlings. The results showed that chill hardening at 12 °C for 2 days markedly lowered osmotic and water potentials, which, in turn, maintained relative higher pressure potential in leaves of *J. curcas* seedlings compared with the control seedlings without chill hardening. In addition, chill hardening gradually increased compatible solutes proline, betaine and total soluble sugar contents compared with the control. When the control and hardened seedlings were subjected to chilling stress at 1 °C for 1–7 days, the chill-hardened seedlings significantly accumulated higher proline, betaine and total soluble sugar contents, which decreased osmotic and water potentials, and maintained higher pressure potential. To further understand the pathways of accumulation of compatible solutes, measurement of activities of Δ^1-pyrroline-5-carboxylate synthetase (P5CS), glutamate dehydrogenase (GDH), ornithine aminotransferase (OAT), arginase, proline dehydrogenase (ProDH) and betaine dehydrogenase (BADH) showed that the chill hardening at 12 °C for 2 days obviously increased the activities of P5CS, GDH, OAT, arginase and BADH, as well as lowered ProDH activity both in leaves and stems of *J. curcas* seedlings to some extent as compared with the control. When the control and hardened seedlings were exposed to chilling stress at 1 °C for 1–7 days, the chill-hardened seedlings generally maintained significantly higher activities of P5CS, GDH, OAT, arginase and BADH. All above-mentioned results illustrated that the chill hardening could induce an accumulation of compatible solutes in leaves of *J. curcas* seedlings and compatible solutes play important roles in chill hardening-induced chilling tolerance.

Keywords Chill hardening · Chilling tolerance · Compatible solutes · *Jatropha curcas* L. · Osmotic adjustment

Abbreviations

ANOVA	Analysis of variance
BADH	Betaine aldehyde dehydrogenase
GDH	Glutamate dehydrogenase
OAT	Ornithine aminotransferase
P5CS	Δ^1-Pyrroline-5-carboxylate synthetase
ProDH	Proline dehydrogenase

Introduction

Plants, due to their sessile and poikilothermic nature, are constantly exposed to a broad spectrum of abiotic and biotic

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Z.-G. Li and P.-X. Ao contributed equally to this work.

P.-X. Ao · Z.-G. Li · M. Gong (✉)
School of Life Sciences, Engineering Research Center of Sustainable Development and Utilization of Biomass Energy, Ministry of Education, Key Laboratory of Biomass Energy and Environmental Biotechnology, Yunnan Normal University, Kunming 650092, Yunnan, People's Republic of China
e-mail: gongming6307@163.com

stresses such as extreme temperature, drought, high salinity and heavy metals stress, as well as mechanical stimulation, and Low temperature is a major environmental factor that affects metabolism, growth, development, distribution and production of chilling-sensitive plant from tropical and subtropical origins (Jan et al. 2009; Janska et al. 2010; Ciarmiello et al. 2011; Li and Gong 2011a). All plants have optimal temperature ranges for their proper metabolism, growth and development, as well as minimum and maximum temperatures for survival. They differ in tolerance to chilling (0–15 °C) and freezing (<0 °C) (Levitt 1980). Chilling-sensitive plants, such as maize, rice, tomato and *Jatropha curcas* L., are sensitive to chilling stress and largely lack the cold acclimation ability (Ruelland et al. 2009; Survila et al. 2010), which can be irreparably damaged when the temperature drops below 10 °C; one of the major causes of chilling injury is 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). To cope with osmotic stress caused by chilling stress, higher plants have developed the mechanism of osmotic adjustment, that is, the accumulation of compatible solutes such as proline, betaine and soluble sugars (Szabados and Savoure 2010; Xie et al. 2011).

A number of studies found that chilling-sensitive plants exposed to low non-freezing moderate temperature for several days or weeks can improve their resistance to subsequent low temperature, which is referred to as chill hardening (Levitt 1980; Prasad et al. 1994). For example, exposure of 3-day-old maize seedlings to 14 °C for 3 days improved survival percentage under chilling stress 4 °C for 7 days (Prasad et al. 1994). Lange and Cameron (1997) also found that sweet basil subjected to chill hardening at 10 °C for 4 h daily for 2 days also increased average shelf life at 5 °C. Interestingly, cold shock pretreatment at 1 °C for 4 h followed by a 6-h recovery at 26.5 °C enhanced survival percentage of maize seedlings under severe chilling stress at 1 °C for 5 days (Li et al. 2011), but their mechanisms remain elusive.

The *J. curcas*, a chilling-sensitive plant, belonging to the tribe *Jatropheae* in the *Euphorbiaceae* family, is considered as an important energy plant because its seed contains high oil content, and the fuel properties of *J. curcas* biodiesel are close to those of fossil diesel fuel and match the American and European standards (Carels 2009; King et al. 2009; Mukherjee et al. 2011). In addition, due to multiple uses of different plant parts, *J. curcas* has spread beyond its original distribution, which is well adapted to arid and semi-arid climates, and even also grows on a large range of soils provided they are well drained and aerated, does not compete arable land with other oleaginous plants or crop plants (Carels 2009; King et al. 2009; Mukherjee et al. 2011). Therefore, *J. curcas* has attracted a great deal of attention worldwide (Carels 2009; King et al. 2009; Mukherjee et al. 2011). Our previous work also found that the chill hardening at 10 or 12 °C for 1 or 2 days all improved the chilling tolerance in *J. curcas* seedlings (Ao et al. 2013), but its mechanism is not fully clear. The present study, using *J. curcas* seedlings as materials, was aimed to investigate effect of chill hardening on accumulation of compatible solutes and their pathways to illustrate the possible mechanisms of chill hardening-induced chilling tolerance.

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 12 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 into 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), 100 μmol m^{-2} s^{-1} and 16 h photoperiod and sequentially grown for 9 days. Then, 2-week-old seedlings were subjected to chill hardening at 12 °C for 2 days (the control seedlings grown in the climate chamber with above-mentioned parameters). After chill hardening, the control and hardened seedlings were subjected to chilling stress at 1 °C for 1–7 days. The following physiological indexes were determined daily during the process of chill hardening and chilling stress.

Determination of osmotic, water and pressure potentials

To explore the effect of chill hardening and chilling stress on osmotic, water and pressure potentials in leaves of the seedlings, leaf segments were obtained using a cork borer at a thermocouple psychrometers, and measurements were performed at 25 °C, detailed procedures were referred to our previous methods (Gong et al. 1989; Chen and Gong 2011), and all potentials were expressed as MPa.

Measurement of compatible solutes contents

Main compatible solutes proline, betaine (trimethylglycine) and total soluble sugar contents both in leaves and stems were extracted and measured according to our methods described previously (Chen and Gong 2011). Proline was estimated

colorimetrically at 520 nm as ninhydrin complex in toluene. To determine the content of betaine, fresh leaves was ground in a mortar with a pestle in distilled water. The homogenates were transferred into 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 are soluble in 70 % acetone solution and the absorbance was determined at 525 nm. Soluble sugar content was determined using anthrone methods at 620 nm. Proline, betaine and soluble sugar contents were expressed as μmol/g DW, respectively.

Enzymes activities assay

To understand the change in activiteis of Key enzymes involved in proline metabolism, glutamate dehydrogenase (GDH), Δ^1-pyrroline-5-carboxylate synthetase (P5CS), ornithine aminotransferase (OAT), arginase and proline dehydrogenase (ProDH) both in leaves and stems were extracted and assayed according to the methods as described previously (Yang et al. 2009), and the activities of GDH, P5CS, OAT, arginase and ProDH were expressed as nmol NAD^+ mg^{-1} protein min^{-1}, nmol $NADP^+$ mg^{-1} protein min^{-1}, μmol P5C mg^{-1} protein min^{-1}, nmol ornithine mg^{-1} protein min^{-1} and nmol NADH mg^{-1} protein min^{-1}, respectively. Betaine dehydrogenase (BADH), rate-limiting enzyme of betaine synthesis, was extracted and measured on the basis of procedures described previously (Xu et al. 2011) and BADH activity was expressed as μmol NADH mg^{-1} protein min^{-1}.

Statistics analysis

All experiments were repeated at least three times and two replications in each time. The data were processed statistically using software package SPSS version 21.0 (SPSS, Chicago, USA) and the comparison of averages of each treatment was based on the analysis of one-way analysis of variance (ANOVA) and Duncan's multiple range test at the 5 % level of significance. Figures were drawn by SigmaPlot 11.0 (Systat Software Inc., London, UK). Error bars represent standard error and each data in figure represents the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P < 0.05$).

Results

Effect of chill hardening and chilling stress on osmotic, water and pressure potentials in leaves of *J. curcas* seedlings

When 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C, the results showed that chill hardening led to a slight decrease of osmotic and water potentials (Fig. 1a, b), which, in turn, maintained relative higher pressure potential in leaves of *J. curcas* seedlings on the first day (Fig. 1c). During the process of chilling stress at 1 °C, all potentials both in the control and chill-hardened seedlings decreased but chill-hardened seedlings had more lower osmotic and water potentials, and maintained relative higher pressure potential at the early stage compared with the control (Fig. 1). On the 2nd day of chilling stress, osmotic and water potentials both in control and chill-hardened seedlings reduced sharply, but pressure potential in leaves of chill-hardened seedlings was of positive value, indicating that turgor pressure still existed, but the turgor pressure in the control was lost (Fig. 1). These results indicated that the chill hardening could maintain higher pressure potential via reduction in osmotic and water potentials in leaves of *J. curcas* seedlings.

Effect of chill hardening and chilling stress on accumulation of compatible solutes both in leaves and stems of *J. curcas* seedlings

As shown in Fig. 2, under normal conditions, compatible solutes proline, betaine and total soluble sugar contents in leaves of *J. curcas* seedlings were higher than those of stems; in particular, the contents of betaine and total soluble sugars were much more significantly different than those of proline. In addition, chill hardening gradually enhanced the contents of proline, betaine and total soluble sugars both in leaves and stems of seedlings as compared with the control. During the course of chilling stress at 1 °C, the contents of proline, betaine and total soluble sugars in leaves of the seedlings all increased, but compatible solute contents in leaves of chill-hardened seedlings increased more rapidly and reached maximum value on the 3rd and 4th day, respectively (Fig. 2), and then gradually declined and went back to control level. These results suggested that the chill hardening could improve proline, betaine and total soluble sugar contents both in leaves and stems of *J. curcas* seedlings, which, in turn, maintained lower osmotic and water potentials, as well as higher pressure potentials in leaves of *J. curcas* seedlings under chilling stress.

Effect of chill hardening and chilling stress on activities of enzymes involved in compatible solutes metabolism both in leaves and stems of *J. curcas* seedlings

To explore further the pathways of chill hardening-induced accumulation of compatible solutes, in this present work, activities of GDH, P5CS, OAT, arginase, ProDH and BADH both in leaves and stems of *J. curcas* seedlings were determined. The results showed that, in normal conditions,

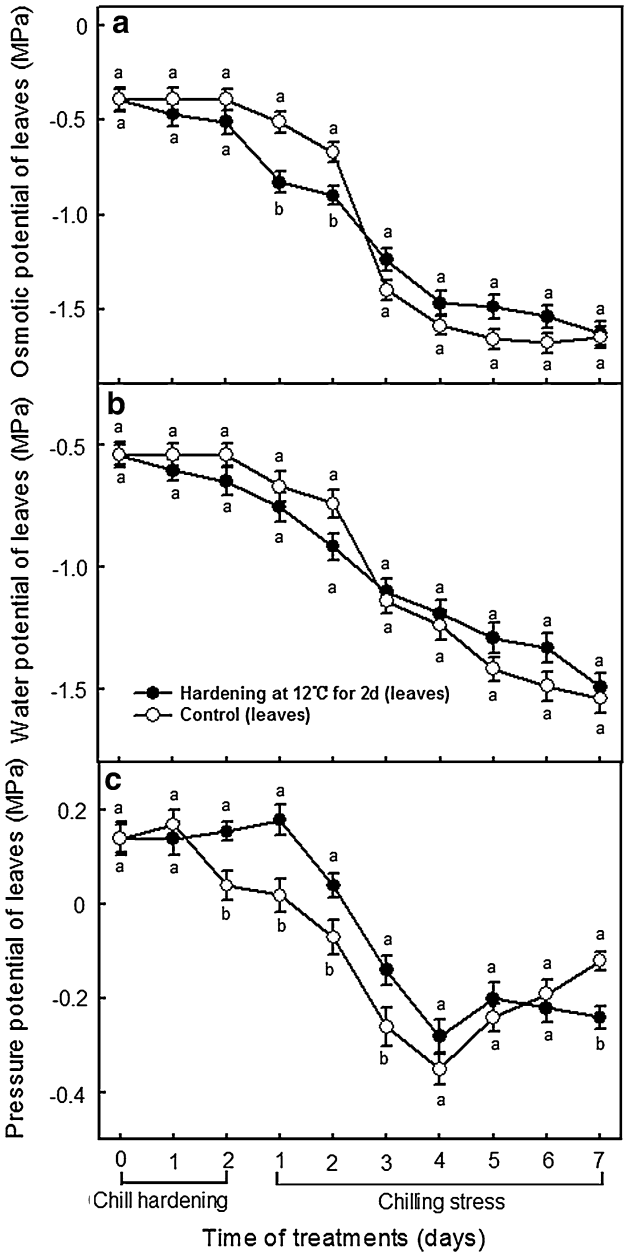

Fig. 1 Effect of chill hardening and chilling stress on osmotic (**a**), water (**b**) and pressure potentials (**c**) in leaves of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Water, osmotic and pressure potentials in leaves of the seedlings were measured daily. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

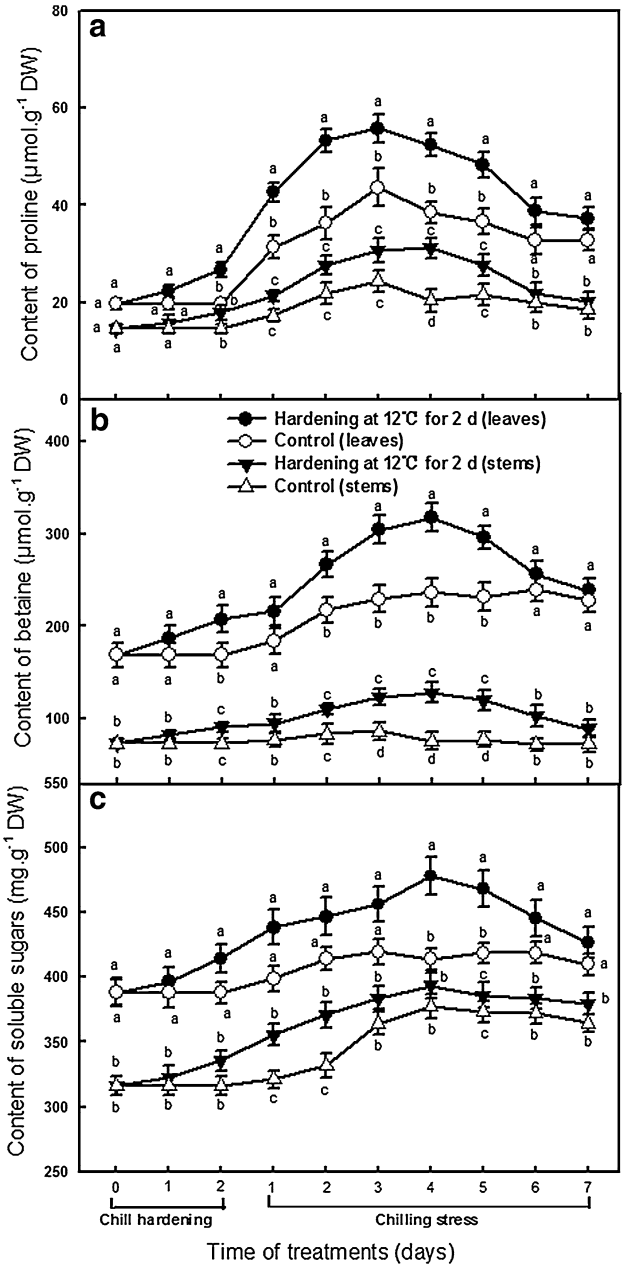

Fig. 2 Effect of chill hardening and chilling stress on contents of proline (**a**), betaine (**b**) and total soluble sugars (**c**) in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Compatible solutes contents both in leaves and stems of the seedlings were assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

five enzyme activities in leaves of *J. curcas* seedlings were higher than those of stems (Figs. 3, 4, 5, 6), similar to contents of compatible solutes (Fig. 2). During the process of the chill hardening, the activities of GDH, P5CS, OAT, arginase and BADH both in leaves and stems of seedlings increased gradually (Figs. 3, 4, 6), especially BADH activity in leaves demonstrated significant difference on the 2nd day of chill hardening ($P < 0.05$; Fig. 6), consisting with the contents of compatible solutes (Fig. 2). Similarly, ProDH activity in leaves declined gradually and reached significant level on the 2nd day of chill hardening

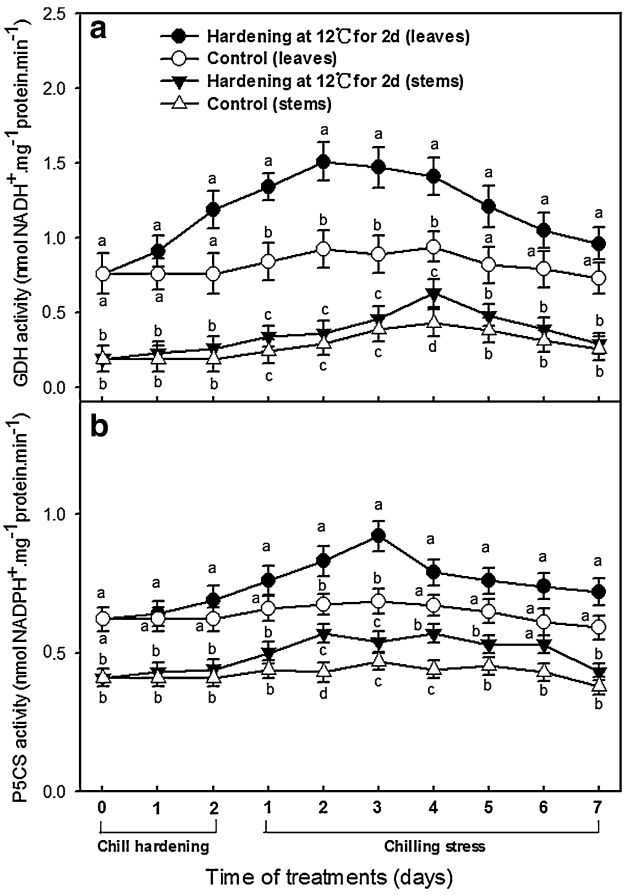

Fig. 3 Effect of chill hardening and chilling stress on activities of GDH (**a**) and P5CS (**b**) both in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Activities of enzymes GDH and P5CS both in leaves and stems of the seedlings were assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

($P < 0.05$; Fig. 5). During the course of chilling stress at 1 °C, the activities of five enzymes GDH, P5CS, OAT, arginase and BADH both in leaves and stems of seedlings sequentially increased and reached maximum value on the 3rd or 4th days of chilling stress, respectively (Figs. 3, 4, 6), and then gradually declined, in accord with the compatible solute contents (Fig. 2); ProDH activity in leaves sequentially declined and demonstrated significant difference on the 2nd and 3rd days of chilling stress($P < 0.05$), but the change in activity of ProDH in stems was not significant (Fig. 5); BADH activity in leaves and stems demonstrated significant difference on the 2nd, 3rd and 4th days of chilling stress, respectively ($P < 0.05$; Fig. 6). These results implied that the chill hardening-induced accumulation of compatible solutes was combinational outcome through increase in biosynthesis and decrease in degradation both in leaves and stems of *J. curcas* seedlings.

Discussion

Though chilling-sensitive plants such as maize, rice, tomato and *J. curcas* are sensitive to low non-freezing moderate temperature stress (chilling stress), and largely lack cold acclimation ability (Ruelland et al. 2009; Survila et al. 2010), their resistance to subsequent severe chilling stress can be improved after they were exposed to moderate chilling temperature for several days or weeks, which is known as chill hardening (Levitt 1980; Prasad et al. 1994). In maize seedlings, chill hardening at 14 °C for 3 days could improve survival percentage under chilling stress at 4 °C for 7 days (Prasad et al. 1994). Average shelf life in sweet basil at 5 °C could increase after they exposed to 10 °C for 4 h daily for 2 days (Lange and Cameron 1997). More interestingly, short-term cold shock at 1 °C for 4 h followed by a 6-h recovery at 26.5 °C also could mitigate increase in electrolyte leakage of primary roots and decrease in vitality of coleoptiles under chilling at 1 °C for 5 days, which, in turn, improved survival percentage of maize seedlings (Li et al. 2011). In *J. curcas* seedlings, our previous work also showed that chill hardening greatly lowered death rate and alleviated electrolyte leakage as well as accumulation of the lipid peroxidation product, malondialdehyde (MDA), of seedlings under severe chilling stress at 1 °C for 1–7 days, indicating that the chill hardening significantly improved chilling tolerance of *J. curcas* seedlings (Ao et al. 2013). These data suggested that chill hardening and shock could improve chilling tolerance of plants, but their mechanisms still remains elusive.

As mentioned above, osmotic stress result from chilling stress is one of the major causes of chilling injury (Ruelland et al. 2009; Leipner and Stamp 2009; Heidarvand and Amiri 2010; Catala et al. 2012; Theocharis et al. 2012). In the present study, *J. curcas* seedlings started wilting (data not shown) due to the loss of turgor pressure after 3 days of chilling stress at 1 °C (Fig. 1c), indicating that chilling stress resulted in osmotic stress. Wilkinson et al. (2001) found that stomata opening under chilling stress is the primary cause of loss of turgor pressure in chilling-sensitive plants, which triggered osmotic stress. To cope with osmotic stress caused by low temperature stress, higher plants have developed a very important osmotic adjustment mechanism, in other words, accumulation of compatible solutes, namely osmolytes such as proline (Pro), betaine (BT) and soluble sugars (Leipner and Stamp 2009; Heidarvand and Amiri 2010; Catala et al. 2012; Theocharis et al. 2012; Fig. 2), which lowered osmotic and water potentials, as well as maintained higher pressure potential (Fig. 1).

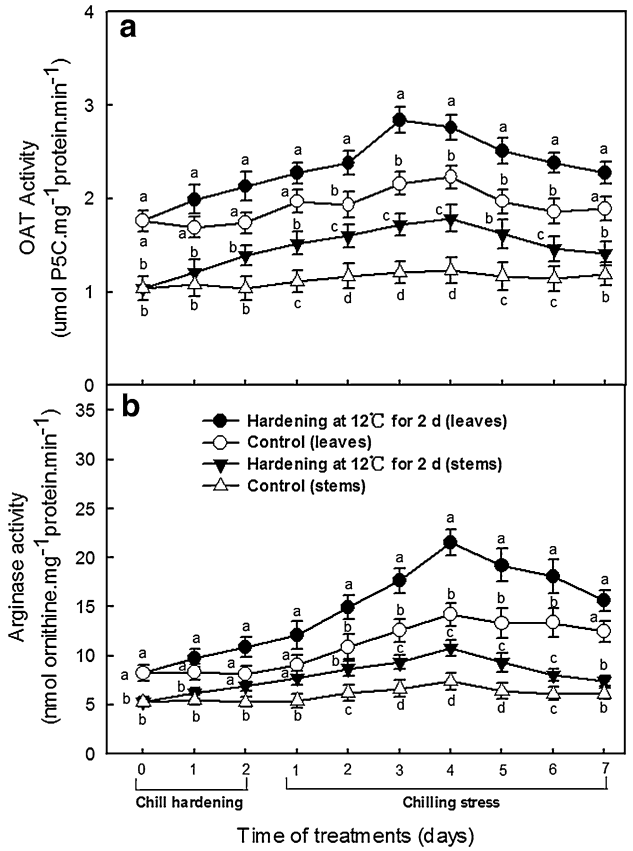

Fig. 4 Effect of chill hardening and chilling stress on activities of OAT (**a**) and arginase (**b**) both in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Activities of enzymes OAT and arginase both in leaves and stems of the seedlings were assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

The rice seedlings with *OsMYB2*-overexpression significantly accumulated proline, followed by increase in resistance to chilling stress at 2 °C for 3 days (Yang et al. 2012). In maize suspension cultures, Songstad et al. (1990) found that cells exposed to 4 °C for 4 weeks inhibited its 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 exhibited symptoms of chlorosis when exposed to a low temperature in the light (Hayashi et al. 1997). Similarly, the transgenic sweet potato with overexpression of BADH gene from *Spinacia oleracea* increased BADH activity as well as accumulation of GB and Pro, followed by enhanced tolerance to chilling stress (Fan et al. 2012). In tobacco seedlings, Cui et al. (2012) found that total soluble sugars increased under chilling

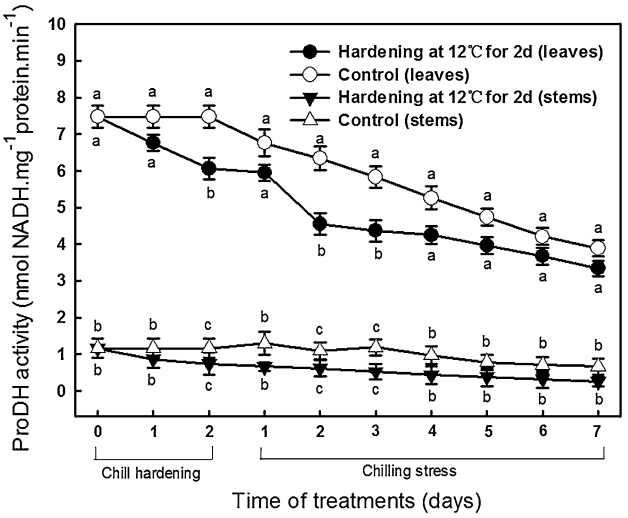

Fig. 5 Effect of chill hardening and chilling stress on activity of ProDH both in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Activities of ProDH both in leaves or stems of the seedlings were assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and each data in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

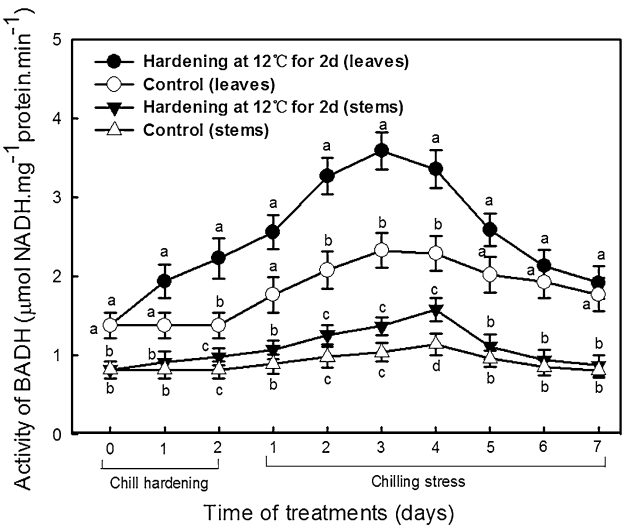

Fig. 6 Effect of chill hardening and chilling stress on activity of BADH in leaves and stems of *J. curcas* seedlings. 2-week-old seedlings of *J. curcas* were subjected to chill hardening at 12 °C for 2 days, and then were exposed to chilling stress at 1 °C for 1–7 days. Activity of the BADH both in leaves and stems of the seedlings was assayed daily during the chill hardening and chilling stress. *Error bars* represent standard error and *each data* in the figures represents the mean ± SE of at least three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Duncan's multiple test

stress and its content in chilling tolerant variety was higher than that of chilling sensitive variety. Again, during the process of chill hardening at 5 °C, soluble sugar contents in cabbage seedlings increased markedly, suggesting that this increase could be related to the acquisition of chilling tolerance (Sasaki et al. 1996). In the present work, the chill hardening improved the activities of GDH, P5CS, OAT, arginase and BADH both in leaves and stems of seedlings (Figs. 3, 4, 6), and lowered the ProDH activity (Fig. 5), which, in turn, induced accumulation of compatible solutes proline, betaine and total soluble sugars via increasing their biosynthesis and decreasing their degradation (Fig. 2), followed by reduced osmotic and water potentials (Fig. 1a, b), ultimately relative higher pressure potential that was maintained in leaves of *J. curcas* seedlings (Fig. 1c). A number of researches found that compatible solutes not only have osmotic adjustment function, that is, helping the cells to maintain their hydrated state and turgor pressure, but also exert very important role in stabilization of proteins, enzymes and biomembrane (interacting with the lipid bilayer); scavenging of reactive oxygen species; redox buffering as well as induction of stress proteins (Ashrak and Foolad 2007; Chen and Murata 2008; Ruelland et al. 2009; Szabados and Savoure 2010; Xie et al. 2011), followed by increase in resistance of plants to chilling stress.

In summary, 2-week-old seedlings of *J. curcas* subjected to chill hardening lowered osmotic and water potentials in leaves, followed by maintained relative higher pressure potential in leaves of *J. curcas* seedlings during the chilling stress. In addition, chill hardening also induced accumulation of compatible solutes proline, betaine and soluble sugars both in leaves and stems of *J. curcas* seedlings via increase in biosynthesis and decrease in degradation, suggesting that compatible solutes play a very important role in chill hardening-induced chilling tolerance of *J. curcas* seedlings.

Author contribution In this study, Zhong-Guang Li carried out conception and writing the article, Ping-Xing Ao and Ming Gong carried out conception, design and coordinated the study. 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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