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Physiological and Metabolic Responses of Jatropha to Chilling Stress

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Abstract

Chilling-susceptibility is the bottleneck for cultivation and commercialization of *Jatropha curcas* L., a novel promising biodiesel tree. To explore its network reactions to cold stress and pick out the most valuable approach for germplasm improvement, four pop systems, consisting of reactive oxygen species (ROS) scavenging enzymes, photosystem, glycol-metabolism and phenylpropanoid metabolism, and typical chilling-induced genes/transcription factors (TFs) were detected in seedlings exposed to 4℃. The results revealed that *J. curcas* has high cold-sensitivity might result from its seriously hysteretic responses (at least 2-3 h delay) to chilling damages. Among its molecular web of cold response, *CBF* TFs reacted most dramatically, up to 44-118 folds above the control. High chilling-responsiveness indicated *CBF*s should be candidates of gene engineering for improvement of chilling tolerance in *J. curcas*. Results also revealed that glycolysis, tricarboxylic acid and hexose monophosphate pathways might generate more ATP, NADH/NADPH for photosystem to maintain the cyclic electron flow. Besides the ROS scavenging system, flavonoids from phenylpropanoid pathway could also protect membrane lipid in cold stress. The studies provided a foundational knowledge of net response of *J. curcas* to chilling stress, and inspired that predictable and rapid response of *CBF* regulon is the direction for improvement of cold tolerance. © 2013 Friends Science Publishers

Keywords: *J. curcas*; Chilling stress; Net response; Photosystem; Transportation factors; Phenylpropanoid metabolism

Introduction

Chilling injury is a major abiotic factor limiting the production, harvest quality and geographical distribution of crops (Stewart and Guinn, 1971; Farooq *et al*., 2009). Chilling mechanism was studied for many aspects, originated from water regime (Zholkevich, 1955) and metabolic changes (Goss *et al.*, 1984), then transferred to membrane phase transitions (Stewart and Guinn, 1971; Moon *et al*., 1995), excessive reactive oxygen species (ROS, like H_2O_2, $^{\bullet}OH$) (McKersie *et al.*, 1993), photo-oxidative damage and photoinhibition (Leipner *et al.*, 2000; Rossini *et al.*, 2006). In summary, several classifiable systems are damaged miserably by cold, and their activity reactions are vital chilling response for plants: ROS scavenging enzymes, photosystem, sugar metabolism and second metabolism (especially phenylpropanoid pathway). Recently, researchers are keen on a group of chilling/dehydration-responsive transcription factors (TFs), e.g., *CBF* and *MYB*, which were involved in cold regulated (COR) genes and stress signal transduction pathways in rice (Yun *et al.*, 2010), *Arabidopsis* (Stockinger *et al.*, 1997) and other plant species.

Based on the above, the concept of net responses to low temperature, in physiological, cellular metabolic and other levels, was putting forward gradually (Weiser, 1970; Korenjak *et al.*, 2004). However, two questions are facing the network response theory: (1) besides cooperation between plant multi-systems, is there the most important one responding to chilling damage, or which system acts most intensely under cold stress ? (2) For genetic breeding or engineering, it is obviously not realistic to operate the object plants together from the above five angles. And it will be handy to screening out a succinct and practical entry point for improvement of chilling tolerance.

Jatropha curcas L. is a perennial, deciduous shrub tree belonging to the Euphorbiaceae family, with the greatest reputation mainly from its biofuel potential (Tang *et al.*, 2011). As a tropical and subtropical plant, *J. curcas* is sensitive to cold stress, could not grow in low temperature or at higher altitude regions. Recent years, chilling-susceptibility is becoming the bottleneck for Jatropha cultivation. Earlier studies showed that the early-stage (0-12 h) acclimation of PS Π and the late-stage (after 24 h) H_2O_2 scavenging might be involved in response mechanisms of Jatropha under cold stress (Liang *et al.*, 2007). Beyond that, few literatures are reported in details on its chilling injury, except for a TF, JcDREB (Tang *et al.*, 2007; 2011). And little attention was paid to distinguish the main point from a large chilling response network. Hence, as investors argued, basic research on *J. curcas* was needed urgently (Sanderson, 2009).

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The objective of this study is to understand the chilling response network for *J. curcas* in physiological and metabolic levels. Through normal experiments, a most valuable point is to pick out among ROS scavenging system, photosystem, glycometabolism genes, phenylpropanoid metabolism, typical chilling-induced genes and TFs. And the results might not only help to understand the cold tolerance and/or cold-sensitive mechanisms of *J. curcas*, but also be instructive for germplasm improvement of this plant species.

Materials and Methods

Plant Material and Cold Treatment Assay

J. curcas L. mature seeds were harvested from Panxi region (27°42' N, 101°57' E), Sichuan Province, China. Young seedlings were cultivated in greenhouse conditions with a 16 h light photoperiod (24000 Lx) and irrigated daily with a half-strength Hoagland solution as described by Devlin (Devlin, 1966). Three-leaf-old seedlings were transferred to a cold chamber at 4℃ with all day light condition. Samples (roots, stems and leaves) were harvested 0, 1, 2, 3, 5, 8, 12, 24 and 48 h after cold treatment, respectively frozen in liquid nitrogen and/or stored at -80℃ or directly used by following analysis.

Assay of Physiological Parameters

Physiological parameters were analyzed by spectrophotometric methods as previously described. Fresh or cold treated leaves were ground with polyvinylpyrrolidone K30 in buffer. Subsequently, the supernatant followed from centrifugation was for physiological analysis. Malondialdehyde (MDA) contents, activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were assayed using the method of (Pinhero *et al.*, 1997). Soluble sugar contents were tested by the method of Cole *et al.* (1966).

Gene Expression Property

Key enzyme genes involved in basic metabolism and phenylpropanoid pathway (a crucial secondary metabolism for stress responses) were searched in Jatropha Genome Database (http://www.kazusa.or.jp/jatropha/). Genes selected are ones participating in basal metabolism pathways (Supplemental Table 1, 2) and ROS scavenging system (Supplemental Table 3). Some typical chilling-induced genes (*MAT1*, *LEA2*, *LEA4*, *USP7*, *COR413* and *KIN7*) and TFs (*MYB1*, *CBF1* and *CBF2*) related to low temperature ere also screened out from the genome database (Supplemental Table 4). The total RNA isolation and quantification RT-PCR were experimented with method as described by (Gao *et al.*, 2012). Quantification was determined with $\Delta\Delta C_T$ method.

Analysis of Flavonoids and Chlorophylls Content in Leaves

Fresh and cold treated leaves were ground with polyvinylpyrrolidone K30 in 70% ethanol, and the supernatant after centrifugation was loaded in a chromatographic column filled by SBC MCI GEL (F style, 70-150 μm; Kepubio, China). The filtrate was used for total flavonoid analysis with spectrophotometric method by absorptions at 510 nm, apigenin used for standard curve. Afterwards, chlorophylls were washed down from chromatograph column by 100% ethanol, then quantified by the absorption at 665 nm (for chlorophyll A) and 649 nm (for chlorophyll B) (Merzlyak *et al.*, 2008).

Survey of Photosynthesis Rates

The photosynthesis rates *in vivo* of detached leaves were detected with a S - 110 Portable Photosynthesis Meter (SINTEK, China). Typical parameters used were: closed loop style; volume 1; blade area 1; 4℃; 24000 Lx. And the photosynthesis rate P_n (μmol CO_2 m^{-2} S^{-1}) was calculated as followed:

$$P_n = - (V/\Delta t) \bullet (273.15/T_a) \bullet (P/1.013) \bullet (1/22.41) \bullet (10000/A) \bullet (C_o - C_i).$$

Among the formula, V, Δt, T_a, P, A, $C_o$and C_i were respectively chamber volume, interval time, air temperature, atmospheric pressure, blade area, terminal and initial concentration of CO_2.

Data Analysis

All chilling treatments of *J. curcas* seedlings were arranged in completely randomized design with five replicates. Detection tests for physiological parameters, qRT-PCR, content of leaf extracts and photosynthesis rates were carried for three times. Data reported here represent the average of three repeats with the ANOVA analysis.

Results

Chilling Injuries of *J. curcas* Leaves and Membrane System

Three-leaf-old *J. curcas* seedlings were exposed to 4℃ for 0 to 48 h. Visible crispate on blade edges emerged three hours later (Fig. 1A). Then the leaves became wilted during treatment. After 48 h, the chilled leaves were completely dried up (Fig. 1B). Accompanied with serious wilting phenomenon, leaves color changed from green to dark (Fig. 1C). Morphological observations of *J. curcas* seedlings cultivated in 4ºC indicated that severe growth inhibitions arose only 3 h after cold treatment (Fig. 1A).

Malondialdehyde (MDA) from degradation of membranes (Ibrahim and Jaafar, 2012; Schmid-Siegert *et al.*, 2012) is usually thought as an index of chilling injury. The present results suggested that MDA content of leaves significantly increased up to 3.2 times comparing with the

controls after 8 h chilling treatment (Fig. 1D). It suggests apparent membrane damages of *J. curcas* leaves. We further investigated mRNA levels of several genes involved in membrane lipid synthesis by qRT-PCR method. The acyltransferase (*AT*) gene displayed a bell-shaped expression pattern, with the peak expression of 5.3 folds higher than the original time after 12 h chilling stress. Nevertheless, The *AT* gene was low transcribed before 5 h (Fig. 1E). Other genes from lipid synthesis pathways (e.g., glycerol-3-phosphate transporter, *G3P*; phosphlipase D, *PLD*) were limitedly affected by cold stress (Fig. 1F and G).

Responses of ROS Scavenging System in *J. curcas*to Cold Stress

To analyze roles of ROS scavenging system of *J. curcas* during chilling stress, related enzymes' activities were measured during 0-48 h (Fig. 2). The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) all showed a M type trend, with a small activity peak at 2, 1 and 3 h, respectively together rising to maximum at 8 h under chilling stress. The highest enzyme activity of POD (8 h point) was 4.0 folds higher than its initial activity. Maximum activities of SOD and CAT were about 2 times higher than their initial time, respectively. In addition, transcripts of the three enzyme genes were affected in the similar trends and with the same intensity (2.3-2.4 folds higher than controls after 8 h of stress) as their protein activity.

Chilling Effects on Photosynthesis Systems

To achieve the effects of chilling on photosynthesis of *J. curcas*, leaf CO_2 concentrations were detected to calculate photosynthesis rate P_n. As showed in Fig. 3, following a period of low abundance, P_n rose 2.1 times higher than control samples in hour 8-12 at 4℃ stress. The photosynthesis rate was declined to about 20% less compared to the control group (Fig. 3A1). Relative to results of (Liang *et al.*, 2007), due to chilling stress, chlorophyll A and B contents of *J. curcas* leaves were all dropped by more than 30% compared to their initial state respectively (Table 1).

Further studies were ongoing to identify

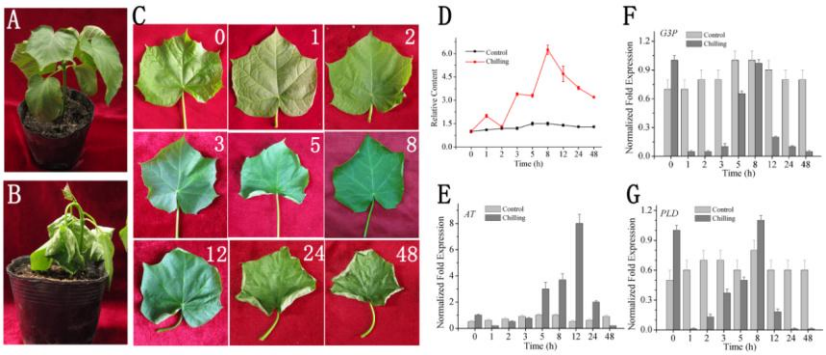

Fig. 1: Chilling damage to leaves and membrane system response of *J. curcas* seedlings
(A) Whole seedlings after 3 hour treatment. (B) Whole seedlings after 48 hour chilling. (C) Leaf damages. Arabic numeral in top-right corners was the chilling time experienced by each whole seedling. Leaves were the second ones from seedling top. (D) Contents of MDA and soluble sugars in chilled leaves of *J. curcas*. (E-G) Transcription profiles of lipid synthesis genes by qRT-PCR. Values represent the means ± SE of three replicates, not significantly different ($p<0.05$) and gene information about their identifier and primers were prepared in Supplemental Table 1 and 2

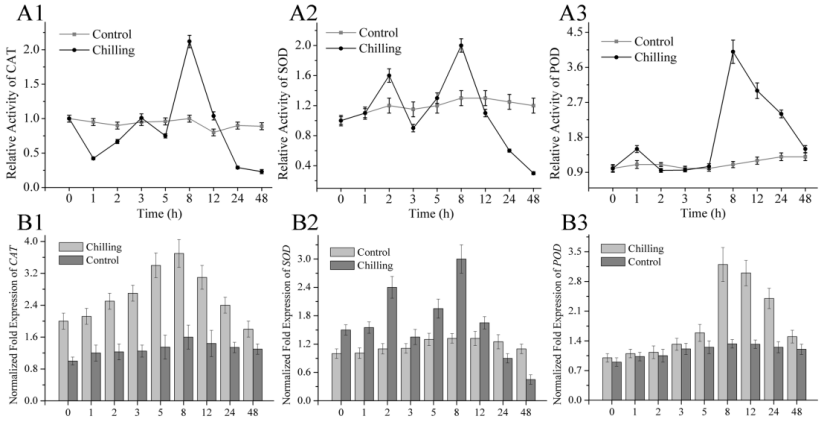

Fig. 2: Activities of ROS scavenge enzymes (A) and genes (B) of *J. curcas* leaves in time-gradient chilling stress
Values represent the means ± SE of three replicates, not significantly different ($p<0.05$) and gene information about their identifier and primers were prepared in Supplemental Table 3

Table 1: Content changes of chlorophylls in chilled leaves of *J. curcas*

Content of chlorophylls (μg chlorophyll /g FW)			Control	Chilling
	Chlorophyll *a*	0h	343.33 ± 25.44	408.94 ± 33.61
		24h	327.15 ± 30.12	284.23 ± 28.51
	Chlorophyll *b*	0h	442.17 ± 36.62	561.43 ± 40.59
		24h	190.19 ± 12.59	366.12 ± 22.17

The third leaf was detected by spectrophotometric method at 665 nm (for chlorophyll A) and 649 nm (for chlorophyll B). Values represent the means ± SE of three replicates, not significantly different ($p<0.05$)

Table 2: Contents of total flavonoids in *J. curcas* leaves exposed to cold temperature

Content of flavonoids (μg Flavonoid /g FW)		Control	Chilling
	0h	140.59 ± 15.1	391.13 ± 25.6
	8h	166.89 ±17.3	343.30 ±24.7
	24h	223.15 ± 23.2	272.75 ± 21.2

The third leaf was detected by spectrophotometric method at 510 nm. Values represent the means ± SE of three replicates, not significantly different ($p<0.05$)

Table 3: Some *CBF* target genes involved in four classifiable systems in *A. thaliana*

Gene Index	References	Function
Sugar metabolism		
At4g33070	1, 2	Pyruvate decarboxylase
At1g10760	1, 3	SEX1 (STARCH EXCESS 1)
At5g20830	1	Sucrose synthase SUS1
At2g24560	1, 3	Carboxylic ester hydrolase
Photosystem		
At5g57110	1	Calcium-transporting ATPase
At1g54410	2	Dehydrin
At2g34850	1	NAD-dependent epimerases
ROS scavenging system		
At3g55610	1, 3	Pyrroline-5-carboxylate synthetase (P5CS2)
At1g62570	1, 3	Disulfide oxidoreductase
At1g08570	1	Thioredoxin
Phenylpropanoid metabolism		
At4g34230	1	Cinnamyl alcohol dehydrogenase 5
At3g57020	1	Strictosidine synthase

1: Vogel *et al.* (2005); 2: Maruyama *et al.* (2004); 3: Gilmour *et al.* (2004)

transcriptional activity of some photosystem genes. And the transcription of ribulose-1,5 bisphosphate carboxylase (*RUBPC*) from Calvin cycle was constantly increased from 0 to 24 h and up-regulated more than 9.5 folds after 24 h chilling (Fig. 3A2). A rieske protein gene (*Rieske*), which bridges phtosyetem I (PS I) and Π (PSΠ), was transcriptionally upregulated by 2.5 folds after 8 h cold acclimation (Fig. 3A3).

Glycometabolism Responses to Low Temperature

It is suggested that plants resistant to environmental stresses by osmotic regulation through accumulating small molecules such as soluble sugars. The content of total reduced sugars was measured in *J. curcas* seedlings under cold treatment. The results suggested that it was gradually increased along with chilling time, in accordance with expectation (Fig. 3B1).

To analyze chilling impacts on glycometabolism of *J. curcas*, plenty of related genes were tested by qRT-PCR method (Supplemental Table 1). After 0-48 h chilling stress, hexokinase (*HK*) gene displayed a M expression model, phosphofructokinase (*PFK*) and citrate synthase (*CS*) a changing V type, with maximum transcription level at 12 or 24 h (Fig. 3B2-4). Amylase, cellulase, and isocitrate dehydrogenase (*iCD*) genes showed bell expression shapes under cold temperature treatment with expression peak at 8 hour (Fig. 3B5, 8 and 9). The expression of glucose-6-phosphate dehydrogenase (*GPD*) gene also showed a bell shape,up to tiptop at 24 h (Fig. 43B6). All the tested genes were regulated differently by cold temperature at their expression peak time. Among these genes, *HK* and *PFK* (from glycolysis) were up-regulated 30.2 and 10.5 folds respectively by chilling than its original state (hour 0). Chilling stress also induced tricarboxylic acid pathway genes (e.g. *CS* and *iCD*) transcription by 4-5 folds. Transcripts of genes participated in degradation of polysaccharides (amylase, cellulase) and hexose monophosphate path (HMS, *GPD*) were raised 2-3 folds higher than their control conditions.

Some glycometabolism shunt pathways (e.g. isocitratelyase, *iCL*, of glyoxylate pathway) and glyconeogenesis genes (like phosphorylase and sucrose

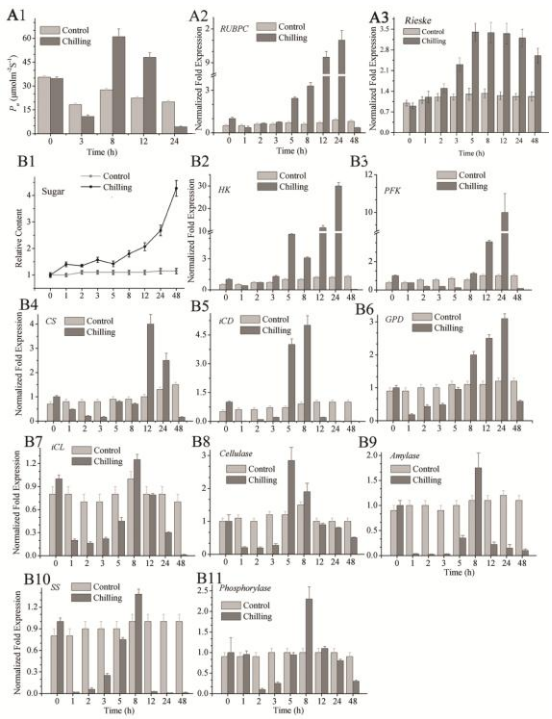

Fig. 3: Effect of cold stress to photosynthesis systems (A) and glycometabolism (B) of *J. curcas*

(A1) The photosynthesis rate P_n ($\mu molCO_2 \bullet m^{-2} \bullet S^{-1}$) and the means ± SE of three repetitive plants were calculated by S -110 Portable Photosynthesis Meter. (A2-A3) Response properties of ribulose-1, 5 bisphosphate carboxylase (*RUBPC*) and *Rieske*genes to chilling stress. (B2-B11) Transcription profiles of genes participated in metabolism pathways of glycolysis. Values represent the means ± SE of three replicates, not significantly different ($p<0.05$) and gene information about their identifier and primers were prepared in Supplemental Table 1 and 2

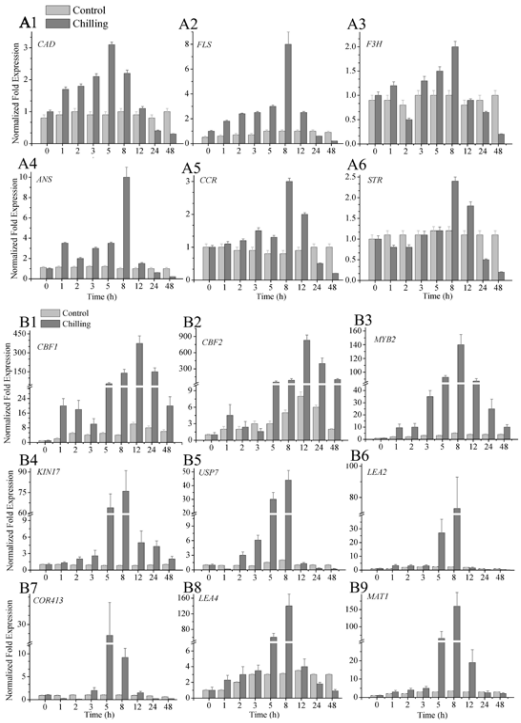

Fig. 4: Expression characters of the phenylpropanoid metabolism (A) *MYB*s, *CBF*s and *COR* (B) genes

Values represent the means ± SE of three replicates, not significantly different ($p<0.05$) and gene information about their identifier and primers were prepared in Supplemental Table 4

synthase, *SS*) were limitedly affected by cold stress (Fig. 3B7, J and K).

Phenylpropanoid Pathway Responses to Chilling Injury

Phenylpropanoid pathway is the typical secondary metabolism, and its products play important roles in responses to cold stresses. Along with trends toward to different products in this pathway, lignins, flavonols, anthocyanins and alkaloids are four types of end products (Supplemental Fig. S1), with key switch genes cinnamoyl CoA reductase (*CCR*), cinnamyl alcohol dehydrogenase (*CAD*), flavonol synthase (*FLS*), Anthocyanidin synthase (*ANS*) and strictosidine synthase (*STR*), respectively (Supplemental Table 2). In this survey, the qRT-PCR data displayed that expression profiles of all these genes were regulated by cold stress in a bell shape with the top transcription level at 5 or 8 h (Fig. 4). Compared with genes toward to lignins (*CCR*; *CAD*) and alkaloids (*STR*), the ones regulated flavonols (*FLS*) and anthocyanins (*ANS*) were significantly increased by about 10 folds higher after 5-8 hour cold treatment than their control states (Fig. 4A1, 4).

Given that flavonols and anthocyanins were classified into flavonoids, to further explore whether the content of end products rose following the expression increase of *ANS* and *FLS*, total flavonoids were extracted from chilled *J. curcas* leaves and measured by spectrophotometric absorbance at 510 nm. It was beyond thought that the total flavonoids after cold stress were decreased by about 13% and 50% after 8 and 24 h cold stress than their original state, respectively (Table 2). On the contrary, in the control state, the content of total flavonoids rose about 19% and 60% in the corresponding time point respectively.

Expression Profiles of TFs and COR Genes Related to Chilling Tolerance

The *CBF*- and *MYB*-like transcription factors had been shown to play predominant roles in freezing tolerance and cold acclimation, by activating downstream genes related to cold responses in plants (Su *et al.*, 2010). Results indicated that the tip transcripts of *MYB2*, *CBF1/2* were dramatically raised by respectively 27, 44 and 118 folds compared to the control after 4℃ treatment. However, it took 8 h for *MYB2* up to highest change in respond to cold condition, and 12 hours for *CBF1* and *CBF2* (Fig. 4B1-3). Moreover, expression levels of cold-regulated genes like *KIN17*, *LEA2/4*, *USP7*, *MAT1* and *COR413* were highlighted up 27-143 times than those before cold processing (Fig. 4B4-9).

But these genes' expression peaks emerged earlier than *MYB2*, *CBF1/2*. Overall, these data implied that compared to basal and secondary metabolism genes, these genes were more sensitive to cold in *J. curcas*.

Discussion

To date, about four classifiable systems of plants are documented well in respond to cold i.e., ROS scavenging enzymes, photosystem, sugar and phenylpropanoid metabolisms. However, molecular web studies demonstrate that CBF pathway may be major components of cold hardening for plants. As TFs, although *CBF*s being different from the above four systems in classification criteria, they are still important directions to improve cold tolerance of agricultural species for practitioners. It is a delightful founding that in *J. curcas* the TF family *CBF*/*MYB* and some *COR* genes respond 27-143 times to cold stress, much more dramatic than not only the four classifiable systems in *J. curcas* (Fig. 3B and Fig. 4). So these *CBF*/*MYB*-like TFs with their downstream genes might play more particular roles in *J. curcas*'s chilling reaction, and researches about them may be a succinct and practical entry point for *J. curcas*'s chilling tolerance.

It deserves note that *MYB2* (27 folds) was lower induced than *CBF1* (44 folds) and *CBF2* (118 folds) in *J. curcas* exposed to cold, indicating that *MYB2* was relatively less associated with chilling stress than *CBF*s. And references have proved *MYB*s could be activated by other stress such as salt, drought, high temperature and light (Su *et al.*, 2010). Interesting, the fact that *MYB32* in *A. thaliana* could also be regulated by *CBF*s tell more importance of *CBF*s than *MYB*s in cold acclimation (Vogel *et al.*, 2005). Similarly, many *COR* genes, which are possessing cold responsive sequences in their promoter the CRT/DRE motif, are also regulated by *CBF*s. By binding to those motifs, *CBF*s recognize and induce (or restrain) transcriptions of *COR* genes. In addition, the binding of *CBF*s to CRT/DRE elements be low temperature-dependent and specific (Xue, 2003). All in all, *CBF*s seem more dominant than *MYB*s and *COR* genes in cold hardening.

As regulons, *CBF*s are more like messengers of cold signal at upstream of various response systems. Their importance can be understood by analyzing genes that are CBF targets. Researches on *A. thaliana* demonstrate that many genes (from the above four systems) are in part dependent on the CBF pathway (Table 3). Thus, it is surmised that the level of CBF proteins might determine the cold response. It also has been demonstrated by many overexpression tests and promoters analysis.

Cold temperatures could lead to oxidative stress, producing elevated level of O_2^- and other ROS, stimulating lipid peroxidation and degradation of cell structures (Xiong *et al.*, 2002). Chilling-tolerant plants (like *Spinaciaoleracea*) respond to stress as soon as cold signals were captured, due to the chilling-adaptive ROS scavenge enzymes (McKersie *et al.*, 1993), function-specific lipid synthesis system (Murata and Yamaya, 1984) and particular cytoskeleton proteins (Ouellet *et al.*, 2001). However, *J. curcas* seedlings' reply displayed long lag time before chilling injury. For instance, the major antioxidant enzymes (SOD, POD and CAT), without exception, were delayed about 2-3 h to fulfill their activity only after chilling damage to leaves had been initiated (Fig. 2). And the hysteretic responses were same to the performances of all tested metabolisms and COR genes in this study (Fig. 3-6). On the other hand, the response intensity of *J. curcas* to chilling stress was not less than chilling-tolerant plants. Like the condition in *Nicotiana* (Bowler *et al.*, 1989), the activity of SOD and CAT rose about 2 folds higher than the initial states in Jatropha exposed to 4℃ stress. And the response intensity of *CBF/MYB* and *COR* genes were even above the situations in *Arabidopsis* (Gilmour *et al.*, 2000). Thus, as a tropical tree, *J. curcas*is indeed a cold sensitive plant species, and the low chilling tolerance might result from its serious response hysteresis in physiological and metabolic levels.

Three ATP or reducing ability-generating metabolisms were heightened more than other basal metabolisms in *J. curcas* exposed to chilling stress (Fig. 3B). Glycolysis is a common metabolism, in which glucoses are degraded into pyruvates and ATP (Kosower, 1962). HMS and TCA pathways generate NADPH or reduced NADH/FADH2 respectively, the latter two are re-oxidized into ATP through the electron transport chain and oxidative phosphorylation of PS I (Campbell, 1999). The inference was further validated by gas chromatography-mass (GC-MS), in which more core aglucones of NAD^+, nicotinamide, were detected in 12 h chilled *J. curcas* leaves (data not shown). In *Khaya ivorensis* under temporal chilling, to protect its photosystem, the ratio of effective quantum yield of cyclic electron flow to effective quantum yield of photosystem Π [Y(CEF)/Y(Π)] significantly increased through NADPH and ATP (Huang *et al.*, 2011). Similarly, it was concluded that *J. curcas* might produce more energy to contend against chilling damage. As the physiological mechanism of energy germinating and election flow remains unclear, more studies were to undergo.

Many middle and end products of phenylpropanoid pathway such as lignins and flavonoids were involved in chilling responses (Merzlyak *et al.*, 2008). However, in *J. curcas*, less research was reported. Our previous studies have indicated an upstream gene, *JcPAL1*, was up-regulated responding to 4ºC treatment (Gao *et al.*, 2012). It was yet a mystery which concrete branch of four trends played the special role in cold responses (Supplemental Fig. S1). Results of this paper showed highly enhanced transcripts of *FLS* and *ANS* (Fig. 4A2 and 4), however with not increase of their end product, revealing that mass flavonoids were consumed in leaves exposed in 4 ºC (Table 2). Flavonoids, often esterified, could interact with other components to function distinguishingly in various systems of plants. By quenching active oxygen species, they inhibit oxidations of

plant oils and lipids such as unsaturated arachidonic acid (AA), which could keep flowability of cell membranes and act as signals under cold stress. Flavonoids also protect AA by inactivating the lipoxygenase, which catalyzes oxo processes of AA. So was in *J. curcas*. In addition, the transcriptional decline (41%) of lipoxygenase was in line with positive roles of flavonoids in chilled *J. curcas* leaves (Supplemental Fig. S2). Therefore, flavonoids of *J. curcas* might participate in modulation of terpenoid metabolism to respond to chilling stress. It deserves further research to find direct relationship between flavonoids and membrane lipids.

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