Responses of *Jatropha curcas* seedlings to cold stress: photosynthesis-related proteins and chlorophyll fluorescence characteristics

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Photosynthesis-related proteins and PSII functions of *Jatropha curcas* seedlings under cold stress were studied using proteomic and chlorophyll fluorescence approaches. The results of chlorophyll fluorescence measurement indicated that electron transport flux per reaction center (ETo/RC) and performance index (PIABS) were relatively sensitive to low temperature, especially at early stage of cold stress. The increase in O–J phase and decrease in J–I phase of chlorophyll fluorescence transient indicated a protection mechanism of *J. curcas* to photoinhibition at early stage of cold stress. Eight photosynthesis-related proteins significantly changed during cold stress were identified using liquid chromatography MS/MS. Results of correlation analyses between photosynthesis-related proteins and chlorophyll fluorescence parameters indicated that (1) ATP synthase and Rieske FeS protein were significantly correlated with electron transport of reaction center in PSII; (2) precursor for 33-kDa protein was positively correlated with fluorescence quenching of the O–J and J–I phases and PIABS during cold stress, which implies that it might be related to multiple processes in PSII; (3) contrary correlations were found between FJ and two enzymes in the Calvin cycle, and the relations between these proteins and PSII function were unclear. The combined study using proteomic approaches and chlorophyll fluorescence measurements indicated that the early-stage (0–12 h) acclimation of PSII and the late-stage (after 24 h) H2O2 scavenging might be involved in the cold response mechanisms of *J. curcas* seedlings.

Introduction

Low temperature is one of the limiting factors adversely affecting the growth and development of plants, especially tropical or subtropical plant species. It has been recognized that the mechanisms of coping with low-temperature stress are complex and multigenic (Hughes and Dunn 1996, Thomashow 1998). Of many physiological processes, photosynthesis is usually sensitive to cold stress, which is a main reason for the reduction or cessation of growth and productivity decrease of plants under low temperature. Plant species have developed different strategies of photosynthesis in response to cold temperatures (Öquist and Huner 2003, Savitch et al. 2002). Some herbaceous annuals could grow during autumn months by enhancing photosynthetic carbon metabolism and changing respiratory metabolism.
(Ensminger et al. 2006). Evergreen tree species have downregulated photosynthesis and inactivation of PSII reaction centers (RCs) and re-organization of light-harvesting complexes may be involved (Ensminger et al. 2006, Ottander et al. 1995, Savitch et al. 2002). It is suggested that during cold stress, an imbalance appears between the source of energy and the metabolic sink and photosynthesis could act as a sensor of this imbalance through the redox state of photosynthetic electron transport components, regulating photophysical, photochemical and metabolic processes of plants (Ensminger et al. 2006, Huner et al. 1996).

Proteome can be defined as the full complement of proteins expressed by the genome of a cell, a tissue or an organism at a specific time point (Aebersold and Goodlett 2001, Renaut et al. 2006). As a powerful tool to study the complex response of plants to environmental stimuli, proteomics is believed helpful to better understand the process of cold tolerance and develop strategies to improve resistance (Renaut et al. 2004, 2006). Using proteomic approaches, changes of proteins within specific function catalog, such as photosynthesis, metabolisms and defenses, could be studied during cold stress.

_Jatropha curcas_ L., deciduous tree species, is a drought-resistant oil species widely distributing in tropical and subtropical areas, especially in Central and South America, Africa, India and Southeast Asia (Schmook and Serralta-Peraza 1997). Different parts of _J. curcas_ have been used for various purposes such as animal feeding, medicine producing and ecosystem restoration of disturbed areas (Heller 1996, Openshaw 2000, Tang et al. 2007). Most importantly, the seeds of _J. curcas_ contain viscous oil that can be used for soap making in the cosmetics industry and as a diesel/kerosene substitute or extender (Openshaw 2000). While _J. curcas_ could withstand slight frost, it is relatively sensitive to lower temperatures (Heller 1996, Luo et al. 2006, Wan et al. 2006). The damage of low temperature may probably cause a sharp decrease in chlorophyll contents and membrane unsaturated fatty acids of _J. curcas_ and even survival of seedlings (Luo et al. 2006, Wan et al. 2006). Studies on photosynthesis responses of _J. curcas_ are necessary for better utilization of this plant species and understanding the response mechanisms of cold-sensitive tree species as exposed to low temperature.

Proteomic changes of _J. curcas_ seedlings were studied and chlorophyll fluorescence transient of leaves was determined in the present study, and our objective is to elucidate the response processes of _J. curcas_ exposed to low temperature. The combination of proteomic approaches and chlorophyll fluorescence transient determination allows finding correlations between protein changes and photosystem functions.

### Materials and methods

#### Plant material and cold treatment

Seeds of _Jatropha curcas_ L. were surface-sterilized using 70% ethanol and 0.1% HgCl₂. Embryos were taken carefully from the seeds and placed on MS medium (Murashige and Skoog 1962) supplemented with 0.6% (w/v) agar. One-week seedlings were then planted in a mixed soil of peat and vermiculite (1:1) in pots and grown at 28°C with a 14-h light (150 μmol photons m⁻² s⁻¹)/10-h dark photoperiod for 5 weeks. Six-week seedlings, which were about 10 cm in height with two fully expanded leaves, were subjected to cold stress. Cold treatment was performed in the growth chamber at 4°C with previous light intensity and photoperiod.

Fresh leaves were taken at 0, 6, 12, 24 and 48 h during cold stress for leaf water content (LWC) measurements and protein extraction. LWC was calculated as follows: LWC (%) = 100 (FW − DW)/FW, where FW and DW were fresh weight and dry weight, respectively, of leaves. Fresh leaves were frozen in liquid nitrogen and kept at −80°C before protein extraction.

#### Chlorophyll fluorescence measurements

Chlorophyll fluorescence transient, reflecting the primary reactions of photosynthesis (Krause and Weis 1991), is a useful tool to study the function of photosynthetic apparatus of plants. The characteristic changes of chlorophyll fluorescence (Kautsky transient) could provide information on photochemical activity of PSII and status of plastoquinone pool (Strauss et al. 2006).

The polyphasic chlorophyll fluorescence transients (OJIP) were measured at 0, 6, 12, 24 and 48 h during cold stress by a plant efficiency analyzer (Hansatech Instruments Ltd., King’s Lynn, Norfolk, UK) according to Strasser et al. (1995). The transients were induced by red light of about 3000 μmol photons m⁻² s⁻¹ provided by an array of six light emitting diodes (peak 650 nm). The fluorescence signals were recorded within a time span ranging from 10 μs to 1 s, with a data acquisition rate of 10² points per second for the first 2 ms and of 1000 points per second after 2 ms. Five values of each OJIP curves were recorded for further calculation of OJIP parameters, i.e. the fluorescence intensity at 50 μs, F₀, when all RCs are open; the maximum measured fluorescence intensity, F_m, when all RCs of PSII are closed and the fluorescence intensities at 300 μs (F₁), 2 ms (F₂) and 30 ms (F₃). Terms and formulas used in the chlorophyll fluorescence analysis are shown in Table 1, according to JIP test (Strasser et al. 2000, Strasser and Tsimilli-Michael 2001, Strauss et al. 2006).
Table 1. Terms and formulas used in the chlorophyll fluorescence analysis.

<table>
<thead>
<tr>
<th>Terms and formulas</th>
<th>Illustrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSII</td>
<td>Photosystem II</td>
</tr>
<tr>
<td>RC</td>
<td>PSII RC</td>
</tr>
<tr>
<td>(F_0)</td>
<td>Minimal recorded fluorescence intensity</td>
</tr>
<tr>
<td>(F_m)</td>
<td>Maximal recorded fluorescence intensity</td>
</tr>
<tr>
<td>(F_r, F_s, F_i)</td>
<td>The fluorescence intensity at K, J and I steps</td>
</tr>
<tr>
<td>(F_v = (F_m - F_o))</td>
<td>Variable fluorescence</td>
</tr>
<tr>
<td>(V_1 = (F_1 - F_o)(F_m - F_o))</td>
<td>Relative variable fluorescence intensity at the J step</td>
</tr>
<tr>
<td>(M_o = 4(F_k - F_o)(F_m - F_o))</td>
<td>Approximated initial slope of the fluorescence transient</td>
</tr>
<tr>
<td>(\phi_{po} = TR_o/ABS = [1 - (F_o/F_m)])</td>
<td>Maximum quantum yield for primary photochemistry</td>
</tr>
<tr>
<td>(\phi_o = ET_o/TR_o = (1 - V_o))</td>
<td>Probability that a trapped exciton moves an electron into the electron</td>
</tr>
<tr>
<td>(ABS/RC = (TR_o/RC)\phi_{po})</td>
<td>Absorption flux per RC</td>
</tr>
<tr>
<td>(TR_o/RC = M_o/V_1 = 4(F_k - F_o)(F_1 - F_o))</td>
<td>Trapped energy flux per RC at (t = 0)</td>
</tr>
<tr>
<td>(ET_o/RC = (TR_o/RC)\cdot \phi_o = (TR_o/RC)\cdot(1 - V_1))</td>
<td>Electron transport flux per RC at (t = 0)</td>
</tr>
<tr>
<td>(RC/CS_o = F_o \phi_{po} V_o/M_o)</td>
<td>Density of RCs (QA-reducing PSII RCs)</td>
</tr>
<tr>
<td>(P_o = (RC/ABS)\cdot\phi_{po}\cdot(1 - \phi_{po})/\phi_o\cdot(1 - \phi_o))</td>
<td>Pi on absorption basis</td>
</tr>
</tbody>
</table>

**Protein extraction**

Proteins were extracted using a modified protocol according to Shen et al. (2003). Five hundred milligrams of fresh leaves was ground into fine powder in liquid nitrogen with a precooled mortar and pestle and homogenized in 2 ml homogenization buffer containing 20 mM Tris–HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT and 1% Triton X-100. The homogenate was transferred into an Eppendorf tube and centrifuged at 15 000 g for 15 min at 4°C. The supernatant was transferred to a new tube and protein was deposited using 1/4 volume 50% cold TCA in an icy bath for 30 min. The mixture was centrifuged at 15 000 g for 15 min at 4°C, and the supernatant was discarded. The pellet was washed with acetone thrice, centrifuged and vacuum-dried. The dried powder was dissolved in sample buffer containing 9 M urea, 4% Nonidet P-40 (NP-40), 2% amphotline pH 3.5–10 (GE Healthcare Bio-Science, Little Chalfont, UK) and 5% 2-mercaptoethanol.

**Two-dimensional gel electrophoresis**

Two-dimensional electrophoresis was carried out according to Shen et al. (2003). The first dimensional IEF was performed in a 13-cm-long glass tube with 3 mm in diameter. The gel mixture contains 4% acrylamide, 8 M urea, 5% ampholine (one part pH 3.5–10, one part pH 5–8) and 2% NP-40. IEF was performed at 200V, 400V and 800V for 30 min, 15 h and 1 h, respectively. About 500 μg of proteins was loaded. After the first dimensional run, gels were incubated in equilibration buffer [0.05 M Tris–HCl pH 6.8, 2.5% SDS, 10% (v/v) glycerol and 5% 2-mercaptoethanol] for 15 min twice. The second dimensional electrophoresis was performed on vertical slab gels (135 × 170 × 1 mm), and a buffer system described in Laemmli (1970) was used to cast 5% stacking gel and 15% resolving gel. After electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue R-250.

**Image analysis**

The stained gels were scanned at 600 dots per inch resolution using a UMAX Power Look 2100XL scanner (Maxium Tech Inc., Taipei, China). The transparency mode was used to obtain a grayscale image. The image analysis was performed using ImageMaster™ 2D Platinum software (GE Healthcare Bio-Science). The optimized parameters were as follows: saliency was 2.0, partial threshold 4 and minimum area 50.

**Protein identification**

Protein spots were excised from the gels manually and cut into small pieces. Protein digestion was performed according to Shen et al. (2003), with slight modification. Each small gel piece with protein was washed with 25% (v/v) ethanol and 7% acetic acid for about 12 h or overnight at room temperature and destained with 50 mM NH₄HCO₃ in 50% (v/v) methanol for 1 h at 40°C. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH₄HCO₃ for 1 h at 60°C and incubated with 40 mM iodoacetamide in 100 mM NH₄HCO₃ for 30 min at room temperature. The gel pieces were minced and lyophilized and then rehydrated in 25 mM NH₄HCO₃ with 10 ng of sequencing grade modified trypsin (Promega, Madison, WI) overnight at 37°C. After digestion, the protein peptides were collected, and the gels were washed with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile three times to collect the remaining peptides. The peptides were desalted by
ZipTipC 18™ pipette tips (Millipore, Bedford, MA) and cocryssallized with one volume of saturated α-cyan-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile containing 1% TFA. The desalted protein samples were then subjected to liquid chromatography (LC) MS/MS.

**LC MS/MS**

LC was performed on a surveyor LC system (Thermo Finnigan, San Jose, CA). The C18 column was obtained from Column Technology Inc. (Fremont, CA). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. The trypic peptide mixtures were eluted using a gradient of 2–98% phase B over 180 min. The MS/MS was performed on an LTQ linear ion trap mass spectrometer (Thermo Finnigan) equipped with an electrospray interface and operated in positive ion mode. The capillary temperature was set to 170°C and the spray voltage was at 3.4 kV. The acquired MS/MS spectra were compared against the NCBI Basiidiomycota protein database using the TURBOSEQUENT program in the BioWORKS 3.1 software suite (Thermo Finnigan). An accepted SEQUEST result had to have an eCn score of at least 0.1 (regardless of charge state). Peptides with a +1 charge state were accepted if they were fully tryptic digested and had a cross correlation (Xcorr) of at least 1.9. Peptides with a +2 charge state were accepted if they had an Xcorr ≥ 2.2. Peptides with a +3 charge state were accepted if they had an Xcorr ≥ 3.75.

**Statistical analysis**

Percentages of spots volume (vol%) were determined from two-dimensional gels using image analysis software IMAGE MASTER (version 5.0; GE Healthcare BIO-Science). The comparisons of vol% differences between control (0 h) and cold treatment (6, 12, 24 and 48 h) were carried out using the Student’s t-test. Fold of each protein spot was defined as the ratio of mean spot vol% of the treatment gels to that of the control gels. Significantly changed (fold > 1.5 or < 0.66, P < 0.05) protein spots were subjected to protein identification.

Spearman correlation coefficients between percentage of volume of photosynthesis-related proteins and chlorophyll fluorescence parameters were calculated using spss 10.0 for Windows (SPSS, Cary, NC).

**Results**

**Leaf water content**

Changes of LWC of *Jatropha curcas* L. during cold stress are shown in Fig. 1. LWC did not change significantly before 12 h and decreased at 24 and 48 h. The LWCs at 24 and 48 h were 85.5% and 52.7% of that of control (0 h), and obvious wilt of leaves of *J. curcas* was found only after 24 h of cold stress.

**Chlorophyll fluorescence transient and parameters**

When plotted on a logarithmic time scale, the steps J and I (Strasser and Govindjee 1992) or I1 and I2 (Schreiber and Neubauer 1987) often appear between the initial O (Fo) and maximum P level (Fp) of the Kautsky transient, and the polyphasic Chl a fluorescence is also called OJIP fluorescence transient. OJIP curves of *J. curcas* leaves treated at 4°C for 0, 6, 12, 24 and 48 h are shown in Fig. 2A. When exposed to saturating actinic light, the Chl a fluorescence curve started from the initial Fo intensity (the origin, o) and increased to the highest intensity (P or Fm). The OJIP transient of *J. curcas* changed remarkably during cold stress. There were obvious increases at the J step at 6 and 12 h and Chl a fluorescence decreased greatly at 24 and 48 h during cold stress. There was an increase in FJ − Fo and a simultaneous decrease in FI − FJ at early stage of cold stress (6 and 12 h) (Fig. 2B, C), which was consistent with the changes of the OJIP curves. Considering that there are no diurnal changes of chlorophyll fluorescence parameters in normally grown *J. curcas* (data not shown), the changes in FJ − Fo and FI − FJ may the results of chilling.

Some parameters in the JIP test during cold stress are shown in Fig. 3. The changes in the maximum quantum yield for primary photochemistry (Fv/Fm) were similar to those in LWC, which showed no significant shifts at 6 and 12 h and decreased sharply at 24 and 48 h. A significant increase in the flux of photons absorbed by the antenna chlorophylls per reaction center (ABS/RC) was found at 48 h of cold treatments, which might be because of the decrease in density of reaction centers (RC/CSO) at the same time. No significant changes were found in the...
trapped energy flux per reaction center (TRo/RC) except a decrease at 48 h. Electron transport flux per reaction center (ETo/RC) significantly decreased at 6 and 12 h and then increased slightly at 24 and 48 h during cold treatment.

Two-dimensional electrophoresis and protein identification

Over 600 reproducible protein spots were detected on each gel of the control and cold treatment with the pH range of 4–8 and the mass weight range of 20 to 120 kDa. Of significantly changed protein spots, eight proteins (shown in Fig. 4) were identified as photosynthesis-related proteins using LC MS/MS (Table 2). Three proteins, i.e. ATP synthase beta subunit (No. 1), precursor for 33-kDa protein of PSII (No. 3), and chloroplast Rieske FeS protein (No. 4), were involved in important protein complexes in the thylakoid membrane of chloroplast. Ribulose bisphosphate carboxylase (RuBisCO) large subunit (No. 2) and triosephosphate isomerase (TPI, No. 5) are enzymes of the Calvin cycle in chloroplast. Cysteine synthase (No. 8) is responsible for cysteine (Cys) biosynthesis. Cys could serve as a precursor for the synthesis of various sulfur-containing metabolites, of which GSH represents the major storage and transport form of reduced sulfur. Catalase (CAT) (No. 6), glutathione reductase (GR) (No. 7) and GSH are all involved in the H2O2-scavenging systems of plant.

Correlation between chlorophyll fluorescence parameters and proteins

In order to evaluate the relationship between photosynthesis-related proteins and chlorophyll fluorescence, Spearman correlation coefficients between chlorophyll fluorescence parameters and photosynthesis-related proteins were calculated (Table 3). ETo/RC, showing the initial rate of the reopening of RCs by re-oxidation of QA2, is a measure of the rate of electron transport after QA. Significant positive correlations were found between ATP synthase beta subunit (No. 1) and ETo/RC, and
significant negatively negative correlations were found between chloroplast Rieske FeS protein (No. 4) and ETo/RC, which indicated that these two proteins might have positive or negative relations with electron transport of photosynthetic apparatus. The PIABS is an indicator of PSII function, integratively considering the three main functional steps, i.e. light energy absorption, excitation energy trapping and conversion of excitation energy to electron transport and of photosynthetic activity (van Heerden et al. 2004). The significant correlation between precursor for 33-kDa protein of PSII (No. 3) and PIABS implied that precursor for 33-kDa protein of PSII might play an important role in PSII functioning. It is generally believed that the O–J phase of chlorophyll fluorescence transients reflects reduction of QA (Hsu and Leu 2003). The positive correlation between the precursor for 33-kDa protein of PSII and FJ implied that an interaction or feedback might exist between function of 33-kDa protein of PSII and reduction of QA. Contrary relations were found between two enzymes and FJ, i.e. significantly positive correlations between TPI (No. 5) and FJ and significantly negative correlations between RuBisCO large subunit (No. 2) and FJ. Significantly positive correlations were also found between precursor for 33-kDa protein of PSII (No. 3) and \( F_{J} - F_{O} \).

There were no significant correlations between chlorophyll fluorescence parameters and proteins involved in H2O2-scavenging systems (No. 6, No. 7 and No. 8).

### Discussion

**Changes of chlorophyll fluorescence parameters and PSII function**

Chlorophyll fluorescence has been proven to be a very useful, non-invasive tool for the study of the photosynthetic apparatus and more specifically the behavior of PSII (Krause and Weis 1991, Liu et al. 2006, Strasser et al. 1995, 2000). Because the shape of the chlorophyll fluorescence transient is sensitive to stress caused by changes in many environmental conditions, the JIP test is suggested as a suitable tool to investigate the behavior of the photosynthetic apparatus under stresses (Krüger et al. 1997, Strauss et al. 2006, Tsimilli-Michael et al. 1998, 1999).

### Table 2. Photosynthesis-related proteins identified in this study

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Mr/pl</th>
<th>No. of matched peptides</th>
<th>Coverage (%)</th>
<th>Name</th>
<th>Organism</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>540/5.26</td>
<td>47.49</td>
<td>ATP synthase beta subunit</td>
<td>Parnassia tenella</td>
<td>AAY19328</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>598/6.33</td>
<td>30.00</td>
<td>RuBisCO large subunit</td>
<td>Orithyia edulis</td>
<td>BAA84557</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>893/6.25</td>
<td>9.42</td>
<td>Precursor for 33-kDa protein of PSII</td>
<td>Pisum sativum</td>
<td>BAA02554</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>242/8.63</td>
<td>13.48</td>
<td>Chloroplast Rieske FeS protein</td>
<td>Pisum sativum</td>
<td>CAA45151</td>
</tr>
<tr>
<td>5</td>
<td>33</td>
<td>526/7.65</td>
<td>15.29</td>
<td>TPI, chloroplast precursor (TIM)</td>
<td>Fragaria ananassa</td>
<td>Q9M4S8</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
<td>173/6.87</td>
<td>27.24</td>
<td>Catalase CAT1</td>
<td>Manihot esculenta</td>
<td>AAD50974</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
<td>896/6.59</td>
<td>10.64</td>
<td>GR</td>
<td>Pisum sativum</td>
<td>Q43621</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>270/5.69</td>
<td>7.69</td>
<td>Cysteine synthase</td>
<td>Glycine max</td>
<td>AAL66291</td>
</tr>
</tbody>
</table>
The J step represents the momentary maximum of 
$[Q_A^-]$, and I step reflects the heterogeneity of PQ pools
(fast reducing and slow reducing) (Govindjee 1995). The
O–J and J–I transients reflect the processes of full
reduction of the primary quinone receptor $Q_A$ and the
electron transfer from $Q_A$ to $Q_B$ (Govindjee 1995, Strasser
et al. 1995). The differences in fluorescence intensity
at O–J and J–I, i.e. $F_I - F_o$ and $F_I - F_P$, could therefore
reflect the energy gaps between $P_680^+Q_A^-$ and
$P_680^+Pheo^-$ and between $Q_A^-$ and $Q_B^-$. Based on
thermoluminescence measurements of PSII, Sane et al.
(2003) suggested that there were higher activation energy
for the $S_2Q_A^-$ redox pair and lower activation energy for
the $S_2Q_B^-$ redox pair under cold-acclimated leaves,
which involved in the protection mechanisms of Arabidopsis
to photo-inhibition under low temperature. The increase in $F_I - F_o$ and decrease in $F_I - F_P$ at 6 and 12 h
during cold stress in our study could therefore be the results of an increasing in the free energy gap between
$P_680^+Q_A^-$ and $P_680^+Pheo^-$ and narrowing of the free
energy gap between $Q_A^-$ and $Q_B^-$ in PSII electron acceptors. As the modification of the redox properties of the acceptor side of PSII may enhance the dissipation of excess light energy and protects PSII from photo-inhibition
and D1 degradation (Ivanov et al. 2003, Sane et al. 2003),
similar protection mechanism may also exist in J. curcas
seedlings during early stage of cold stress.

Comparing with $F_v/F_m$, an important parameter reflecting photosystem responses to abiotic stresses, some chlorophyll fluorescence parameters such as PI are more sensitive to chilling temperature (van Heerden et al. 2004). Our results also showed that $F_I - F_o$, $F_I - F_P$, $ET_r/RC$ and $PI_{ABS}$ were more sensitive to chilling, especially at early stage (6 and 12 h) during cold stress. $ABS/RC$, $TR_o/RC$ and $ET_r/RC$ are parameters showing specific energy fluxes of the $Q_A^-$ reducing PSII RC. $ABS/RC$ and $TR_o/RC$, indicating absorption and trapped energy flux per RC
respectively, were relatively insensitive to cold treatment
within 12 h in the present study. The decrease in $F_v/F_m$, as the ratio of $TR_o/ABS$, at 48 h during cold stress could probably be because of the decreased capability of RC in energy trapping. Compared with $F_v/F_m$, $ABS/RC$ and $TR_o/RC$, $ET_r/RC$ and $PI_{ABS}$ were relatively sensitive to chilling in the present study, which indicates that inhabitation in the electron transport might be a main reason for the decrease in PSII function at early stage of chilling.

<table>
<thead>
<tr>
<th>Protein spots</th>
<th>$ET_r/RC$</th>
<th>$PI_{ABS}$</th>
<th>$F_I - F_o$</th>
<th>$F_I - F_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP synthase beta subunit</td>
<td>0.90*</td>
<td>0.10</td>
<td>-0.60</td>
<td>0.60</td>
</tr>
<tr>
<td>Rubisco large subunit</td>
<td>0.60</td>
<td>-0.60</td>
<td>-0.90*</td>
<td>-0.60</td>
</tr>
<tr>
<td>Precursor for 33-kDa protein</td>
<td>-0.10</td>
<td>0.90*</td>
<td>0.90*</td>
<td>0.90*</td>
</tr>
<tr>
<td>Chloroplast Rieske FeS protein</td>
<td>-1.00**</td>
<td>-0.20</td>
<td>0.50</td>
<td>-0.20</td>
</tr>
<tr>
<td>TPI, chloroplast precursor (TIM)</td>
<td>-0.60</td>
<td>0.60</td>
<td>0.90*</td>
<td>0.60</td>
</tr>
<tr>
<td>CAT</td>
<td>0.40</td>
<td>-0.60</td>
<td>-0.60</td>
<td>-0.60</td>
</tr>
<tr>
<td>GR</td>
<td>0.20</td>
<td>0.00</td>
<td>-0.30</td>
<td>0.00</td>
</tr>
<tr>
<td>Cysteine synthase</td>
<td>-0.30</td>
<td>0.30</td>
<td>-0.20</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 3. Correlations between some OJIP parameters and identified proteins involved in photosynthesis. * and ** indicate significant correlations at $P < 0.05$ (critical value is 0.878) and $P < 0.01$ (critical value is 0.959) levels, respectively.

### Relationship between photosynthesis-related proteins and PSII functions

**Precursor for 33-kDa protein**

While the 33 kDa component appears to play a central role in the stabilization of the manganese cluster and is essential for efficient and stable oxygen evolution, its function within the photosystem is still unclear (Bricker and Frankel 1998). It has been found that J–I and J–P transients could reflect the damage of oxygen-evolving complex (OEC) (Govindachary et al. 2004). It is reasonable that the amount of precursor for 33-kDa protein, a component of OEC, was positively correlated with fluorescence quenching of the J–P phase in our study. The positive correlation between the precursor for 33-kDa protein and fluorescence quenching of the O–J phase and $PI_{ABS}$ implied that the protein might also be directly or indirectly related to other processes in PSII, e.g. re-oxidation of $Q_A$ and energy flux in RCs.

**ATP synthase and chloroplast Rieske FeS protein**

As an important component of thylakoid membrane, ATP synthase is closely related to the proton gradient, which was suggested as the factor controlling electron transport (Foyer et al. 1990). Our results that changes in $ET_r/RC$ were significantly correlated with ATP synthase beta subunit also implied the interactions between ATP synthase and electron transport of PSII.

Chloroplast Rieske FeS protein is an important part of Cyt $b_6/f$ complex. In a study on electron transport activity of isolated PSII core complex, Suh et al. (2000) found that the photoinactivation rate of PSII increased linearly with increasing concentration of Cyt $b_6/f$. Suh et al. suggested that the Rieske center of Cyt $b_6/f$ might play an important role in photo-inhibition processes. The negative correlation between $ET_r/RC$ and Rieske FeS protein in the our study implied that the decrease of electron transport of PSII at the early stage of chilling might be because of a Rieske FeS protein involved photoinhibition process.
Enzymes in the Calvin cycle

While it was suggested that CO₂ fixation and PSII function were related (Hollinderbäumer et al. 1997), the relationships between enzymes in the Calvin cycle and PSII function were not consistent in different studies. Kingston-Smith et al. (1997) found in maize that the maximum catalytic activities of the Calvin cycle enzymes decreased following chilling, while in another study, Hurry et al. (1995) found increased activities of these enzymes during cold stress. Some researchers believed that the inhibition of CO₂ fixation and photorespiration led to a substantial decrease in the photochemical quenching, which is determined by the reduction state of QA in PSII (Hollinderbäumer et al. 1997, Krause and Weis 1991). Our results indicated that F₁ − Fₐ, a parameter reflecting reduction state of QA₂, was also correlated with two enzymes in the Calvin cycle, i.e. RuBisCO large subunit and TPI. Considering the conflicting correlations between F₁ − Fₐ and RuBisCO large subunit (negative) and between F₁ − Fₐ and TPI (positive), the interactions between enzymes in the Calvin cycle and PSII function are expected to be relatively complex and should be tested in further studies.

Changes of H₂O₂-scavenging systems

Low temperature stress usually induces H₂O₂ accumulation in cells (O’Kane et al. 1996, Suzuki and Mittler 2006), which could cause oxidative damage to cells (Apel and Hirt 2004). An increase of content and activity of reactive oxygen species (ROS)-scavenging enzymes was often found during cold stress (O’Kane et al. 1996, Saruyama and Tanida 1995, Sato et al. 2001).

The decrease of GR and increase of CAT in the present study implied that H₂O₂ scavenging of J. curcas under cold stress might depend much on CAT rather than on the GR-related pathway. The decrease in provision of GSH, as a result of decreased content of cysteine synthase, might be a reason for the inactivity of the GPₓ-GR pathway.

Our results that no significant correlations were found between proteins involved in H₂O₂-scavenging systems and chlorophyll fluorescence parameters could be because of the complementarity of the CAT and GPₓ-GR pathways in H₂O₂-scavenging systems. Significantly negative correlations (data not shown) was found between contents of GR (No. 8) and CAT (No. 6) (r = −0.669, P < 0.01). While the photosynthesis and PSII function of plants could be affected by H₂O₂ and ROS produced during cold stress, they might be responsive to effects of the whole H₂O₂-scavenging system rather than individual enzymes in the H₂O₂-scavenging systems.

Conclusions

The results of chlorophyll fluorescence measurement indicated that ETₒ/RC and PI₂ABS were relatively sensitive to chilling compared with Fv/Fm, ABS/RC and TRₒ/RC, especially at early stage of cold stress. The increase in F₁ − Fₐ and decrease in F₁ − Fₐ at early stage of cold stress in the present study support the proposition of Sane et al. (2003) that there are an increase in the free energy gap between P680⁺QA⁻ and P680⁺Pheo⁻ and a decrease in the free energy gap between QA and QB in PSII electron acceptors during cold acclimation.

Results of correlation analyses indicated that amount of some photosynthesis-related proteins were closely related to PSII functions. ATP synthase and chloroplast Rieske FeS protein were significantly correlated with electron transport of RC in PSII. Precursor for 33-kDa protein was found positively correlated with fluorescence quenching of the O–J phase and J–I phase and PI during cold stress, which implies that the 33-kDa protein of PSII might be related to multiple process in PSII and might play an important role in photosystem functioning. Negative correlation between F₁ − Fₐ and RuBisCO large subunit and positive correlation between TPI and F₁ − Fₐ were found during cold stress, and the roles of these proteins in PSII functioning are unclear.

The combined study using proteomic approaches and chlorophyll fluorescence transient determination indicated that the early-stage (0–12 h) acclimation of PSII and the late-stage (after 24 h) H₂O₂ scavenging might be involved in the cold response mechanisms of J. curcas seedlings.

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