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Effects of abiotic stresses on the expression of *Lhcb1* gene and photosynthesis of *Oenanthe javanica* and *Apium graveolens*

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Abstract

The effects of abiotic stresses on the expression of *Lhcb1* gene (coding light-harvesting chlorophyll-protein complex II), and on photosynthetic rate were studied in one *Oenanthe javanica* (cv. Baguazhou Shuiqin) and three *Apium graveolens* cultivars (Liuhe Huangxinqin, Jinnan Shiqin, and Ventura). The *Lhcb1* genes were cloned and we predicted that Lhcb1 proteins were most probably able to form homo-trimers. Each monomer contained five helical segments, of which three were likely transmembrane helices, and 15 putative chlorophyll-binding sites. The abiotic stresses affected the *A. graveolens* similarly in all the cultivars, however, the *O. javanica* photosynthesis was not significantly affected. The expression of the *Lhcb1* gene was up-regulated under the cold, heat, salt, and drought stresses in the cvs Liuhe Huangxinqin and Jinnan Shiqin. The *Lhcb1* was up-regulated under the heat, salt, and drought stresses in the cv. Ventura, whereas had no significant changes under the cold stress. No significant changes of the *Lhcb1* expression were found under the cold and salt stresses and even little down-regulation following the heat and drought stresses in *Oenanthe javanica*. The expression of *Lhcb1* may be a useful indicator of photosynthetic activity.

Additional key words: chlorophyll, light harvesting complex, net photosysnthetic rate, salinity, stomatal conductance, temperature, transpiration rate, water stress.

Introduction

The light-harvesting complex (LHC) consists of chlorophyll (Chl) *a*, Chl *b* and Chl *a*/*b* binding proteins. As outer antenna, the light-harvesting Chl *a*/*b* binding proteins (LHCP) surround the reaction center of photosystem II (PS II) which includes the major LHCP II and the minor LHCP II and normally a complex with Chl and xanthophylls (Green and Durnford 1996, Jansson 1999). The minor LHCP II associate with PS II more closely than the major LHCP II – both are encoded by the *Lhcb* gene family (Green and Durnford 1996). The major LHCP II are encoded by the *Lhcb1*, *Lhcb2*, and *Lhcb3* genes, and the minor LHCP II by *Lhcb4*, *Lhcb5*, and *Lhcb6*, respectively (Teramoto *et al.* 2001, Xia *et al.* 2012). In addition, *Lhca1*, *Lhca2*, *Lhca3*, and *Lhca4* encode four antenna proteins which are LHCPs connected to PS I (Liang *et al.* 2008). All the proteins encoded by the genes *Lhcb1-6* and *Lhca1-4* belong to the Chl *a*/*b*-bind superfamily. None of these 10 proteins have been lost during the 350 million years of plant evolution (Ganeteg *et al.* 2004). This strongly indicates that each of the 10 proteins has a specific function.

The major function of LHCP is to capture and transfer light energy to the reaction center of PS II and take part in regulating the distribution of excitation energy between PS II and PS I. Moreover, LHCP may be involved in photoprotection by dissipating excess excitation energy under high irradiance (Kovacs *et al.* 2006, Wei *et al.* 2006). Plants with those genes mutated often lack visibly changed phenotype suggesting that the LHCPs are important only under certain conditions or have overlapping functions (Ganeteg *et al.* 2004). Plants without *Lhcb1* and *Lhcb2* are pale green and show decreased Chl content and elevated Chl *a*/*b* ratio (Andersson *et al.* 2003). Previous reports show that the

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Abbreviations: Chl - chlorophyll; c_i - internal CO_2 concentration; E - transpiration rate; g_s - stomatal conductance; LHC - light harvesting complex; LHCP - light-harvesting chlorophyll *a*/*b* binding proteins; P_N - net photosynthetic rate; PS - photosystem.
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members of the LHCP family play important roles in plant adaptation to environmental stresses (Ganeteg *et al.* 2004, Xu *et al.* 2012). The *LHCP* genes are regulated by multiple environmental and developmental factors, including irradiance (Humbeck and Krupinska 2003), temperature (Seki *et al.* 2002), salinity (Seki *et al.* 2002), drought (Hazen *et al.* 2005, Guo *et al.* 2009), oxidative stress (Nott *et al.* 2006, Staneloni *et al.* 2008), abscisic acid (Staneloni *et al.* 2008), and pathogens (Manickavelu *et al.* 2010). In contrast to the model plants and major crops, there has been little molecular biology research done in the *Apiaceae* family.

In this study, we cloned four *LHCP* genes from three *Apium graveolens* cultivars and one *Oenanthe javanica* cultivar and used for detailed analyses of sequences, performing an evolutionary analysis and researching the dynamic changes in gene expression and photosynthesis under different stresses (cold, heat, salt, and drought). As far as we are aware, this study represents the first report of a relationship between the *Lhcb1* gene expression and photosynthesis under different stresses in higher plants.

Materials and methods

Plants: *Apium graveolens*, 2n=2x=22, is a typical species of the *Apiaceae* family. Liuhe Huangxinqin is a local cultivar from east China (about 80 cm in height with yellow-green leaves), Jinnan Shiqin is a cultivar from north China (about 90 cm in height), and Ventura is a cultivar introduced from the USA (about 80 cm in height with thick green stalks). *Oenanthe javanica*, 2n=2x=22, originates from east Asia (Iovene *et al.* 2008, Zhao *et al.* 2010). Baguazhou Shuiqin is an aquatic perennial root plant from north China. Newborn plants are generated from stolons in autumn. The cultivar names are correspondingly indicated as Q1 - Q4 in the following text. Plants were grown in pots containing a soil/ *Vermiculite* mixture (3:1) in a controlled growth chamber (a 16-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1}, day/night temperatures of 25/16 °C and a relative humidity of 70 %). Two-month-old plants were irrigated with double distilled (dd) H_2O (control), 200 mM NaCl (salt treatment), or 20 % PEG 6000 (drought treatment) every day. Cold and heat treatments were performed by placing pots in a chamber set at -4 or 38 °C, respectively. After 0.5, 1, 2, 4, 6, 8, 12, 24, 48, and 72 h, the plants were harvested, quickly immersed in liquid nitrogen, and stored at -70 °C.

Cloning and sequencing: Total RNA was extracted from leaves using an *RNAprep* pure plant kit (*Tiangen-bio*, Beijing, China). Single-strand cDNA was synthesized from 1 000 ng of RNA with an oligo dT primer using a *PrimeScript RT* reagent kit (*TaKaRa*, Dalian, China) according to the manufacturer's protocol. A PCR amplification was performed using the primer pair 5'-ATGGCTGCTTCAACAATGG-3' and 5'-TCACTT TCCGGGAACAAAGTTAG-3' to obtain the open reading frame of the *Lhcb* gene. The PCR conditions were 95 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 60 s; and finally 72 °C for 10 min. The PCR product was ligated into a *pMD-18* vector (*TaKaRa*) and used to transform an *Escherichia coli* strain DH5α. After the PCR identification of an extracted plasmid, DNA sequencing was entrusted to *GenScript* (Nanjing, China).

Data analysis: The amino acid sequences were searched from *NCBI* and *RAP-DB* (Rice Annotation Project Database). Accession numbers are listed in Suppl. Table 2. The nucleotide and amino acid sequences were analyzed using the *BLAST*, *Clustal W*, and *BioEdit v. 7.0*. Transit peptide was predicted in the following website: http://www.cbs.dtu.dk/services/ChloroP/ (Emanuelsson *et al.* 1999). The transmembrane (TM) regions and orientation were predicted by *TMpred* program (Bertaccini and Trudell 2002). The alignment report was produced using *DNAMAN 6.0*. The molecular phylogenetic tree was built using *MEGA5* with the neighbor-joining (NJ) method (Tamura *et al.* 2011). The basic properties of proteins were analyzed by the related software from http://www.expasy.org (Gasteiger *et al.* 2003). Three-dimensional structure modeling was performed using *Swiss-Model* (http://swissmodel. expasy.org/) (Kiefer *et al.* 2009). The results of modeling were analyzed with *Swiss-viewer 4.0* (Ho and Brasseur 2005). The relative expression ratio was calculated by the $\Delta\Delta C_T$ method (Pfaffl 2001).

Real-time PCR analysis: The qPCR systems were performed on *ABI 7500* (*Applied Biosystems*, Foster city, USA) with *SYBR Premix Ex Taq* (*TaKaRa*). As reference gene, the *actin* gene was amplified together with the target gene. The primer pairs of the *actin* gene were 5'-CTTCCAGCCATCTATGATTGG-3' and 5'-GCCACC ACCTTGATCTTCATG-3' in *A. graveolens*, 5'-AGA GGTTCCGCTGTCCAGAAGT-3' and 5'-TGGGAG CAAGGGCAGTGATTTC-3' in *O. javanica*. Based on the high identity of the four gene nucleotide acid sequences, one pair of qPCR primers with the same sequence regions of the four genes was designed. The primer pair of target gene was 5'-GTTACC GTGTTGCTGGTGGA-3' and 5'-GAGAACATGGCT AGTCTTCCA-3'. The PCR conditions were 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 54 °C for 30 s, and finally 65 °C for 15 s.

Photosynthetic parameters: Net photosynthetic rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (c_i), and transpiration rate (E) were determined in a sunny day from 9:00 to 11:00 using a *LI-6400XT* portable photosynthesis system (*LI-COR,*

Lincoln, NE, USA). An air flow rate was 500 μmol·s^{-1}, an irradiance 700 μmol·m^{-2} s^{-1}, a temperature 20 °C, a relative humidity 70 %, a CO_2 concentration 400 μmol mol^{-1}, and a leaf chamber area 11 cm^2.

Results

Four *LHCP* genes were cloned from three *A. graveolens* cvs. Liuhe Huangxinqin (Q1), Jinnan Shiqin (Q2), Ventura (Q3), and one *O. javanica* cv. Baguazhou Shuiqin (Q4). The identity of the multiple alignments of the four gene nucleotide sequences was 95.76 % (Fig. 1 Suppl.). The identity of the multiple alignments of the deduced amino acid sequences was 97.37 %. The lengths of the genes were 801, 795, 795, and 795 bp, containing complete open reading frames that encoded 266, 264, 264, and 264 amino acids, respectively. After the initiation codon ATG, transit peptides with 34, 32, 32, and 32 amino acid residues were predicted (Fig. 2 Suppl.). Pairwise amino acid sequence comparisons were performed among the four deduced proteins from the four plants (Q1 - Q4) and the six members of LHCP proteins from *Arabidopsis thaliana*. The four deduced proteins showed high similarity with each other; and Lhcb1 from *A. thaliana* showed high identity with them (88.12 %). Thus, the four genes cloned from *A. graveolens* and *O. javanica* were inferred to be the *Lhcb1* gene. Interestingly, *Lhcb2* from *A. thaliana* also had a high similarity with them.

The LHCP protein is located in chloroplast thylakoid membrane (Galka *et al.* 2012). The TM regions and orientation of Lhcb1 from the one *O. javanica* and three *A. graveolens* plants were predicted (Fig. 3 Suppl.). Three possible TM regions were found in the proteins, and the distributions of them were quite similar in the four Lhcb1. As example, the three TM regions in Q2 were distributed in the amino acid residues of 98 - 118 (outside to inside), 153 - 173 (inside to outside) and 218 - 234 (outside to inside). In photosynthetic eukaryotes, the TM1 and TM3 are highly conserved (Durnford *et al.* 1999).

The multiple alignments of amino acid sequences of Lhcb1 proteins from *A. graveolens*, *O. javanica*, and other nine plants without transit peptide are shown in Fig. 2 Suppl. The sequences of these Lhcb1 proteins were very similar. The identity of the multiple alignments was 92.77 % indicating that the Lhcb1 protein was highly conserved and may play a particular role in functions related to adaptation to harsh environments. The 15 putative Chl-binding sites are shown in Fig. 2 Suppl (Wei *et al.* 2006). The conserved motif (WYGPDR) is the presumed phosphatidylglycerol binding site required for trimer formation in higher plants (Nussberger *et al.* 1993, Hobe *et al.* 1995). Another motif (AD) for the phosphatidylglycerol binding site was also conserved (Wei *et al.* 2006).

A comparison of composition and physical and chemical characterizations of amino acid sequences of Lhcb1 among the different plants is shown in Table 1 Suppl. The molecular mass of the protein including transit peptide was 27.5 - 28.6 kDa. The theoretical pI was 4.99 - 5.96. The percentage of basic amino acids was 8 - 10 %, of acidic amino acids 9 - 11 %, of aromatic amino acids 10 - 11 %, and of aliphatic amino acids 17 - 20 %. The insolubility was 74.5 - 84.4 % indicating a low protein solubility.

A phylogenetic tree showing relationships among the six members of the LHCP proteins associated with PS II (including transit peptides) was constructed using the NJ method – the four proteins we discussed were on the branch of Lhcb1. Accession numbers of plants are listed in Table 2 Suppl. According to the result, all proteins were on the branch of their own subfamily except for Lhcb1 from *Physcomitrella. patens*, which was on the branch of the Lhcb2 subfamily and next to the Lhcb2 from *P. patens*. The Lhcb1 and Lhcb2 from *Selaginella. moellendorffii* were also close to each other. The evolutionary distances of all the Lhcb1 and Lhcb2 proteins from the different plants were short. These proteins were possibly generated by more recent gene duplications. Both *P. patens* and *S. moellendorffii* are ancient species, the changes in their evolutionary process may be smaller and more recent. The tree was constructed to represent the main events during the evolutionary process of the LHCP protein associated with PS II (Fig. 1).

The alignment without transit peptide showed that Lhcb1 from Jinnan Shiqin and the protein from *Spinacia oleracea* shared the highest similarity of 96.4 %. The protein from spinach (PDB ID:1rwt_F) (Liu *et al.* 2004) was selected as template to model the three-dimensional structure using *Swiss-Model*. The polypeptide main chain of the template was continuously traced from Ser 14 to Gly 231 (Liu *et al.* 2004). The protein structure generated was a trimer which consisted of three monomers from Ser 46 to Gly 263. This model revealed some basic structural features of Lhcb1, including three TM α-helices (helices A, B, and C), two short amphipathic helices (helices D and E), and 15 putative Chl-binding sites (Fig. 2).

A Ramachandran plot is a 2D plot of the Φ - Ψ torsion angles of the protein backbone. It provides a simple view of the conformation of a protein. There are four basic types of Ramachandran plots, depending on the stereo-chemistry of the amino acids: generic, glycine, proline, and pre-proline (Ho and Brasseur 2005). According to the *PdbViewerManual v. 3.7*, the plot delimits the allowed regions, and most of the amino acids of any given protein should be plotted in blue area (regions of maximum tolerable limits of steric strain). In Ramachandran plot of Lhcb1, 94.5 % of the amino acids of the putative protein structure are in the blue area of the Ramachandran diagram which indicates that the protein

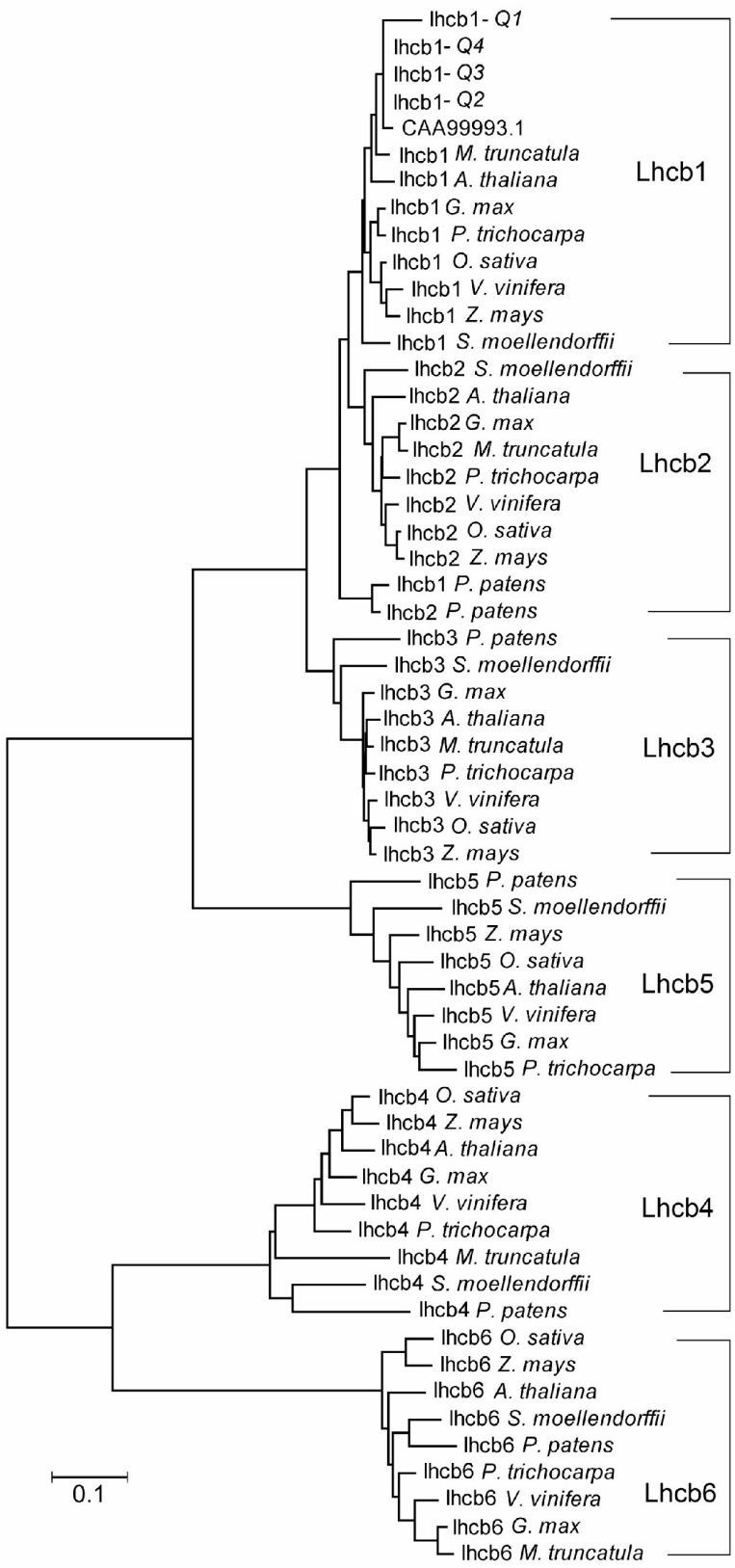

Fig. 1. The phylogenetic tree of amino acid sequences with bootstrap values (% of 1000 replicates) showing relationships among the six members of the LHCP proteins associated with PS II. Q1 - Liuhe Huangxinqin, Q2 - Jinnan Shiqin, Q3 - Ventura, and Q4 - Baguazhou Shuiqin.

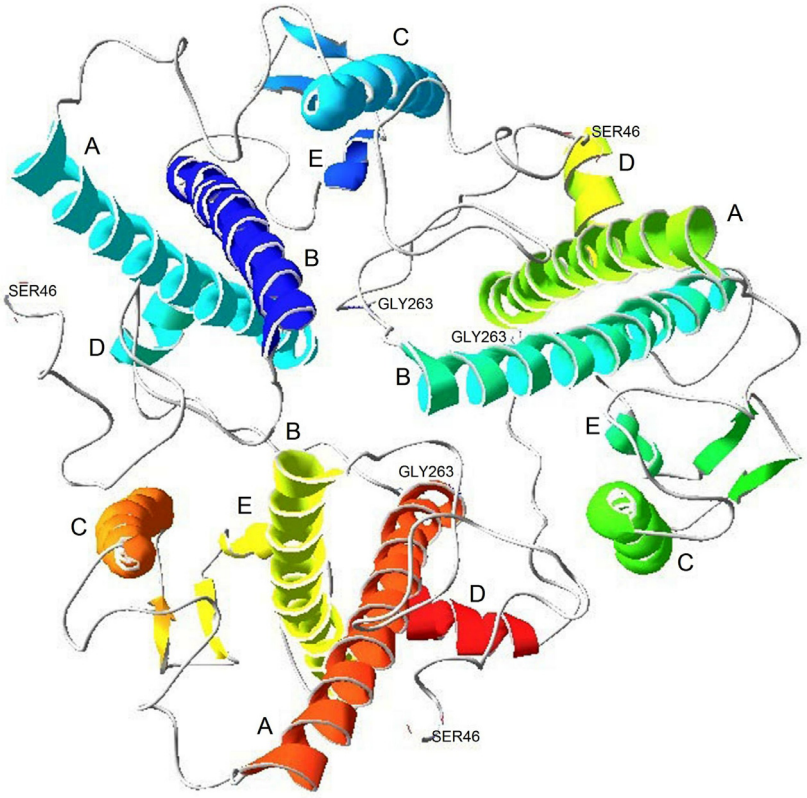

Fig. 2. The three-dimensional structures of LHCP from Q2 (Jinnan Shiqin). Color changing from the *blue* (N terminus) to *red* (C terminus); *capitals* indicate different helices; a β-strand is shown as *green arrow*.

structure is stable. The energy of the total structure was -16 364.8 kJ mol^{-1}. These results indicate that the three-dimensional structure of Jinnan Shiqin LHCP is reliable.

We compared the expressions of the *Lhcb1* genes in the four plants (Q1 - Q4) under different abiotic stresses. Following the cold treatment, the *Lhcb1* of Q1 was up-regulated twice with maxima at 0.5 h and 2 d. The *Lhcb1* of Q2 was up-regulated twice at 4 and especially at 12 h. There were no remarkable differences in the expressions of *Lhcb1* from Q3 and Q4. Following the heat treatment, the *Lhcb1* of Q1 and Q2 were up-regulated at 0.5 h, and then they were reduced to an extremely low level, the change of Q2 was milder than that of Q1; the *Lhcb1* of Q3 was up-regulated at 1 h and then also decreased to an extremely low level; and the *Lhcb1* of Q4 was down-regulated (Fig. 3).

Following the NaCl treatment, the *Lhcb1* of Q1 was up-regulated twice at 6 h and 2 d; the *Lhcb1* of Q2 was up-regulated twice at 1 h and 1 d; and the *Lhcb1* of Q3 was up-regulated twice at 4 h and 1 d. The first up-regulations were more intensive then the second ones. There were no distinct differences in the expressions of *Lhcb1* from Q4 in response to the salt stress.

Following the drought treatment, the *Lhcb1* of Q1 was up-regulated twice at 1 h and 1 d; the *Lhcb1* of Q2 was up-regulated twice at 2 h and 2 d, and the former was more intensive; the *Lhcb1* of Q3 was up-regulated at 2 h, then decreased and subsequently rebounded at 2 d; and the *Lhcb1* of Q4 was down-regulated (Fig. 3).

The *Lhcb1* gene expressions under the abiotic stresses (Fig. 3) were compared with basic photosynthetic parameters (Fig. 4). The basic photosynthetic parameters of Q4 plants which suffered abiotic stress showed no significant change (data not shown) The expression of *Lhcb1* in Q1 and Q3 plants had similar trends as P_N, g_s, and E, and an opposite trend as c_i (Fig. 4 *A,C,D,F*). But photosynthesis was weakened when the gene expression increased in Q2 (Fig. 4*B,E*). Although the gene expression in this cultivar increased following the cold and heat stresses, the plants were not adapted to the environment, as reflected in the decreased photosynthesis. Nevertheless, the expression of *Lhcb1* might be a useful indicator of these stresses.

Discussion

During plant life, various abiotic stresses have detrimental effects on plant biomass and yield (Yamaguchi-Shinozaki and Shinozaki 2006, Li *et al.* 2012, Xue *et al.* 2012, Kim *et al.* 2013, Nakashima and

Yamaguchi-Shinozaki 2013). Plants without *Lhcb1* and *Lhcb2* show photo-inhibition when subjected to a stress which appeared in pale green colour, a decreased Chl content and elevated Chl *a*/*b* ratio (Andersson *et al.* 2003, Ganeteg *et al.* 2004). Previous reports showed that the members of the LHCP family could be regulated by drought, high-salinity, and cold (Seki *et al.* 2002, Guo *et al.* 2009, Loukehaich *et al.* 2012), but the results are not uniform. Moreover, no researchers studied the relationship between a *Lhcb1* gene expression and

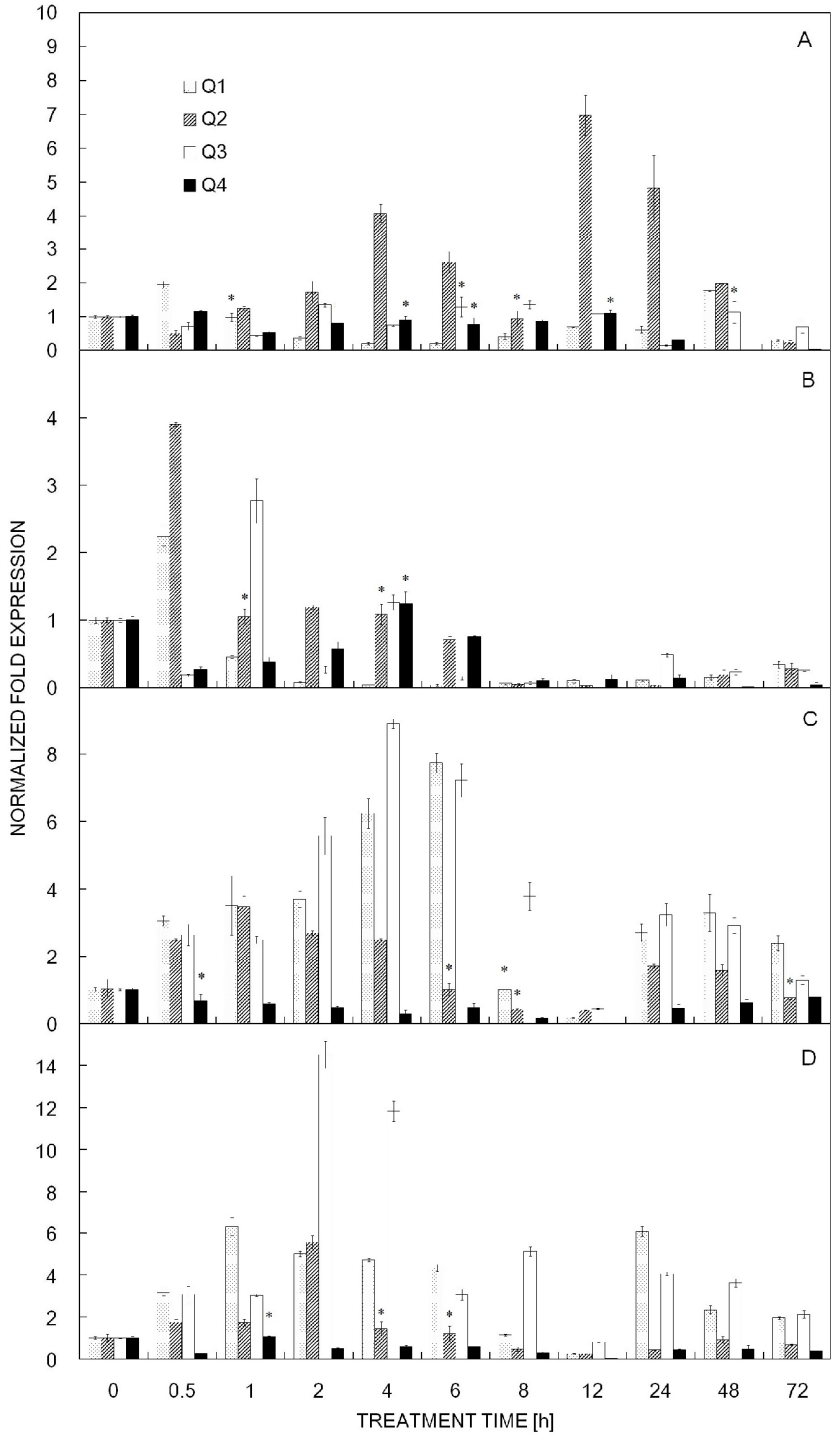

Fig. 3. The expression of *Lhcb1* from four plants under abiotic stresses: cold (*A*), heat (*B*), salinity (*C*), and drought (*D*). Q1 - Liuhe Huangxinqin, Q2 - Jinnan Shiqin, Q3 - Ventura, and Q4 - Baguazhou Shuiqin; * indicate no significant difference ($P > 0.05$) between the treated and untreated plants.

photosynthesis under different stresses. Here, we cloned *Lhcb1* sequences from one *O. javanica* and three celery cultivars, explored the relationship between *Lhcb1* gene expression and photosynthesis under different stresses. The cultivars Liuhe Huangxinqin, Jinnan Shiqin, Ventura, and Baguazhou Shuiqin had their own characteristics with significant differences in phenotypes. However, the pairwise amino acid sequence comparisons revealed a high similarity among them. The multiple alignments, comparison of composition, and physical and chemical characteristics also show a high similarity among the Lhcb1 proteins. This indicates that the Lhcb1 protein is highly conserved and may play a particular role in functions associated with adaptation to harsh environments. The phylogenetic tree of the relationships among the six members of the LHCP proteins associated with PS II (including the transit peptides) shows that Lhcb1 and Lhcb2 were generated by gene duplications which happened more recent than Lhcb4, Lhcb5, and Lhcb6. The evolutionary processes experienced by Lhcb4, Lhcb5, and Lhcb6 were simpler than by Lhcb1 and Lhcb2. *P. patens* and *S. moellendorffii* are ancient species, the changes in their evolutionary process were smaller than in other plants. Moreover, Lhcb1 and Lhcb2 shared the closest genetic relationship (with a high homology) which is consistent with previous research (Teramoto *et al.* 2001, Xia *et al.* 2012).

Dynamic changes in the gene expression under the

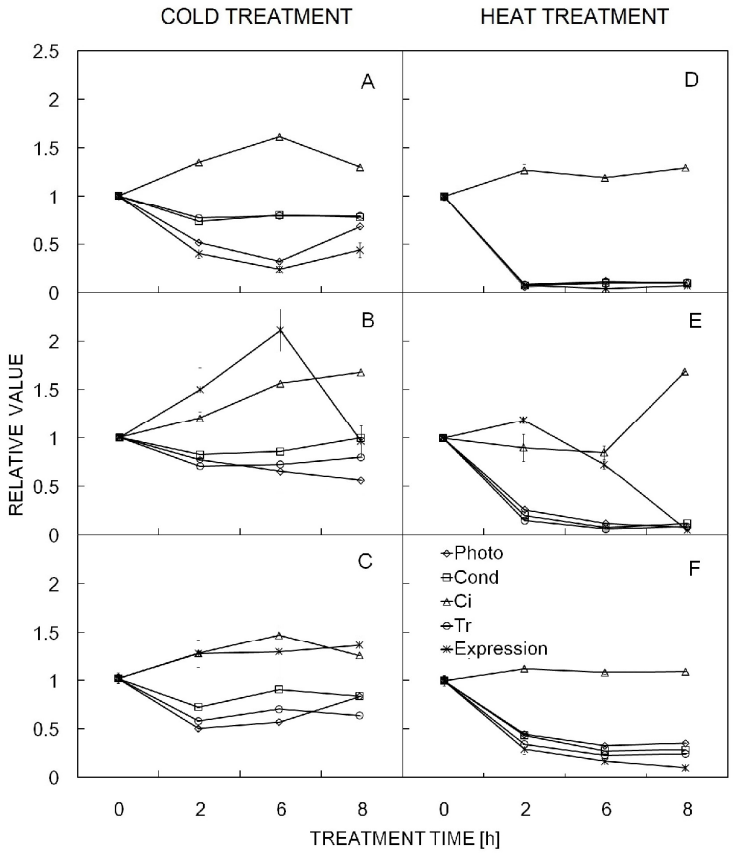

Fig. 4. Basic photosynthetic parameters and the *Lhcb1* gene expression of three *A. graveolens* cultivars determined under cold and heat treatments (a relative value of treated to untreated plants (Photo - net photosynthetic rate, Cond - stomatal conductance, c_i - intercellular CO_2 concentration, Tr - transpiration rate). Q1 - Liuhe Huangxinqin (*A, D*), Q2 - Jinnan Shiqin (*B, E*), and Q3 - Ventura (*C, F*). Except expression of Q3 at 2h and 6h, all values showed significant difference at $P<0.05$ between treated and untreated plants.

abiotic stresses, supported by the photosynthetic measurements, revealed that the expression of *Lhcb1* from Liuhe Huangxinqin and Jinnan Shiqin was up-regulated under the cold, heat, salt, and drought stresses. The *Lhcb1* from Ventura was up-regulated under the heat, salt, and drought stresses, and had no significant changes under the cold stress. The *Lhcb1* from Baguazhou Shuiqin showed no significant changes under the cold and salt stresses, and was a little down-regulated following the heat and drought stress. Loukehaich *et al.* (2012) found that 27 chlorophyll *a*/*b* binding proteins were up-regulated in plants overexpressing a universal stress protein gene (*SpUSP*) under drought conditions. However, Guo *et al.* (2009) investigated differentially expressed genes among three barley cultivars under a drought stress using microarrays, and the Chl *a*/*b* binding

protein was down-regulated in two cultivars but up-regulated in another cultivar. Seki *et al.* (2002) monitored the expression profiles of 7 000 *Arabidopsis* genes under drought, cold, and salinity, and 13 Chl a/*b* binding proteins were down-regulated following the stresses. Some proteins were actually up-regulated at some time points which were observed from their supplementary data (Seki *et al.* 2002). Compared with the studies described above, the present study was more concentrated on the target gene, and provided much more detailed analysis with more time-points.

The correlation between the gene expression and photosynthetic basic parameters not only indicates that the data are reliable, but also implies that *Lhcb1* played an important role in the plant growth. The small difference in *Lhcb1* nucleotide sequences of the four plants results in remarkable differences of photosynthesis. This is in agreement with a previous study, which indicates that single nucleotide polymorphisms in *Lhcb1* are significantly associated with agronomic traits in barley (Xia *et al.* 2012).

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