RESEARCH NOTE

Cloning and expression of a ζ-carotene desaturase gene from *Lycium chinense*

ZHAODI LI[1], GUANGXIA WU[1], JING JI[2]*, GANG WANG[2], XIAOWEI TIAN[1] and HAILING GAO[2]

[1]*School of Chemical Engineering and Technology, and* [2]*School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China*

[Li Z., Wu G., Ji J., Wang G., Tian X. and Gao H. 2015 Cloning and expression of a ζ-carotene desaturase gene from *Lycium chinense*. *J. Genet*. **94**, 287–294]

Introduction

ζ-Carotene desaturase (ZDS) is a key enzyme in the carotenoid biosynthetic pathway which catalyses the conversion of ζ-carotene into lycopene. In this study, a cDNA encoding ZDS was isolated from *Lycium chinense* by rapid amplification of cDNA ends approach (RACE) and designated as *LcZDS*. The full-length cDNA sequence was 2042 bp containing a 1767 bp putative open reading frame (ORF) which encoded a 588 amino acid whose molecular weight was 64.69 kDa computationally. The putative protein sequences showed a high degree identity compared with known ZDS from other plants. Phylogenetic analysis depicted that LcZDS has a closer relationship with ZDS of higher plants and chlorophyta than with those of other species. Quantitative real-time PCR assay revealed that the expression of *LcZDS* was the highest in maturing fruits and was upregulated by different abiotic stresses in *L. chinenese*. In addition, expression of this gene in *Escherichia coli* produced a single polypeptide which could successfully catalyze ζ-carotene into lycopene via neurosporene.

Carotenoids are structurally diverse group of natural pigments synthesized by all higher plants, algae, some bacteria and fungi (Sandmann 2001). In higher plants, carotenoids represent a class of yellow, orange and red lipid-soluble pigments, providing distinct colours and nutritional quality to flowers, fruits and vegetables. Carotenoids participate in light-harvesting processes and protect photosynthetic apparatuses from photooxidation (Tracewell *et al.* 2001; Szabó *et al.* 2005; Dong *et al.* 2007). Carotenoids can also confer resistance to abiotic stress in plants by scavenging reactive oxygen species (Kim *et al.* 2012). In animals, carotenoids are indispensable components of antioxidant systems (Palozza and Krinski 1992), which play essential roles in reducing the risk of certain forms of cancers (Rao and Agarwal 2000).

The biochemistry of carotenoid biosynthesis has been clearly illustrated. The process starts with the condensation of two geranylgeranyl diphosphate molecules to produce phytoene, catalysed by phytoene synthase (PSY) (Chamovitz *et al.* 1992). Phytoene experienced four desaturations to generate red-coloured lycopene catalysed by carotene desaturase via the intermediates of phytofluene, ζ-carotene and neurosporene (Cunningham and Gantt 1998). In bacteria and fungi, these sequential desaturations were achieved by a single gene product, CRTI, while in cyanobacteria and higher plants, two related enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) participates together in this procedure (Sandmann 2001). Subsequently, lycopene was cyclized twice by two individual cyclases, lycopene ε-cyclase and lycopene β-cyclase, yielding α and β carotenes, which were further processed to other types of carotenoids, such as lutein, violaxanthin and zeaxanthin (Hirschberg 2001).

ZDS plays a crucial role in carotenogenesis which catalyses the desaturation of ζ-carotene into neurosporene and then to lycopene, providing a precursor for cyclization reaction. Heterologous overexpression in *E. coli* revealed the indispensable function of ZDS in the formation of lycopene (Bartley *et al*. 1999). Mutation of *ZDS* gene from *Arabidopsis* resulted in impaired carotenoid biosynthesis and subsequent spontaneous cell death from the increased content of superoxide (Dong *et al.* 2007). Additionally, Lao *et al.* (2014) reported that *ZDS* in *Dunaliella bardawil* was regulated in response to salt stress. As *ZDS* was essential for carotenoid biosynthesis, cell growth and stress responses, it has been isolated and characterized in maize (Matthews *et al.* 2003), *D. salina* (Ye and Jiang 2010), and persimmon (Zhao *et al.* 2011). As yet the cDNA sequence for *ZDS* has not been isolated from *L. chinense*.

*For correspondence. E-mail: jijing@tju.edu.cn.
Zhaodi Li and Guangxia Wu contributed equally to this work.

Keywords. ζ-carotene desaturase; carotenoid biosynthesis; abiotic stress; *Lycium chinense*.

L. chinense is a kind of Chinese traditional fruit crop with various important biological activities. The fruits are rich in different sorts of active ingredients, such as betaine, polysaccharide, vitamins, unsaturated fatty acids, especially carotenoids (with a concentration of about 0.03–0.5%) (Peng *et al.* 2005; Inbaraj *et al.* 2008). Further, *L. chinense* is also a stress resistant plant that has great capability of environmental adaption. Previous studies on *L. chinense* have mainly focussed on identification of chemical compositions and extraction of active ingredients (Weller and Breithaupt 2003), whereas the molecular mechanisms of carotenoid accumulation and abiotic stress responses in *L. chinense* were relatively scarce. In this study, we report the isolation and characterization of *L. chinense ZDS* gene. In this study, we reported the isolation and characterization of *L. chinense ZDS* gene, as well as its ability to convert ζ-carotene into lycopene. Moreover, the expression pattern of *LcZDS* in different tissues and under stress conditions were also investigated.

Materials and methods

Plant growth and stress treatment

L. chinense was grown in greenhouse under a photoperiod of 12 h at light intensity of 300 μmol m^{-2} s^{-1} at room temperature 22 $\pm$ 2°C with 75% relative humidity. Various plant tissues including root, stem, leaf, flower and fruit were separated from the same plant for tissue-specific assay. Fruit samples were harvested in 10 days postanthesis (DPA), 20 DPA, 30 DPA and 45 DPA, the key ripening stage for colouration.

Prior to stress treatments, developed seedlings were carefully uprooted uniformly to avoid injury, rinsed with water and transferred to Hoagland's solution to culture for seven days under the same conditions as described above. For high salt and ABA treatments, the *L. chinense* seedlings were cultured in solutions containing either 300 mM NaCl or 100 μM ABA for time periods (0, 3, 6, 9, 12, 24 h). For drought stress, the roots of *L. chinense* seedlings were placed on paper towels and sampled at the designated time. The 4th pair of leaves from the apex of the growing shoot were harvested from different sets of plants and immediately frozen in liquid nitrogen before further analysis. Each treatment was repeated at least thrice independently.

Cloning the full-length sequence of LcZDS cDNA

Total RNA was extracted from fresh *L. chinense* leaves using RNeasy plant Mini kit (Qiagen, Valencia, USA) following the manufacturer's instructions. The concentration and purity of total RNA was quantified by SMA1000 UV Spectrophotometer (Merinton Technology, Beijing, China). The extracted RNA was reverse transcribed into cDNA using the 3′-Full RACE Core Set ver. 2.0 (TaKaRa, Osaka, Japan). The obtained first-strand cDNA was used as template to amplify *LcZDS* fragment with 5′ gene specific primer (RACE-*LcZDS* F: 5′-ATGGCTACTTCTTCAGCTTATTT-3′) designed according to transcriptomic sequence of *L. chinense* sequenced using BGI (BGI, Beijing, China). PCR reaction was performed under the following conditions: denaturation at 94°C for 4 min; 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 2 min; and a final extension at 72°C for 8 min. The amplified product was ligated into pMD18-T vector (TaKaRa, Osaka, Japan) after purification and cloned into *E. coli* strain DH5α followed by sequencing.

Bioinformatics analysis

Physical and chemical characteristics were analysed using ExPASy (http://expasy.org/). The conserved domains were searched with the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Multiple sequence alignment was performed by ClustalX and ESPript (Gouet *et al.* 2003). The SignalP Server and Wolf Psort programs were employed to identify signal peptide and predict their protein localization (http://www.genscript.com/psort/wolf_psort.html). Phylogenetic analysis was constructed with MEGA4 based on the neighbour-joining (NJ) method.

Expression analysis by qRT-PCR

To evaluate expression levels of *LcZDS* in different tissues and under stress conditions, qRT-PCR was performed (Roche lightcycler 480, Basel, Switzerland). Total RNA was extracted from the samples and used for cDNA synthesis following the aforementioned procedures. First-strand cDNA synthesis was performed from 1 μg total RNA with PrimeScript^{TM}RT Master Mix (Takara, Osaka, Japan). Each PCR reaction contained 5 μL of cDNA, 10 μL of SuperReal PreMix Plus (Tiangen, Beijing, China), 0.6 μL of each primer and nuclease-free PCR-grade water to a final volume of 20 μL. The constitutively expressed *L. chinense actin* gene, amplified with the primers *Actin*F and *Actin*R, was used as an internal control to normalize the relative expression level of the analysed gene in *L. chinense*. Quantitative analysis was performed with PCR conditions of 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 60°C for 15 s and 72°C for 20 s. Each sample was experimented in triplicate. Finally, the data analyses were performed according to the comparative C_T method (Livak and Schmittgen 2001).

LcZDS primers: *LcZDS*F1, 5′- TGGTTGCTATAATAATCT GTTC-3′ and *LcZDS*R1, 5′-GTGTGAGTATGGTCCTTC-3′.

L. chinense actin primers: *Actin*F, 5′-GGAAACATAG TGCTCAGTGGTG-3′ and *Actin*R, 5′-GCTGAGGGAAGC CAAGATAG-3′.

Expression and purification of LcZDS

The *LcZDS* coding region was amplified by PCR, cut with *Bam*HI and *Xho*II, at sites introduced via the *LcZDS*F2 and *LcZDS*R2 primers (*LcZDS*F2: 5′-CGGGGTACCATGG CTACTTCTTCAGCTT-3′; *LcZDS*R2: 5′-CTAGTCTAGAT TAGACAAGACTCAACTCATC-3′), purified and cloned

in a similarly digested pET-28a(+) (Invitrogen, Carlsbad, USA) to form the recombinant plasmid pET-28a-*LcZDS* carrying a 6× His-tag at the N-terminal.

E. coli BL21 transformed with pET-28a-*LcZDS* was grown in liquid LB medium selected by 100 mg/L kanamycin at 37°C, with agitation (180 rpm), until it reached an OD of 0.5–1. The cells were then induced by addition of 1 mM IPTG and cultivated for a further 4 h for the target protein expression. The total bacterial proteins were extracted using Bacterial Protein Extraction Kit (Comwin, Beijing, China) and the LcZDS protein was purified with the 6× His-Tagged Protein Purification Kit (Comwin, Beijing, China). The prepared samples were then loaded onto a 12% SDS-PAGE and separated on a Mini-Protean Tetra (Bio-Rad, Hercules, USA) according to the method of Laemmli (1970).

Functional assay of LcZDS in E. coli

Plasmid pACCRT-EBP encoding *Erwinia uredovora* crtE (GGPP synthase), crtB (phytoene synthase) plus *Synechococcus* crtP (phytoene desaturase), mediated the formation of ζ-carotene, whereas plasmid pACCRT-EBI$_{RC}$ encoding *Erwinia uredovora* crtE, crtB, plus *Rhodobacter capsulatus* crtI (phytoene desaturase), was responsible for the synthesis of neurosporene (Linden *et al.* 1993). Transformation of *E. coli* BL21with the plasmids pACCRT-EBP or pACCRT-EBI$_{RC}$ resulted in a yellow or a deep yellow colouring of the *E. coli* cells, respectively. For heterologous complementation assay, *E. coli* BL21 cells harbouring pET-28a-*LcZDS* were cotransformed with plasmids pACCRT-EBP or pACCRT-EBI$_{RC}$. The cotransformed cells were then plated on LB agar plate in presence of chloramphenicol (50 mg/L), kanamycin (100 mg/L) and 1 mM isopropylthio-β-D-galactoside (IPTG). After two days of growth at room temperature, the plates were screened for a colour change from yellow to red. Cells with either pACCRT-EBP/pET-28a(+), or pACCRT-EBI$_{RC}$/pET-28a(+) were grown similarly with appropriate antibiotics serving as negative controls.

Carotenoid extraction and HPLC analysis

For *E. coli* BL21 cells, transformants were grown in liquid LB medium harbouring 100 mg/L kanamycin and 50 mg/L chloramphenicol at 37°C for about 6 h with agitation (180 rpm), then induced with 1 mM IPTG at late-log growth phase, and further incubated at 28°C for 2 d with mild

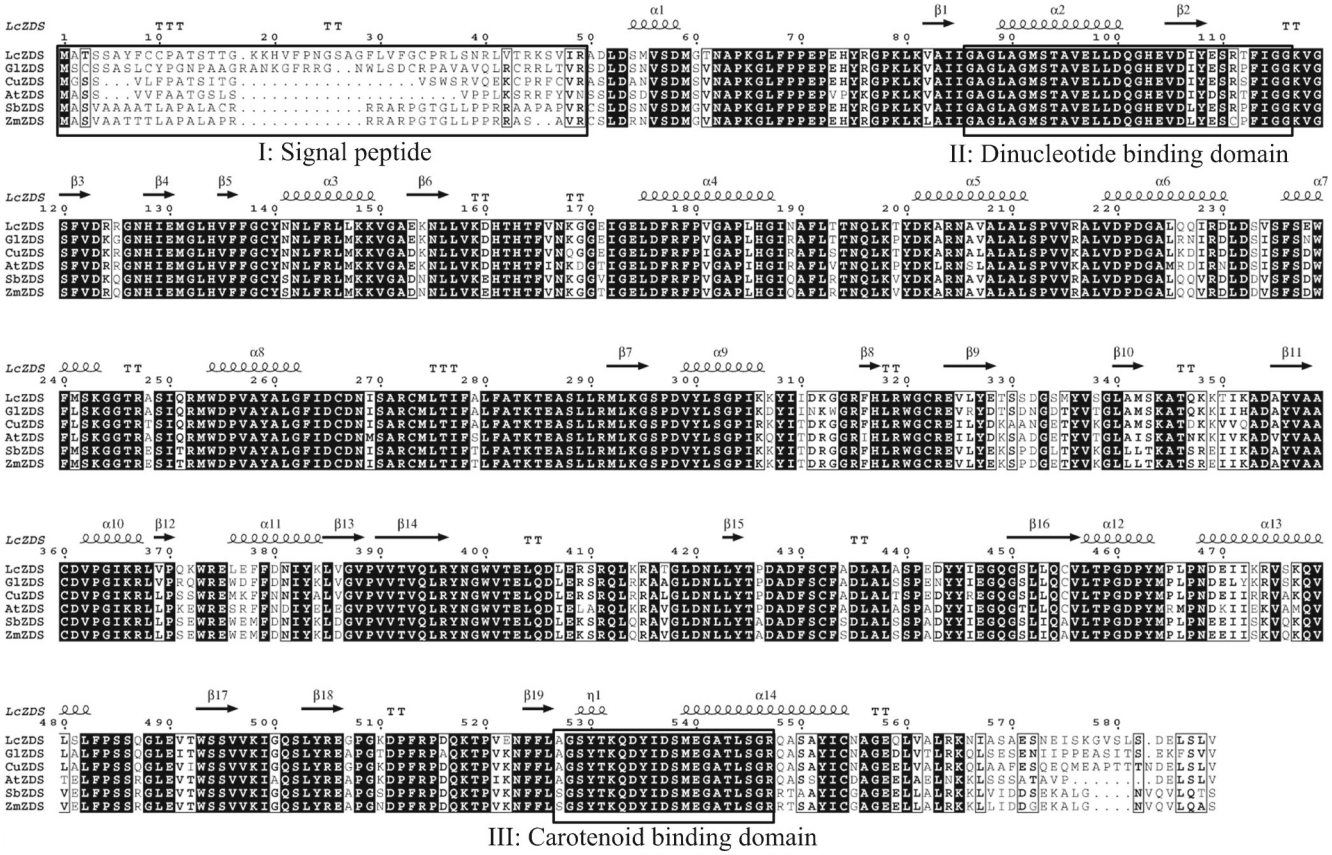

Figure 1a. Multiple sequence alignment of predicted amino acid sequence of LcZDS and other plant ZDS. The black boxes represent conserved motifs in the ZDS protein. The secondary structure and numbering are shown above the alignment. α-Helices, η-helices and strict β-turns are marked with α, η and TT, respectively. LcZDS, *L. chinense* ZDS; NtZDS, *Nicotiana tabacum* ZDS (AEG73891.1); SlZDS, *Solanum lycopersicum* ZDS (ABD67160.1); ZmZDS, *Zea mays* ZDS (ACG46735.1); GlZDS, *Gentiana lutea* ZDS (BAA88843.1); AtZDS, *Arabidopsis thaliana* ZDS (NP_187138.1).

agitation (100 rpm) to maximize carotenoid production. Pigmented cells were finally harvested by centrifugation and freeze-dried before extraction of total carotenoids. For carotenoid extraction, the prepared samples were extracted with methyl alcohol including 6% potassium hydroxide (KOH) solution by heating for 20 min at 60°C. After partitioning into 20 mL 10% ether in petrol, the upper phase was collected and dried down. For high-performance liquid chromatography (HPLC) separation, the dried extracts were dissolved in acetone and separated on a standard C-18 reversed-phase column with acetonitrile/methanol/2propanol (85:10:5, v/v) as eluent at a flow of 1 mL/min. Spectra were recorded online with a Thermo Photodiode Array detector. Carotenoids were identified by comparing typical retention times and absorption spectra with standard samples (Zhao *et al.* 2014).

Statistical analysis

Triplicate analyses were performed and the data were subjected to analysis of variance by ANOVA and Duncan's multiple range test for statistical significance ($P < 0.05$) in mean comparison based on SPSS (ver. 15.0, Chicago, USA).

Results and discussion

Isolation and sequence analysis of LcZDS cDNA

On the basis of the sequence information of *L. chinense* transcriptome, a 2042 bp fragment was successfully obtained by 3′rapid amplification of cDNA ends (RACE) with the 5′ gene-specific primer. The resulting sequence was deposited into GenBank with accession number KJ174516 and designated as *LcZDS*. The deduced protein encoded 588 amino acid residues with a calculated molecular mass of 64.69 kDa and a theoretical isoelectric point (pI) of 8.073. The putative amino acid sequence of LcZDS was compared with those of known carotenoid dehydrogenase homologous proteins (figure 1a). It showed that LcZDS had high homology with ZDS sequences of other plants.

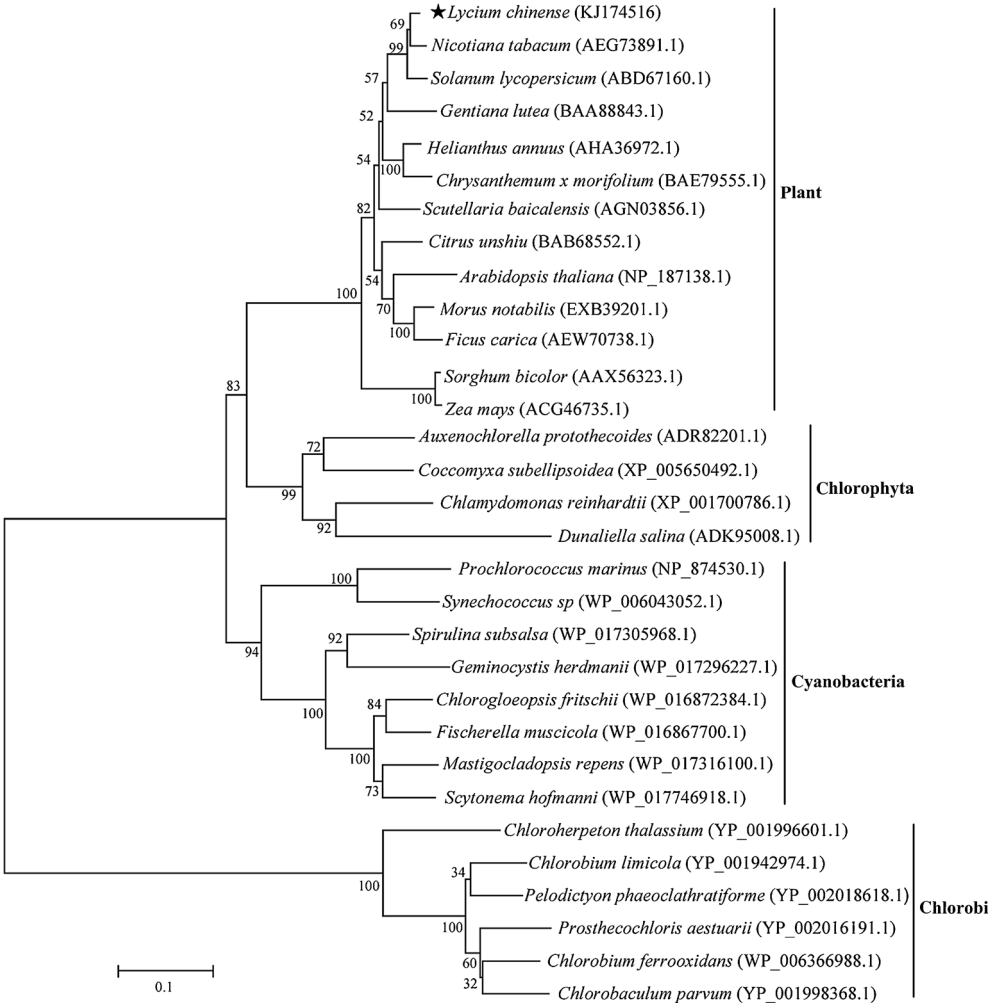

Figure 1b. Phylogenetic tree of ZDS from various species. The ⋆ symbol represents the position of the *L. chinese* ZDS on the phylogenetic tree. Numbers associated with the branches are bootstrap values ($n = 100$). The accession numbers of reference proteins of other species are in parentheses. Branch lengths expressing evolutionary distance are measured by its scale being 0.1.

Analysis using ChloroP and WoLF PSORT programs showed that there was a signal peptide of 49 amino acid residues in this polypeptide which was responsible for the targeting of LcZDS protein to chloroplast. Conserved domain prediction of NCBI database revealed that the enzyme contained a nicotinamide adenine dinucleotide (phosphate) (NAD(P))-binding Rossmann-fold domain cluster (superfamily) and a carotene 7,8-desaturase domain model (multidomain), further indicating that the obtained protein was a carotenoid desaturase ZDS. Phylogenetic tree for ZDS proteins was constructed using NJ method by MEGA4 software (figure 1b). Four defined clusters of ZDS could be verified. It was clearly observed that LcZDS had a closer relationship with those of higher plants and chlorophyta than with those of cyanobacteria and chlorobi.

Expression patterns of LcZDS in different tissues and under stress conditions

Quantitative real-time PCR analysis was employed to reveal the expression pattern of *LcZDS* in different tissues including root, stem, leaf, flower and fruits at four ripening stages (figure 2). The results indicated that *LcZDS* was expressed at the highest level in maturing fruits, at relatively high levels in leaf and fruit stage 3, at low levels in flower, stem, fruit stage 1 and fruit stage 2, and was barely detectable in root. Intriguingly, during four ripening stages of fruits, the transcript levels of *LcZDS* gradually increased from stages 1 to 3, and then rapidly reached a peak at stage 4. These would be in accordance with other model in which ZDS expression increased gradually as the fruits approached maturity (Yan *et al.* 2011). Hence, it was plausible to explain that higher levels of gene expression observed in fruit and leaf led to the higher activity of the enzyme in converting ζ-carotene to lycopene (red) and subsequent carotenoids. The above results suggested that the expression of *LcZDS* was tissue specific.

The expression pattern of *LcZDS* gene in response to abiotic stresses, including ABA, NaCl and drought, was continually monitored from the start of treatment to 24 h after treatment in leaves of the *L. chinense*. As shown in figure 2, drought and NaCl treatments rapidly induced the expression of *LcZDS*, and the NaCl treatment resulted in a relatively persistent effect from 6 to 12 h, then the transcript levels

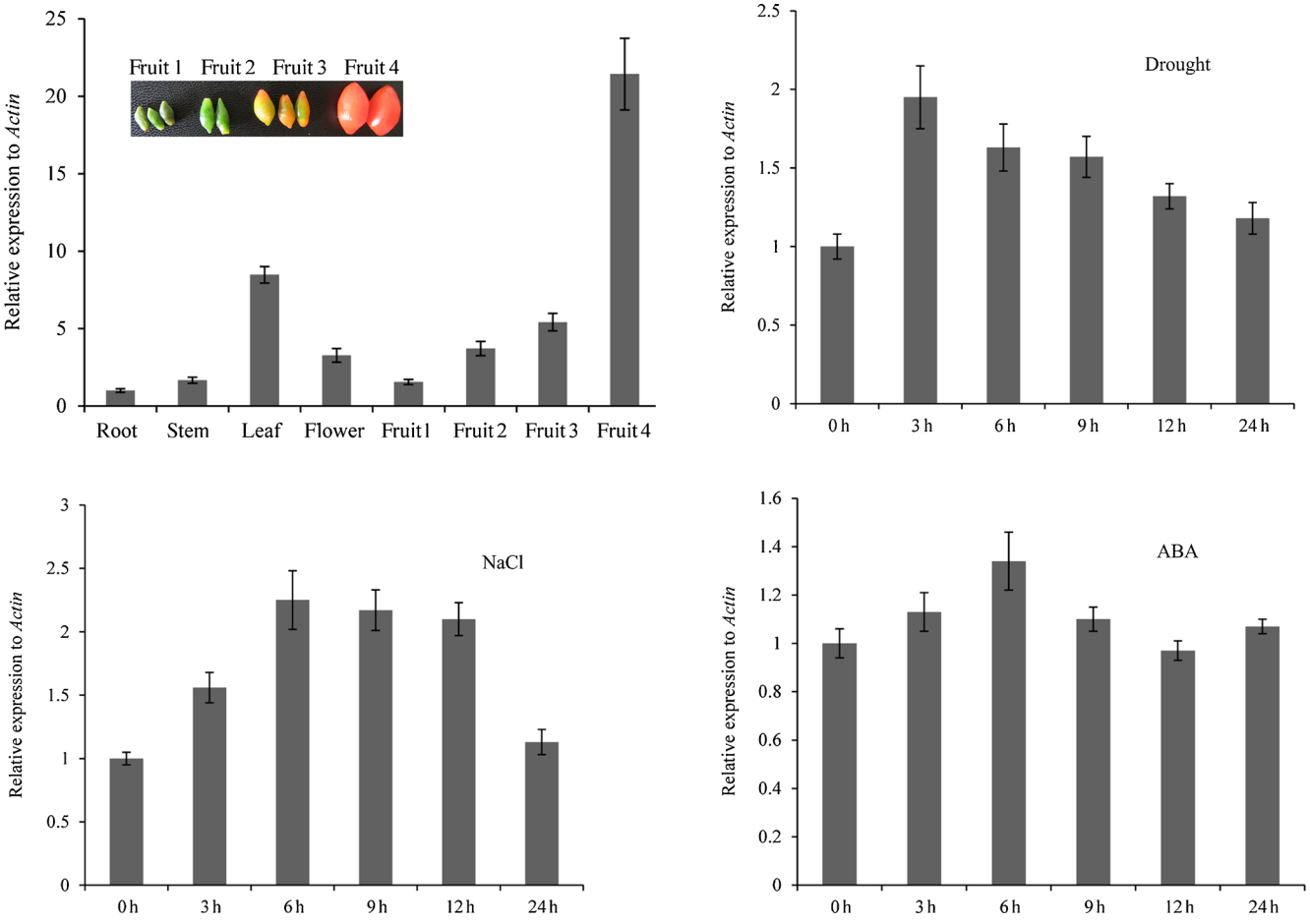

Figure 2. Expression patterns of *LcZDS* in different tissues and under stress conditions. Fruit 1, fruit stage 1 (10 DPA); fruit 2, fruit stage 2 (20 DPA); fruit 3, fruit stage 3 (30 DPA); fruit 4, fruit stage 4 (45 DPA). The *Actin* gene was used as a control to normalize the transcript level. Error bars exhibit the standard deviation of triplicate experiments.

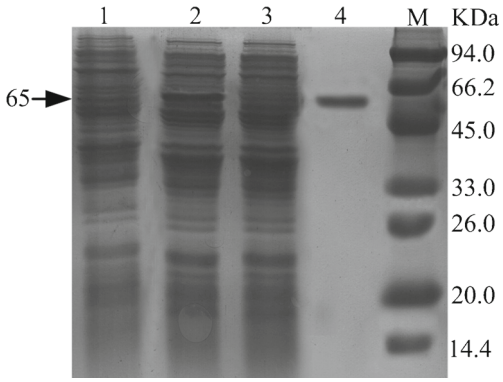

Figure 3. SDS-PAGE of LcZDS recombinant protein. M, protein marker; lanes 1 and 2, cell extracts from uninduced or induced bacteria harbouring pET-28a-*LcZDS*; lane 3, cell extract from induced bacteria containing pET-28a(+) empty vector; lane 4, the purified recombinant LcZDS.

subsequently decreased. In contrast, ABA exerted a negligible effect on the expression of *LcZDS*. The transcript level of *LcZDS* increased slightly at 6 h after ABA treatment and then declined back to its initial level. Consistent with our report, Du *et al.* (2010) indicated that the transcript of *CHYB* in rice was induced by drought and salt treatment and slightly induced by ABA. These results demonstrated that *LcZDS* was stress responsive and might play important roles in the plant stress response.

Expression of recombinant LcZDS protein

We constructed the recombinant plasmid pET-28a-*LcZDS* to express target protein for investigating the molecular weight. Expression of ZDS in IPTG-induced *E. coli* BL21 resulted in a very prominent additional protein band with an apparent molecular mass of 65 kDa, which was absent in empty vector cells. Further, the noninduced cells of pET-28a-*LcZDS* had a lighter stripe at the corresponding site. The purified recombinant LcZDS protein displayed a single band on the SDS-PAGE (figure 3).

Functional identification of LcZDS in E. coli

Colour complementation assay has been proven to be an effective and convenient method for identification and functional characterization of carotenoid biosynthetic genes (Cunningham *et al.* 1994). These experiments are based on the colour differences among carotenoids: ζ-carotene is yellow, neurosporene is deep yellow and lycopene is red (Araya-Garay *et al.* 2011). In the selected medium, the bacterial colonies of *E. coli* harbouring pACCRT-EBP and pET-28a-*LcZDS* grew well along with colour change from yellow to red. As excepted, the colour of cells containing pACCRT-EBI$_{RC}$ and pET-28a-*LcZDS* turned from deep yellow to red. The negative controls did not show changes in their colours (figure 4). These changes suggested a variation in the carotenoid composition from ζ-carotene or neurosporene to lycopene.

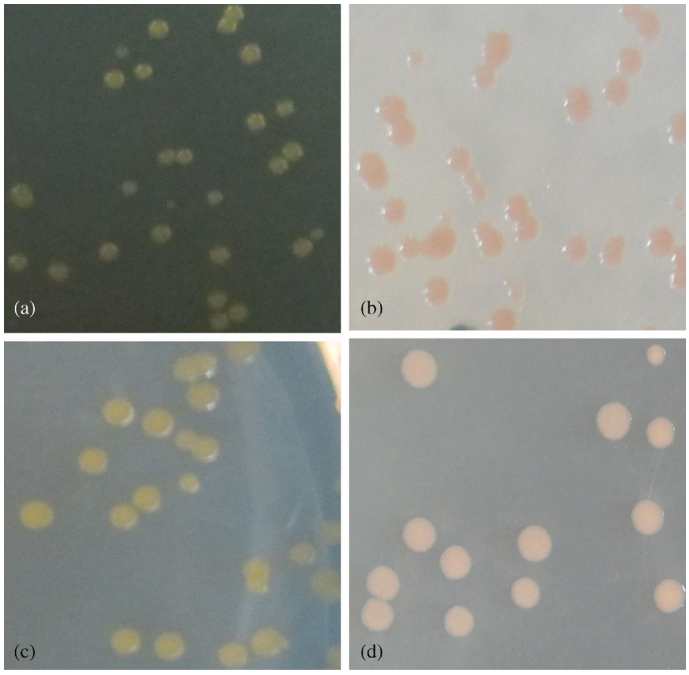

Figure 4. Colour complementation analysis. Colour difference of cells transformed with (a) pACCRT-EBP and pET-28a(+), (b) pACCRT-EBP and pET-28a-*LcZDS*, (c) pACCRT-EBI$_{RC}$ and pET-28a(+), or (d) pACCRT-EBI$_{RC}$ and pET-28a-*LcZDS*.

Table 1. Contents (μg/g dry weight) and compositions of carotenoids in four recombinant *E. coli* strains.

E. coli strain	ζ-Carotene	Neurosporene	Lycopene
pACCRT-EBP+ pET-28(a)+	207.63±19.25a	ND	ND
pACCRT-EBP+ pET-28a-*LcZDS*	128.41±15.37b	26.33±2.85c	53.96±6.14a
pACCRT-EBI$_{RC}$+ pET-28(a)+	ND	231.63±25.72a	ND
pACCRT-EBI$_{RC}$+ pET-28a-*LcZDS*	ND	180.44±17.53b	34.27±3.85b

Each value represents the mean ± standard error (SE) of three samples. Different letters in a column denote statistically significant differences ($P < 0.05$); ND, not detected.

High-performance liquid chromatography (HPLC) analysis was conducted to determine the compositions and contents of carotenoids extracted from transformants. The result demonstrated that neurosporene and lycopene contents of recombinant *E. coli* strain harbouring plasmids pACCRT-EBP and pET-28a-*LcZDS* were 26.33 and 53.96 μg/g of dry weight cells, respectively (table 1). Whereas lycopene content of the recombinant *E. coli* harbouring plasmids pACCRT-EBI$_{RC}$ and pET-28a-*LcZDS* was 34.27 μg/g of dry weight cells. Taken together, these results suggested that the polypeptide encoded by the *LcZDS*cDNA clone was a functional ζ-carotene desaturase which was able to act on both ζ-carotene and neurosporene, and converted ζ-carotene into lycopene via neurosporene in *E. coli*.

In summary, obtaining *ZDS* cDNA from *L. chinense* and examining its transcript levels in different tissues and under stress conditions would help in understanding the regulation mechanisms of *ZDS* in carotenoid accumulation and stress response. Further studies on introduction of *LcZDS* gene into heterologous host would provide a novel genetic material to enrich carotenoid contents and increase tolerance to environmental stress.

Acknowledgements

This work was supported financially by the National Natural Science Foundation of China (numbers 31271419 and 31271793) and the National Science and Technology Key Project of China on GMO Cultivation for New Varieties (number 2014ZX08003-002B).

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Received 13 September 2014, in revised form 7 November 2014; accepted 18 November 2014
Unedited version published online: 20 November 2014
Final version published online: 5 June 2015