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Cloning and Expression Analysis of 9-*cis*-Epoxycarotenoid Dioxygenase Gene 1 Involved in Fruit Maturation and Abiotic Stress Response in *Lycium chinense*

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Abstract Abscisic acid (ABA) plays a crucial role in plant adaptations to environmental stress, growth, and development, such as seed dormancy and germination. 9-*cis*-epoxycarotenoid dioxygenase (NCED) is a rate-limiting enzyme in regulation of ABA biosynthesis in plants. To understand the potential role of NCED in fruit ripening and stress tolerance, a *NCED* gene (*LcNCED1*) was cloned from the leaves of *Lycium chinense*. *LcNCED1* has an ORF of 1824 bp, which encodes a peptide of 607 amino acids. The deduced amino acid sequence of the LcNCED1 protein shares high identity with other NCEDs. Tissue distribution analysis reveals that *LcNCED1* is abundantly expressed in leaves, stems, and flowers. In fruits, the expression level of *LcNCED1* is in accordance with the accumulation of ABA. In addition, ABA accumulation in leaves was associated with enhanced expression of *LcNCED1* induced strongly by abiotic stresses (drought, salt, and $CdCl_2$). Collectively, our results indicated that *LcNCED1* might play a key role in the regulation of fruit ripeness and abiotic stress adaptation in *L. chinense* possibly through regulation of ABA biosynthesis.

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Introduction

In plants, abscisic acid (ABA) plays a key role in environmental stress adaptations and developments such as seed maturation, dormancy, and fruit development (Rodrigo and others 2006; Thompson and others 2000b; Zeevaart and Creelman 1988). Because of these important biological functions, the effectors that regulate ABA biosynthesis in plant tissues have received much research interest. Recently, biochemical and genetic approaches have shown that 9-*cis*-epoxycarotenoid dioxygenase (NCED)—which cleaves C40 *cis*-epoxycarotenoids at the 11,12 double bond to produce C15 xanthoxin, the direct precursor of ABA—is a rate-limiting enzyme in ABA biosynthesis (Chernys and Zeevaart 2000; Tan and others 2003; Tan and others 1997). The first *NCED* gene, *vp14*, was identified from a viviparous mutant of maize which exhibited a defect in ABA biosynthesis (Tan and others 1997). Further studies indicated that recombinant VP14 protein could catalyze the cleavage of 9-*cis*-xanthophylls into ABA in vitro (Chernys and Zeevaart 2000; Schwartz and others 1997). Since then, *NCED* genes have been identified in several plant species, including tomato (*Solanum esculentum*) (Burbidge and others 1999), cowpea (*Phaseolus vulgaris*) (Iuchi and others 2000), avocado (*Persea americana*) (Chernys and Zeevaart 2000), *Arabidopsis* (*Arabidopsis thaliana*) (Iuchi and others 2001), stylo (*Stylosanthes guianensis*) (Yang and Guo 2007),caragana (*Caragana korchinskii*) (Wang and others 2009), and grape (*Vitis vinifera*) (Soar and others 2004).

The abiotic stress-induced expression of NCED genes and their involvement in stress-induced ABA biosynthesis have been studied in several plant species (Burbidge and others 1999; Qin and Zeevaart 1999; Rodrigo and others 2006; Thompson and others 2000a). For example, in *Citrus* leaves, the *CsNCED1* mRNA transcript is greatly increased by dehydration, the ABA concentration significantly increased after 4 h of water stress, and by 6 h, it was 18 times higher than that at the beginning. In contrast, in nonstressed leaves, ABA concentrations did not increase, and *CsNCED1* transcripts could not be detected (Rodrigo and others 2006). Moreover, transgenic plants overexpressing *NCED* genes greatly increased ABA concentrations in plants and resistance to abiotic stress (Aswath and others 2005; Iuchi and others 2001; Qin and Zeevaart 2002; Thompson and others 2000b). These studies provided strong evidence for the regulatory role of NCED in ABA biosynthesis in plants under stress conditions.

In addition, NCED is also engaged in fruit development through regulation of ABA biosynthesis. Fruit ripening is a complex developmental process coordinated by the interaction of plant hormones which play a crucial role in the regulation of metabolic and physiological changes (Srivastava and Handa 2005). The phytohormone ABA may be associated with the regulation of nonclimacteric fruit ripening (Coombe 1992; Davies and others 1997; Giovannoni 2001; Zhang and others 2009b) and climacteric fruit ripening (Vendrell and Buesa 1989). In nonclimacteric fruits such as strawberries, the ABA concentration gradually accumulates with sugar accumulation during the late stage of fruit development (Jiang and Joyce 2003; Manning 1994). In climacteric fruits, ABA promotes transportation and accumulation of assimilation products to fruits during fruit ripening (Martínez-Madrid and others 1996). To date, many investigations have indicated that NCED is implicated in the ripening of several fruits, such as *P. americana* (Chernys and Zeevaart 2000); *Citrus sinensis* (Rodrigo and others 2006); and *V. vinifera* and *Prunus persica* (Zhang and others 2009b).

Lycium chinense is an important material for traditional Chinese medicine, as it contains many active chemical components, such as betaine, carotenoids, polysaccharides, and thiamine. As carotenoid levels in *L. chinense* fruits appear to be high enough as a precursor for ABA biosynthesis, the cleavage of 9-*cis*-xanthophylls is probably the regulatory reaction in ABA production. To understand the potential roles of NCED in fruit ripening and abiotic stress tolerance in *L. chinense*, we isolated a *NCED* cDNA (termed *LcNCED1*) from *L. chinense* leaves and characterized its gene expression patterns under abiotic stress conditions and during the development of fruits. Our results showed that the expression of *LcNCED1* in *L. chinense* fruits during natural maturation and abiotic-stressed leaves was significantly upregulated. More importantly, the upregulated expression of *LcNCED1* was consistent with the great accumulation of ABA, indicating that *LcNCED1* may play an important role in *L. chinenese* fruit ripening and abiotic stress adaptation probably through regulation of ABA biosynthesis.

Materials and Methods

Plant Materials

Tissues were collected from *L. chinense* trees which were grown on the campus of Tianjin University, Tianjin, China. *L. chinense* fruits at different developmental stages (based on days post-anthesis (DPA) defined later) were periodically collected from August to October, 2012. The first stage corresponded to small, green, and hard fruits (stage 1, very young green fruits, 8 DPA); the second stage (stage 2, young green fruits, 15 DPA) was composed of bigger and green fruits which were still hard; fruits at the breaking stage (first sign of color) represented the third developmental stage (stage 3, semimature green–red fruits, 25 DPA); and the fourth stage (stage 4, ripe fruits, 34 DPA) was composed of soft and red fully ripe fruits. For flowers, only petals were selected. All samples were quickly frozen in liquid N_2 and stored at −80 °C for RNA isolation.

RNA Extraction and cDNA Cloning

Total RNA was isolated from 100 mg of *L. chinense* fresh leaves using a RNeasy Plant Mini Kit (Qiangen) and quantified with a spectrophotometer at optical densities of 260 and 280 nm and stored at −80 °C. The first strand cDNA synthesis was accomplished using 1 μg total RNA with a 3′RACE adaptor primer provided by the 3′-Full RACE Core Set Ver.2.0 (TaKaRa, Osaka, Japan). To obtain the carboxyl terminal sequence of *LcNCED1*, a gene-specific primer RACE-*LcNCED1* (5′-ATGGCAACTACTT CTTCTCCTGC-3′) was designed based on the *L. chinense* transcriptomic sequences, and the 3′ RACE-PCR was carried out using the first strand cDNA obtained above as the template in a 25 μl mixture with the primer pair RACE-*LcNCED1* and 3′ RACE outer primer (5′-TACCGTC GTTCCACTAGTGATTT-3′). The PCR procedure was set as per the following: denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, followed by a 8-min extension at 72 °C. The PCR product was cloned to the pMD-18T vector for sequencing. Three independent clones were selected and sequenced on both strands which showed 100 % sequence identity.

DNA Sequence Analysis

Sequence identity of LcNCED1 was determined by a homology search of the NCBI database using the BLAST program. Multiple alignments of the deduced amino acid sequences and phylogenetic analysis were carried out by DNAMAN 6.0 and MEGA 4.1 software, respectively. The molecular weight and isoelectric point of LcNCED1 were calculated through the online tools (http://www.bioinformatics.org/sms/). The subcellular localization of the LcNCED1 protein was predicted by the iPSORT algorithm (http://hc.ims.u-tokyo.ac.jp/iPSORT/).

Dehydration Treatment

Lycium chinense seedlings were grown from cutting propagation under greenhouse conditions. For dehydration treatment, the seedlings were removed from soil carefully to avoid injury and subjected to dehydration on 3 MM Whatman paper at room temperature and approximately 60 % humidity under dim light (300 lux), and the leaves were collected 0, 0.5, 1, 3, 6, and 12 h after the dehydration treatment (Iuchi and others 2000). The samples were frozen immediately in liquid nitrogen and stored at −80 °C for later semiquantitative RT-PCR experiments.

Stress and Phytohormone Treatments

For high salt, ABA and $CdCl_2$ treatments, plants were removed from soil as described previously in the dehydration treatment, and grown in Hoagland's nutrient solution for 3 days. The plants were then transferred to Hoagland's solutions supplemented with 300 mM NaCl, 100 μm ABA, and 500 μM $CdCl_2$, respectively. In each case, the plants were subjected to the stress treatments for 0, 1, 3, 6, 9, 12, and 24 h. The leaves sampled at each time point were frozen in liquid nitrogen, and stored at −80 °C for later RNA extraction.

Semiquantitative RT-PCR Analysis of *LcNCED1* Gene Expression

LcNCED1 mRNA expression was evaluated in vegetative organs (leaves, stems, and roots) and in reproductive tissues (flowers and fruits) by semiquantitative RT-PCR analysis. One microgram of total RNA was used for cDNA synthesis with TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China) according to the manufacturer's instructions. After reverse transcription, *LcNCED1* was amplified by PCR with specific primers (F1: 5′-TGGCGATGGTATGGTACATGCT-3′ and R1: 5′-CAACGAATTTCTCCGTTATCGCG-3′) designed from the conserved region of *LcNCED1* cDNA sequence. A parallel amplification with the gene-specific primer pair (AF1: 5′-GGAAACATAGTGCTCAGTGGTG-3′ and AR1: 5′- GCTGAGGGAAGCCAAGATAG-3′) for the *actin* gene was performed as an internal control for each assay. Each PCR was carried out in triplicate.

Determination of ABA Concentration

ABA concentration analysis was performed according to the methods of Zhang and others (2009b) with slight modification. In brief, 1 g of tissue was quickly frozen in liquid N_2 and ground into fine powder. ABA was extracted with 30 mL of cold 80 % methanol containing 0.5 g polyvinylpolyrrolidone at 4 °C for 24 h in dark, and then centrifuged at 4000 rpm for 10 min. The upper phase containing ABA was collected and filtered using a C18 filter, then extracted with ethy acetate (pH 2.5). After the removal of ethyl acetate by vacuum drying, the pellets containing ABA were dissolved in anhydrous ethanol, and finally filtered through a 0.45-μm filter. ABA was analyzed by high-performance liquid chromatography (HPLC) using a Kromasil C18 column (250 × 4.6 mm, 5 μm). The mobile phase for HPLC assay was methanol:water (45:55, V/V) with a flow rate of 1 mL min^{-1}. ABA concentrations in samples were quantified with an external standard (Sigma).

Carotenoid Analysis

Carotenoids were measured by HPLC as previously described by Zhao and others (2014) with small modification. Freeze-ground *L. chinense* tissue (0.5 g) was suspended in 20 mL methanol with 10 % potassium hydroxide, and then incubated at 60 °C for 20 min. The carotenoids were extracted by 50 % ether in petrol ether. Total carotenoid concentration was determined by spectrophotometer. The maximum absorbance peaks were registered, and the total carotenoid concentration was calculated by measuring the absorbance at 450 nm using the extinction coefficient of β-carotene, $E^{1\ \%} = 2500$.

For HPLC analysis, the solvent in samples was evaporated under a stream of N_2 gas at 37 °C and dissolved in 1 mL acetone and passed through a 0.45-μm nylon filter. Twenty μL of aliquot was then injected immediately. The samples were separated on a nucleosil 100-3 C18 column at column temperature of 32 °C, flow rate of 1 mL min^{-1}, and with the mobile phase consisting of acetonitrile/methanol/isopropanol (85:10:5, volume ratio).

Determination of Ethylene Release

Ethylene release was measured by the methods of Zhang and others (2009b). In brief, harvested fruits were kept under

ambient conditions overnight to reduce harvest shock. Fruits of *L. chinense* were then incubated in 1-L airtight containers at 20 °C for 1 h. One mL of the gas phase was taken out from the container for ethylene determination using a gas chromatograph fitted with a flame ionization detector and an activated alumina column (3 m * 4.5 mm). Measurement conditions were as follows: column temperature, 60 °C; detector temperature, 200 °C; carrier gas (N_2) flow rate, 40 mL min^{-1}; and hydrogen pressure of 0.6 kg cm^{-2}.

Determination of Soluble Sugar

Soluble sugar in fruits of *L. chinense* was measured by the methods of Jia and others (2011). In brief, samples (0.5 g) were ground to powder with liquid nitrogen and extracted with 10 mL of 80 % ethanol in a water bath for 3 min at 80 °C, and then centrifuged at 10,000×*g* for 10 min; the supernatant was collected in a 100-mL triangular flask. The residues were mixed with 10 mL of 80 % ethanol, incubated in a water bath at 80 °C for 20 min, and then centrifuged at 10,000×*g* for 20 min. The entire process was repeated twice, and the collected supernatants were combined. The supernatant was then evaporated and washed twice with 20 mL of distilled water; the volume was finally adjusted to 50 mL, and 2 mL of the adjusted solution was used for LC-18 solid-phase extraction. Afterward, the extraction was passed through a 0.45-μm membrane, and the soluble sugar concentration was determined using HPLC (LC-20AT) equipped with a RID-10A detector. The parameters were set as follows: acetonitrile/ultrapure water (70/30, v/v) as mobile phase, a flow rate of 1 mL min^{-1}; a column (NUCLEOSIL NH_2 100A) temperature of 30 °C; and an injection volume of 20 μL. The sugar concentration in the samples was calibrated with the standards of D-(+)Glc, D-(−)Fru and Suc (Sigma-Aldrich).

Results

Isolation of *LcNCED1* Gene

A full-length cDNA sequence, *LcNCED1*, was cloned from the leaves of *L. chinense* by RT-PCR. *LcNCED1* has an ORF of 1824 bp, which encodes a peptide of 607 amino acids with an estimated molecular weight of 67.52 kDa and the isoelectric point of 6.40. The cDNA sequence has been deposited in the NCBI nucleotide sequence database under accession number KJ123695.

As shown in Fig. 1, multiple sequence alignment of selected NCED1 proteins shows that LcNCED1 protein shares 90, 90, 76, and 70 % sequence identities with the NCED1 s from *Solanum tuberosum* (NM_001288174), *S. esculentum* (Z97215), *V. vinifera* (NM_001281270), and *Zea mays* (NM_001154055), respectively. Sequence analysis revealed that LcNCED1 has four conserved histidine residues which are essential for the coordination of the iron cofactor in the carotenoid cleavage dioxygenase family (Schwartz and others 1997; Tan and others 1997). LcNCED1 also possesses a conserved RPE65 domain, a characteristic feature of enzymes involved in apocarotenoid biosynthesis (Kloer and Schulz 2006). In addition, the N-terminal region of the LcNCED1 protein has a typical structural feature of transit peptides that are involved in chloroplast targeting, suggesting that the LcNCED1 protein may be localized in plastids. Phylogenetic analysis showed that LcNCED1 was homologous to other known NCEDs (data not shown), and displayed a closer relationship with StNCED1 and SeNCED1, two NCED members involved in ABA biosynthesis (Destefano-Beltrán and others 2006; Iuchi and others 2001; Thompson and others 2000b). Finally, LcNCED1 also shows some homology in amino acid sequence with the CCD class (data not shown), which is involved in some flavor and aroma compounds and strigolactone (SL) biosynthesis in plants (Booker and others 2004; Ibdah and others 2006; Schwartz and others 2001; Simkin and others 2004a, 2004b).

Expression Patterns of *LcNCED1* Gene

The expression patterns of the *LcNCED1* gene in different tissues (roots, stems, leaves, and flowers) and fruits at four developing stages were studied by semiquantitative RT-PCR. Fig. 2a shows that the dominant expression of *LcNCED1* was found in stems, leaves, and flowers, and lower expression was found in roots.

In fruits, *LcNCED1* gene expression increased slowly at the first and second stages and reached maximal expression at the third stage, then declined greatly at the fourth stage (Fig. 2b). Associated with the decreased expression of *LcNCED1* at the fourth stage, *L. chinense* fruits colored and softened rapidly, and ripened fully. Interestingly, the concentration changes of ABA were consistent with the expression pattern of *LcNCED1* during fruit development (Fig. 2b, c). These results indicated that expression of *LcNCED1* during fruit development and ripening may contribute importantly to the accumulation of ABA. The expression pattern of *NCED1* during fruit development has also been observed in other plants (Chernys and Zeevaart 2000; Rodrigo and others 2006; Zhang and others 2009b).

Ethylene and Sugar Concentration Changes During *L. chinense* Fruit Development

ABA was shown to trigger ethylene emission (Buesa and others 1994; Zhang and others 2009b) and promote sugar

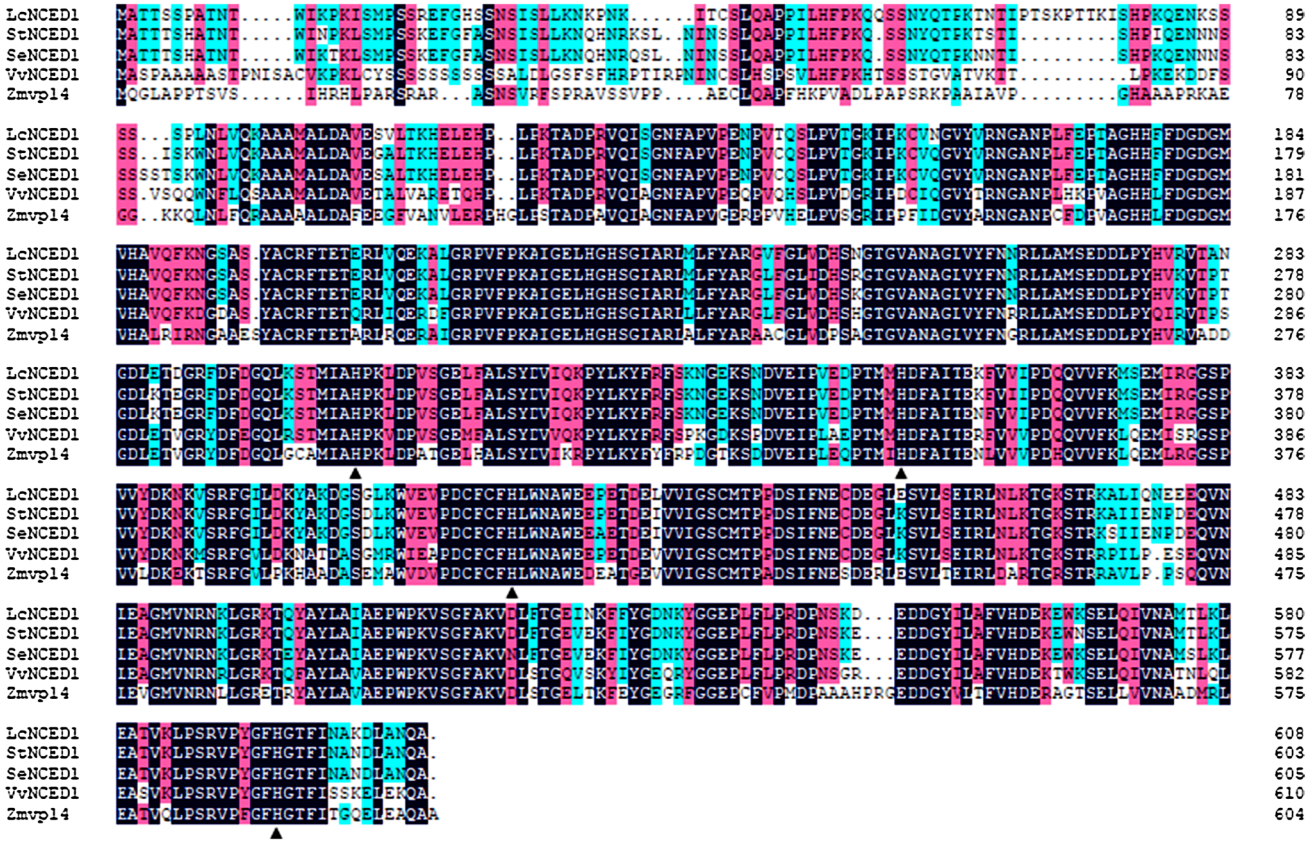

Fig. 1 Alignment of the predicted amino acid sequence of LcNCED1 and NCED1 proteins from other plants. The GenBank accession numbers are *Solanum tuberosum* (StNCED1, NM_001288174), *Solanum esculentum* (SeNCED1, Z97215), *Vitis vinifera* (VvNCED1, NM_001281270), and *Zea mays* (Zmvp14, NM_001154055), respectively. The *triangle* indicates the four histidine residues

metabolism and accumulation in fleshy fruits (Pan and others 2005; Richings and others 2000; Sun and others 2012a). During *L. chinense* fruit development, ethylene production was lower at the first three stages (Fig. 2c). After the third developmental stage, ABA concentration began to decrease in *L. chinense* fruits, and the concentration of ethylene increased considerably, which is associated with decrease in rapid fruit firmness (Fig. 2c). Sugars are not only contributing to the soluble solids, but are also essential to the flavor intensity in fruits. During *L. chinense* fruit development, the concentration of glucose and fructose increased rapidly, especially after the third stage (Fig. 2d). The increased glucose and fructose concentrations in fruits may be due to the enhanced expression of *LcNCED1* which significantly promoted ABA biosynthesis, because ABA has been shown to promote sugar accumulation during the late stage of fruit development (Jia and others 2011; Jiang and Joyce 2003; Richings and others 2000).

LcNCED1 Expression and ABA Accumulation During Abiotic Stress in *L. chinense*

To explore the involvement of *LcNCED1* in stress-induced ABA biosynthesis, we measured the accumulation of endogenous ABA levels and the expression of the *LcNCED1* gene in *L. chinense* plants during drought stress conditions. As shown in Fig. 3a, in leaves, the expression of the *LcNCED1* gene was strongly induced by drought stress as early as 3 h after dehydration, peaked at 6 h, and then declined at 12 h. At the same time, ABA concentration in leaves also accumulated gradually after dehydration (Fig. 3b). These results suggest that the increased ABA biosynthesis may be due to the enhanced expression of *LcNCED1* under the water-deficit stress condition.

We further investigated the effect of salt stress on *LcNCED1* mRNA expression. As shown in Fig. 4a, b, the expression of *LcNCED1* and ABA accumulation in leaves

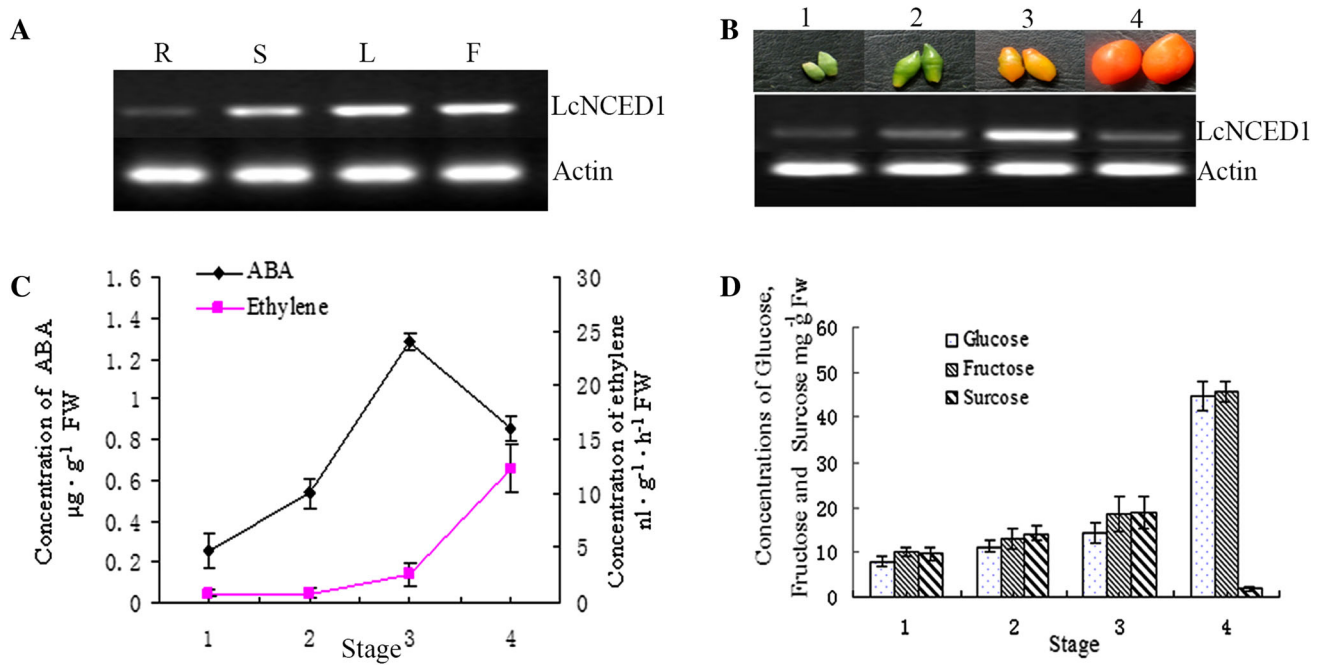

Fig. 2 Expression patterns of the *LcNCED1* gene in different tissues of *L. chinense* by semiquantitative RT-PCR. **a** Expression of *LcNCED1* in R (roots), S (stems), L (leaves), and F (flowers). **b** Expression of *LcNCED1* in fruits at different developing stages (1, very young green fruits; 2, young green fruits; 3, semimature green–red fruits; and 4, ripe fruits). **c** Concentration changes of endogenous ABA and ethylene production during fruit development and ripening in *L. chinense*. **d** Concentration changes of sugars during *L. chinense* fruit ripening (Color figure online)

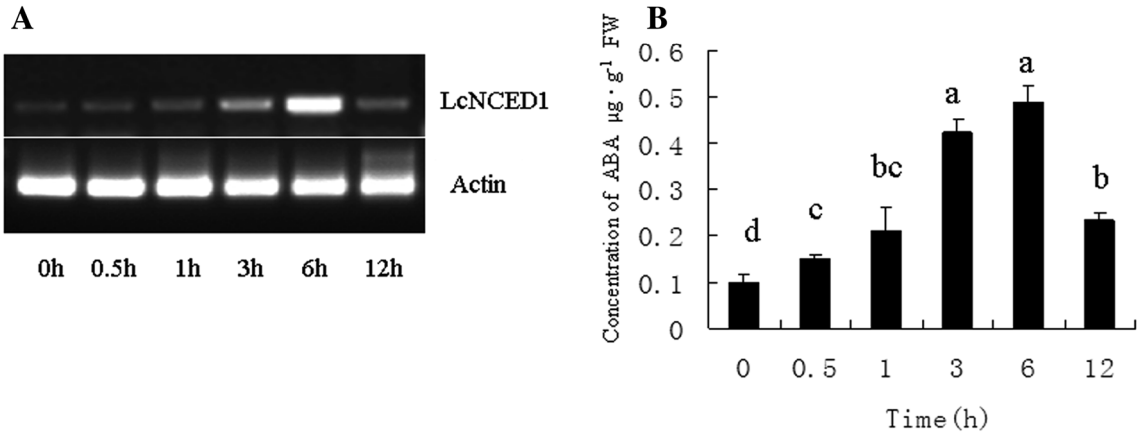

Fig. 3 ABA concentration and *LcNCED1* transcript in the leaves of *L. chinense* under dehydration condition. **a** The expression level of *LcNCED1* mRNA transcripts. **b** Concentration of ABA. Values are presented as mean ± standard deviation of three measurements. Data denoted with *different lowercase letters* indicate significant difference ($P < 0.05$) among treatments

of *L. chinense* were induced strongly by salt stress. The expression of *LcNCED1* was gradually increased at 3 h after the salt treatment, and reached the highest expression level at 9 h (Fig. 4a). ABA concentration also increased slightly during the salt treatment and reached the highest level at 9 h (Fig. 4b). These results indicated that salt stress induced *LcNCED1* gene expression and ABA accumulation in *L. chinense*.

To test the effect of hormone treatment on *LcNCED1* gene expression, *L. chinense* plants were subjected to ABA treatment. As shown in Fig. 4c, the *LcNCED1* gene expression was significantly upregulated by ABA treatment. *LcNCED1* mRNA expression increased at 3 h after exogenous ABA administration and peaked at 9 and 12 h, suggesting a positive feedback regulation of ABA in *LcNCED1* expression. Associated with the increased

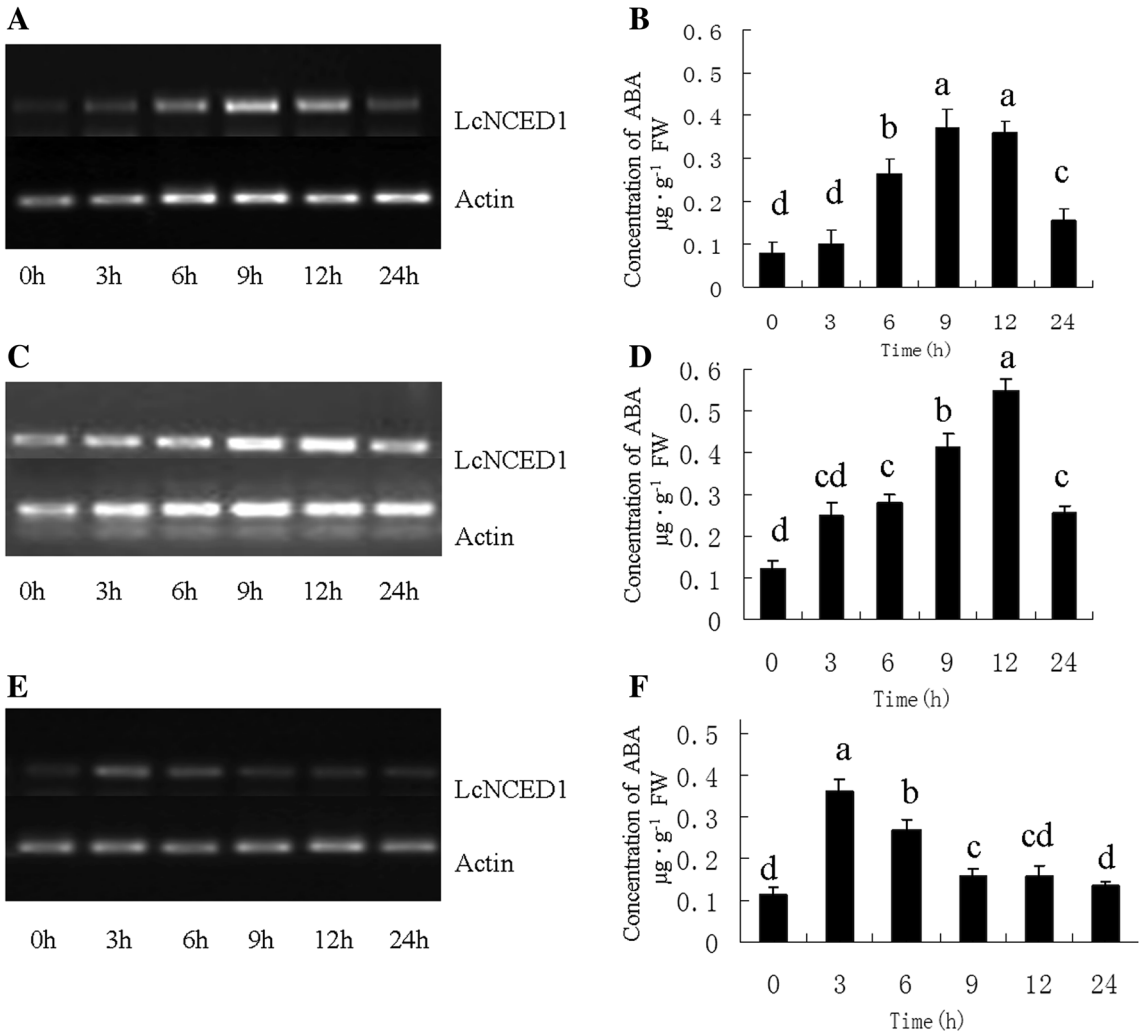

Fig. 4 ABA concentration and *LcNCED1* expression level in *L. chinense* leaves treated with NaCl, ABA, and $CdCl_2$. The induction of *LcNCED1* expression treated by NaCl (**a**), ABA (**c**), and $CdCl_2$ (**e**), respectively; the effects of different treatments of NaCl (**b**), ABA (**d**), and $CdCl_2$ (**f**) on ABA concentration. Values are presented as mean ± standard deviation of three measurements. Data denoted with *different lowercase letters* indicate significant difference ($P < 0.05$) among treatments

expression of *LcNCED1*, ABA also accumulated persistently in *L. chinense* leaves and reached the highest level at 12 h (Fig. 4d).

Heavy metal ($CdCl_2$) stress also induced the expression of *LcNCED1* and ABA accumulation (Fig. 4e, f). *LcNCED1* mRNA transcript expression was induced to the highest level after 3 h of $CdCl_2$ stress; ABA rapidly accumulated on the same course.

Discussion

It has been well established that during development (fruit ripening) and physiological changes (wilting), ABA biosynthesis in plants is regulated by NCED expression levels (Chernys and Zeevaart 2000; Iuchi and others 2000; Rodrigo and others 2006). In the present study, a *NCED* cDNA, named *LcNCED1*, was isolated from the leaves of *L. chinense*. Sequence similarity and phylogenetic analyses of LcNCED1 with known NCED1 proteins indicate that LcNCED1 represents a new member of the carotenoid cleavage dioxygenase family.

Interestingly, we found that the *LcNCED1* gene expression level was correlated to ABA accumulation (Fig. 2b, c) during *L. chinense* fruit natural maturation, indicating that in fruits, ABA biosynthesis may be regulated by *LcNCED1*. It has been suggested that ABA can significantly stimulate ethylene production in fruits (Riov and others 1990; Zhang and others 2009a), because increasing ABA to a certain level can promote the transformation of ACC to ethylene (Hermann and others 2007; Lara and Vendrell 2000a, 2000b). Thus, ABA may act on

upstream metabolic events of ethylene action/perception and then inhibit or activate the general metabolic events initiating the ripening process. The accumulation of ABA preceded the production of ethylene, suggesting that endogenous ABA, but not ethylene, was critical for the beginning of ripening (Zhang and others 2009a).

In addition, sugars, such as Glc (glucose), may act as signal molecules and play key roles in plant development and stress responses (Leon and Sheen 2003; Rook and others 2006). The interaction of the Glc signal with ABA is responsible for the induction of senescence and pigment biosynthesis, and Glc modulates the transcription of genes involved in ABA biosynthesis (Jia and others 2011). Thus, the significant increase in sugar (Glc and Fru) concentrations during development of *L. chinense* fruits may be associated with the concentration changes of ABA (Fig. 2d). To date, several investigations have indicated that NCED is engaged in fruit development through regulation of ABA biosynthesis (Chernys and Zeevaart 2000; Rodrigo and others 2006; Zhang and others 2009a). In avocado, *PaNCED1* and *PaNCED3* are both strongly induced during fruit ripening, and ABA levels in ripe fruits are 30-fold higher than the levels in unripe fruits (Chernys and Zeevaart 2000). The suppressed expression of *SlNCED1* in tomato, however, resulted in a firmer texture of fruit and longer shelf life through downregulation of the biosynthesis of ABA which affects cell wall catabolism during tomato fruit ripening via downregulation of the expression of major catabolic genes of the cell wall (Sun and others 2012a; Sun and others 2012b).

We further analyzed the putative substrate concentrations of LcNCED1 in different tissues of *L. chinense*. The most abundant 9-*cis*-xanthophylls were found in leaves and ripe fruits, whereas in roots, 9-*cis*-xanthophylls were not detected (Table 1). These results indicate that the lower expression level of *LcNCED1* in roots may be the reason that there are not enough substrates for ABA biosynthesis in roots (Fig. 2a; Table 1). During fruit ripening, the *LcNCED1* expression level was in accordance with concentrations of 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin, indicating that *LcNCED1* gene expression may be induced by their substrate concentration. After the third development stage, although 9-*cis*-xanthophyll concentrations were slightly increased (Table 1), *LcNCED1* expression declined rapidly, indicating that, due to the abundant expression of *LcNCED1* at the beginning of fruit maturation, ABA rapidly accumulates and continuously remains at a high level to complete physiological and biochemical reactions during fruit ripening (Fig. 2b, c) (Zhang and others 2009a). It is suggested that 9′-*cis*-neoxanthin is the main precursor of xanthoxin in plants due to the relatively low abundance of 9-*cis*-violaxanthin and high Km for the cleavage in green tissues (Iuchi and others 2000; Schwartz and others 2003). However, analysis of the concentration and composition of 9-*cis*-xanthophylls in different tissues of *L. chinense* showed that the endogenous level of 9-*cis*-violaxanthin is much higher than that of 9′-*cis*-neoxanthin in all tested tissues (Table 1). Which 9-*cis*-xanthophyll is the major substrate for LcNCED1 needs to be addressed in the future study.

ABA is involved in dehydration stress responses because it can promote stomatal closure to reduce water loss by transpiration (Zeevaart and Creelman 1988), and induce the expression of stress-related genes such as *rab18, kin1*, and *rd29b* (Iuchi and others 2001; Zeevaart and Creelman 1988). As the putative rate-limiting enzyme for ABA biosynthesis, the NCED1 gene was strongly induced in leaves and stems by drought stress, which was associated with endogenous ABA accumulation (Iuchi and others 2000; Qin and Zeevaart 1999; Yang and Guo 2007). However, the responses to other abiotic stresses are complicated. The expression of cowpea *VuNCED1* was strongly induced by drought stress and high-salt stress, but not by cold and heat (Iuchi and others 2000). In addition, *PvNCED1* expression was induced by water stress at 7 °C, but not at 2 °C (Qin and Zeevaart 1999). In the present study, dehydration and salt stress induced marked *LcNCED1* expression and endogenous ABA accumulation in leaves of *L. chinense*. These results indicated that the induction of *LcNCED1* is mainly responsible for ABA biosynthesis under drought and salt conditions. In addition, several studies indicated that over-expression of *NCED* genes in transgenic plants enhanced ABA accumulation and increased the tolerance to drought (Iuchi and others 2001; Qin and Zeevaart 2002; Wan and Li 2006) and salt stress (Aswath and others 2005). By contrast, repression of *AtNCED3* downregulated endogenous ABA levels in antisense transgenic mutants (Iuchi and others 2001).

Many biosynthetic pathways are regulated by their end products. ABA has long been thought to negatively regulate ABA accumulation by activating its degradation (Wan and Li 2006). In *Arabidopsis, AtNCED3* gene expression could be induced by ABA (Cheng and others 2002; Xiong and others 2002). However, it has been reported that the *VuNCED1* gene was not induced by exogenous ABA application in cowpea (Iuchi and others 2000). In our studies, the mRNA transcript of *LcNCED1* was also upregulated strongly by application of exogenous ABA, indicating that the *LcNCED1* expression level may be subject to feedback regulation by ABA.

Finally, $CdCl_2$ also induced a slight accumulation in *LcNCED1* mRNA and ABA accumulation, which provided the first evidence that $CdCl_2$ increases ABA levels through a stimulation of *NCED* gene expression.

In conclusion, a *NCED* gene (*LcNCED1*) was isolated from the leaves of *L. chinense*. Expression analysis reveals that the dominant expression of *LcNCED1* was found in

Table 1 Concentration of total carotenoids, 9-*cis*-violaxanthin, and 9′-*cis*-neoxanthin in different tissues of *L. chinense*

Tissues	9′-*cis*-neoxanthin (μg g^{-1} DW)	9-*cis*-violaxanthin (μg g^{-1} DW)	Total carotenoid (μg g^{-1} DW)
Roots	N.D.	N.D.	6.17 ± 0.3f
Stems	1.24 ± 0.3c	6.19 ± 0.5c	55.54 ± 4.8d
Leaves	4.56 ± 0.6a	26.13 ± 1.1a	403.2 ± 33.1b
Flowers	0.92 ± 0.1d	5.28 ± 0.7cd	81.48 ± 5.1d
Fruit 1	0.4 ± 0.1e	3.93 ± 0.4e	37.71 ± 5.5e
Fruit 2	0.58 ± 0.1e	4.42 ± 0.7de	80.23 ± 10.2d
Fruit 3	2.42 ± 0.6b	18.76 ± 1.2b	186.51 ± 21.5c
Fruit 4	2.18 ± 0.3bc	20.93 ± 1.2b	808.05 ± 68.2a

Fruit1, Fruit2, Fruit3, and Fruit4 indicate the four stages (1, very young green fruits; 2, young green fruits; 3, semimature green–red fruits; 4, ripe fruits) of fruit development. Values are presented as mean ± standard deviation of three measurements. Data denoted with different lowercase letters indicate significant difference ($P < 0.05$) among treatments

N.D. not detected

stems, leaves, flowers, and fruits, and lower expression of *LcNCED1* was in roots. During fruit ripening, the expression level of *LcNCED1* is in accordance with concentrations of ABA, indicating that *LcNCED1* may play a key role in fruit maturation through regulation of ABA biosynthesis. In addition, water deficit, salt stress, and $CdCl_2$ stress induced *LcNCED1* expression in a pattern consistent with the accumulation of ABA, suggesting that *LcNCED1* is also involved in stress tolerance.

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