Molecular cloning and characterization of a novel microsomal oleate desaturase gene *DiFAD2* from *Davidia involucrata* Baill.

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Abstract

In the conversion of oleic acid to linoleic acid, δ^{12}-fatty acid desaturase (δ^{12}-FAD) is involved. Based on the conserved oligo amino acid residues of the *FAD2* genes from other plants, a new full-length cDNA (*DiFAD2*) encoding a δ^{12}-FAD was cloned from *Davidia involucrata* Baill. Sequence analysis indicated that the *DiFAD2* gene had an open reading frame (ORF) of 1 149 bp, coding for 382 amino acids residues of 44.3 kDa, pI of the deduced protein was 8.8. The deduced amino acid sequence of the cloned *DiFAD2* showed high identities to those genes of other plant δ^{12}-FAD. RT-PCR showed that *DiFAD2* was expressed in all tissues and expression was abundant in young stems. Expression of *DiFAD2* is not enhanced by low temperature and the altered polyunsaturated fatty acid content in leaves treated with low temperature may be due to the post-transcriptional regulation of the *DiFAD2* gene or the other *FAD2* gene family regulation.

Additional key words: amino acid residues, open reading frame, *Saccharomyces cerevisiae.*

Introduction

Fatty acids in plants play vital roles. They are major structural components of membrane lipids, provide a substantial reserve of free energy and serve as key precursors for the biosynthesis of messengers in signal transduction mechanisms that influence plant growth, development and responses to various environmental factors (Pirtle *et al*. 2001, Wei *et al*. 2004, Li *et al*. 2006). Fatty acid desaturases (FADs) are enzymes responsible for the insertion of double bonds into fatty acyl chains, following the removal of two hydrogen atoms. These desaturation processes take place in both the plastidial membranes and the endoplasmic reticulum (ER) membranes *via* two different pathways (Thelen 2002). *FAD2* gene encodes the δ^{12}-FAD converting oleic acid to linoleic acid, which appears to be important in the metabolic pathway of lipid synthesis (Okuley *et al*. 1994), since polyunsaturated membrane phospholipids are essential for maintaining cellular function and plant viability at low temperature (Browse 2001). A knowledge of the tissue-specific and development-specific regulation of fatty acid compositions of the membranes is crucial in understanding the mechanisms underlying heat and cold tolerance, desiccation, salt tolerance, and disease resistance in higher plants (Heppard *et al.* 1996, Pirtle 2001, Nakamura *et al*. 2004). With better understanding of fatty acid metabolic pathway, many desaturase genes were cloned from plants such as *Arabidopsis*, *Helianthus annuus, Zea mays*, *Glycine max* and *Sapium sebiferum* (Okuley *et al*. 1994, Martínez-Rivas *et al*. 2001, Tao *et al*. 2006, Li *et al*. 2006, Niu *et al*. 2007). However, little information is available on the regulation of fatty acid biosynthesis of *Davidia involucrate* Baill., though its seeds have a high oil content (48 %; m/m).

Thus, in order to explore the regulatory mechanism of oleate desaturation of *D. involucrata,* we cloned a microsomal oleate desaturase gene (*DiFAD2)* from *D. involucrata* and demonstrated its function by expression in yeast (*Saccharomyces cerevisiae*). Meanwhile, the expression pattern of this gene was investigated in different tissues and in response to low temperature.

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Abbreviations: *DiFAD2 - Davidia involucrata* δ^{12}-fatty acid desaturase; *Di18S rRNA - Davidia involucrata 18S rRNA*; ORF - open reading frame; PCR - polymerase chain reaction; Mr - molecular mass; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcriptase-polymerase chain reaction; PUFA - polyunsaturated fatty acids; ER - endoplasmic reticulum.
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Materials and methods

The *Davidia involucrata* Baill. 60-d-old seedlings were collected in Dujiangyan, Chengdu, Sichuan, China. Plants were grown in growth chambers with 12-h photoperiod (irradiance of 400 μmol m^{-2} s^{-1}), air humidity 80 % and day/night temperatures of 28/22, 18/12, 12/8 and 8/4 °C. Leaf, stem, and root tissues were collected from the seedlings grown at different temperatures after 7 d and stored at -80 °C.

RNA was extracted from young roots, stems, and leaves as described by Zhang *et al*. (2006). RNA quality and concentration were checked by agarose gel electrophoresis (*Sigma*, St. Louis, USA) and by spectrophotometry. Total RNA was then treated with Rnase-free DNAse I (*Invitrogen*, USA) and stored at -80 °C untill for RACE and RT-PCR experiments.

RNA (2 μg) was used as template in first strand cDNA synthesis using SuperscriptTM II RNase H-Reverse Transcriptase (*Invitrogen*), according to the manufacturer's protocol. with the reverse transcription primer oliglo (dT):5'-GTCGACCTCGAGTTTTTTTTTTTT TTTTT-3'. cDNA used as a template for RT-PCR amplification with *DiFAD2* primers:P1: 5'-CG(ATC)CG (TCG)CACCA(CT)TCCAACAC-3' and P2: 5'-CCC(GT) (ATCG)A(GA)CCA(AG)TCCCATTC-3'. PCR products (451bp) were cloned and sequenced. Partial sequence for *DiFAD2* that showed high sequence identity to *DiFAD2* like genes were identified. The full-length cDNA clone was obtained by 3' and 5' RACE method. Based on the nucleotide sequence of the above partial cDNA sequence, the gene-specific primers were designed for the 3'and 5' RACE amplification of full length cDNA of *DiFAD2*. The primers of 3P1: 5'-CTGATGCTGGTGTTATTGC-3' and 3P2:. 5'-CTGCCTCACTATGATTCGTCC-3'; 3RP: 5'-GTCAACGATACGCTACGTAACG-3', 3RNP: 5'-TACGTAACGGCATGACAGTG-3' were used for 3' RACE , 5P1: 5'-GCAGCAATAACACCAGCATC-3', 5P2: 5'-CGGGTTGTTCAAGTATTGGGAGTA-3'; AP1: 5'-ACGACTCACTATAGGGCTTTTTTTTTTTTMN-3' (N=A,C,G or T;M=A,G or, AP2; 5'-GTAATACGACTCA CTATAGGGC-3') were used for 5'RACE，the reverse transcription primer(RT): 5'- AGAAAGCCATTCA CAATCAGC-3'. Then the full-length cDNA was amplified with two primers: P3: 5'-TCAAGCTTA CAATGGGAGCCGGTGG CCGAATGT-3' and P4: 5'-ACGGATCCTAACAACATTTTCTTCATCCTGG-3', and the resulting PCR products were subcloned into pMD-18T vectors (*TaKaRa*, Dalian, China) for sequence analyses.

The coding region of *DiFAD2* was amplified with gene-specific primers P3 and P4, and subcloned into pYES2.0 (*Invitrogen*) to obtain the expression construct *pYDiFAD2*. The fidelity of the cloned product was confirmed by sequencing (*Sunbiotech*, Beijing, China). *Saccharomyces cerevisiae* strain Invsc1 (*Invitrogen*) was transformed with pY DiFAD2 and pYES2.0 using the lithium acetate method as described by Wei *et. al*. (2004). Transformants were selected and induced as described by Tonon *et al*. (2004) and Wei *et al*. (2004). Briefly, transformants were plated on SC-Ura selective medium containing 0.2 % yeast synthetic drop-out medium without uracil (*Sigma*), 0.17 % yeast nitrogen base without amino acids and ammonium sulfate (*Sigma*), 0. 5 % $(NH_4)_2SO_4$, 2 % dextrose, 2 % agar and cultured at 30 °C for 3 d. Then transformants were grown at 28 °C in synthetic minimal medium (0.3 % yeast synthetic drop-out medium without uracil, 0.26 % yeast nitrogen base without amino acids and ammonium sulfate, 0.75 % $(NH_4)_2SO_4$, 2 % raffinose). Expression of the gene was induced by supplementation with galactose at 20 g dm^{-3} at 22 °C for 3 d, when A_{600} of the cultures reached 0.2 - 0.3.

RT-PCR analysis was performed as described by Peng *et al*. (2008). Total RNA was extracted at different plant tissues according to the protocol described above. Single-strand cDNA was synthesized with the first-strand cDNA Synthesis kit with oligo(dT) primer. PCR was performed using undiluted and four dilutions (1/125, 1/50, 1/25, 1/5; v/v) of cDNAs as follows: the reactions were heated to 95 °C for 4 min, followed by 25 cycles at 94 °C or 30 s, 54 °C for 30 s, 72 °C for 1 min, then a single step at 72 °C for 7 min. As a marker for constitutive expression, the *Di18S rRNA* gene was amplified with the primers Di18S rRNA1: 5'-GGCATTCGTATTTCATAGT CAG-3' and Di18S rRNA2: 5'-GTTATTGCCTCAAACT TCCGTG-3'. *DiFAD2* cDNA was amplified using primers DiFadF: 5'- CGACGGCACCATTCCAACAC-3' and DiFadR: 5'-GAATGGGACTGGTTGAGGG-3'.

Total fatty acids of yeast cells and seeds were extracted and transmethylated as described by Tonon *et al*. (2002). Most fatty acid methyl esters (FAMEs) were analyzed by CP-7638 GC (*Varian*, Palo Alto, USA) and GC-MS (Shimadzu, Kyoto, Japan). FAME standard mixtures were purchased from *Sigma*. Fatty acid analysis of yeast transformants: sample FAMEs were dissolved in hexane for GC-FID (*Varian*) analysis with a 25 m× 0.53 mm × 1.0μm (film thickness) capillary column. The oven was programmed for 100 °C for 2 min, increase 10 °C min^{-1} to 180 °C, and 4 °C min^{-1} to 260 °C. Each value represents the means of duplicate measurements.

Results

A 451 bp cDNA fragment was generated by RT-PCR with the degenerate primers. A 568 bp 5'RACE product and a 550 bp 3'-RACE product were amplified and subjected for sequence analysis (data not shown). *DiFAD2* is 1389 bp in length and contains an ORF of 1 149 bp, a 32 bp 5'-untranslated region, and a 208 bp 3'-untranslated

region. (GenBank accession no. EU275211), which encodes a 382 amino acid residues (Fig. 1). Using pI/Mr tool (http://www.expasy.org/tools/ pi_tool.html) pI and Mr were 8.8 and 44.3 kDa, respectively.

In order to investigate the function of the putative *DiFAD2,* we examined its expression in *S. cerevisiae*. The results showed two novel fatty acid peaks in the FAME chromatogram from *pYDiFAD2*, which were absent from the yeast containing the empty vector pYES2.0 (Fig. 2*A*,*B*). Moreover, the increase in 16:2 and 18:2 fatty acids caused a decrease in 16:1 and 18:1 fatty acids (Fig. 2). These results indicate that *DiFAD2* encodes a δ^{12}-FAD, which can convert 16:1 and 18:1 into 16:2 and 18:2 fatty acids, respectively, in yeast. Thus, the newly isolated *D. involucrata DiFAD2* gene has been functionally identified because it encodes a microsomal oleate desaturase that catalyzes the desaturation of the endogenous oleate to linoleate.

To investigate the physiological role of the *DiFAD2* gene, we examined the transcript level by semiquantitative RT-PCR using *DiFAD2* gene-specific primers. *DiFAD2* was expressed in all tissues examined and was expressed abundantly in rapidly expanding tissues such as very young stems (Fig. 3).

The composition of saturated and unsaturated fatty acids of both membrane and storage lipids varies depending on environmental temperature. The level of unsaturation of membrane fatty acids, as well as seed storage lipids, has been shown inversely correlated with growth temperature (Heppard *et al*. 1996). The elevated PUFA content in leaves grown at low temperature were not due to enhanced expression of *FAD2* in soybean (Heppard *et al*. 1996). To test whether the increase of PUFA contents in leaves by low temperature is related to enhanced *DiFAD2* expression, we determined the level of transcripts by semi-quantitative RT-PCR analysis. The transcript level of *DiFAD2* gene was relatively constant in leaves at different growth temperatures, and the low temperature did not result in enhanced *DiFAD2* expression (Fig. 4).

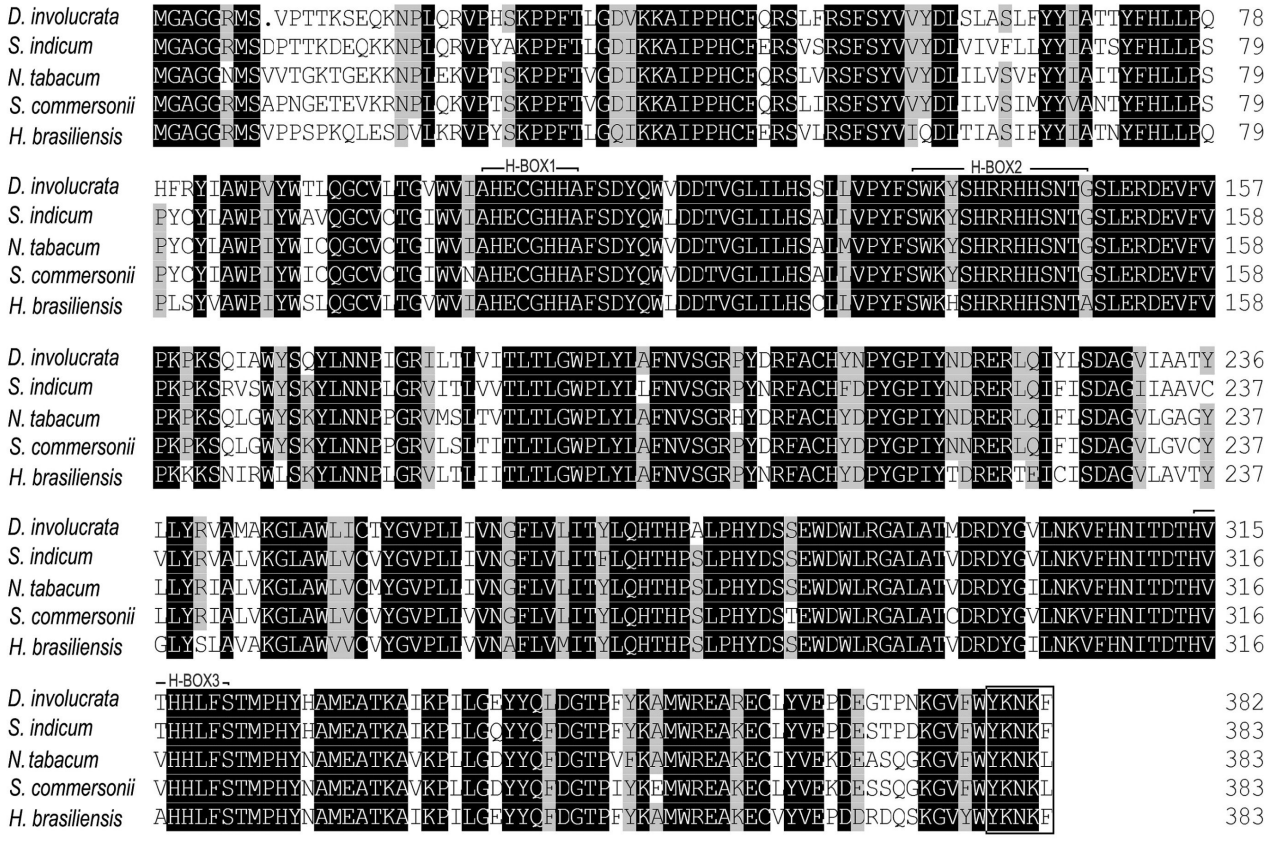

Fig. 1. Amino acid sequence of *Davidia involuctata DiFAD2* comparison with the other plant Δ^{12}-FADs. The sequences were aligned with the *DNAMAN 3.0* with the multiple alignment parameters gap penalty 8, gap extension penalty 2, and PAM protein mass matrix with 100 bootstrap trails. The identical amino acid residues were indicated with *black background* and the different amino acid residues were indicated with *white background*. *Gray shade* indicated 70 % or more conservation among all the aligned sequences. The taxa of origin for these genes were noted. Accession numbers for the sequences were as follows: *D. involuctata* δ^{12}-FAD (EU275211), *S. indicum* δ^{12}-FAD (AAF80560), *H. brasiliensis* δ^{12}-FAD (AAY87459), *S. commersonii* δ^{12}-FAD (CAA63432), *N. tabacum* δ^{12}-FAD (AAT72296).

By comparison of the fatty acid composition of plants grown at 4 - 28 °C, no marked change was detected in the fatty acid profile. In the seedlings grown at low temperature, 18:3 content exhibiting gradual increase (Table 1). There was also a decrease in the proportion of 16:1 and 18:2, but both of 18:0 and 18:1 remained relatively constant. The 22:5 and 22:6 fatty acids showed increase.

Discussion

DiFAD2 protein has three conserved histidine-rich motifs, H-BOX1(105-111), H-BOX2 (134-146) and H-BOX3 (315-322), which are contained in the putative protein (Fig. 1). These residues are shown to be necessary for the function of all membrane-bound fatty acid desaturases. They are likely involved in the catalysis of desaturation reactions (Sakai and Kajiwara 2005). To predict whether signal or plastidial transit peptides are present in the N-terminal regions of the above polypeptides, two different algorithms (PSORT and TargetP) for amino acid sequence analysis were introduced. For the DiFAD2 polypeptide not any indication of plastidial leader peptide was detected at the N-terminus, whereas a DiFAD2 amino acid-enriched retrieval signal (-YKNKF) was present at the C-terminus (Fig. 1), which is both necessary and sufficient for maintaining localization of the enzymes in the ER (McCartney *et al*. 2004).

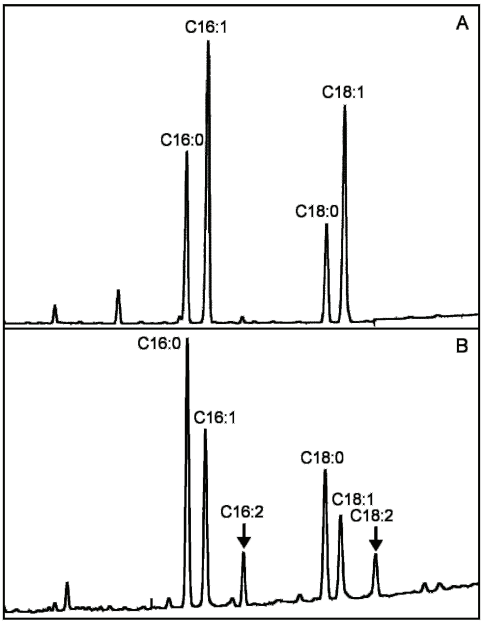

Fig. 2. Identification of the major fatty acids of pYES2.0 and pYDiFad2 yeast transformants by GC analysis. *A* - *S. cerevisiae* transformed with control vector pYES2.0. *B* - *S. cerevisiae* transformed with recombinant plasmid pYDiFad2. Each value represents the means of duplicate measurements.

Table 1. Leaf lipid fatty acid composition of *Davidia involucrata* grown at different temperature. Values represent the mol % of total leaf fatty acids and are the mean ± SD (n = 4).

Acyl group	28/22 °C	18/12 °C	12/8 °C	8/4 °C
C14:0	2.11±0.23	2.12±0.34	2.01±0.24	2.01±0.16
C16:0	13.13±1.43	12.23±1.56	10.21±1.44	10.06±1.21
C16:1	0.34±0.49	≤ 1	≤ 1	≤ 1
C18:0	5.86±0.96	5.87±0.87	5.34±0.76	5.65±0.98
C18:1	17.58±2.05	16.76±1.98	17.45±2.12	16.76±2.32
C18:2	24.93±2.65	21.54±3.76	20.56±3.89	19.09±4.21
C18:3	16.93±3.96	18.98±4.32	25.34±3.65	26.98±3.54
C20:2	3.09±0.56	3.98±0.38	2.34±0.77	2.02±0.43
C22:5	4.83±0.68	4.98±0.94	4.87±0.86	4.67±0.87
C22:6	11.78±1.65	11.95±1.98	12.36±2.04	12.56±2.54

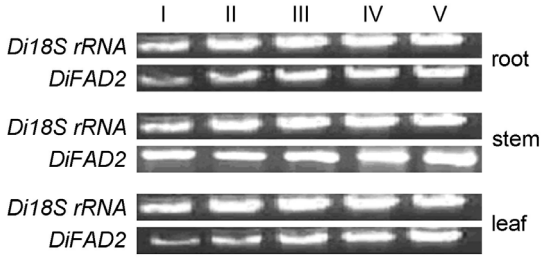

Fig. 3. RT-PCR analysis of *DiFAD2* gene expression pattern. The amplification length of 18sRNA is 385 bp, The amplification length of *DiFAD2* is 451 bp. Specific primers were used to amplify a 451 bp (DiFAD2) fragment with cDNA from root, stem, leaf. I - V denote the five 5-fold cDNA (1/125, 1/50, 1/25, 1/5, 1/1; v/v) serial dilutions from low to high separately. Di18S rRNA was used as a reference gene PCR procedure: 95 °C for 4 min, followed by 25 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, then a single step at 72 °C for 7 min.

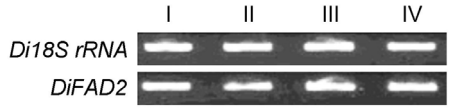

Fig. 4. RT-PCR analysis of *DiFAD2* gene expression pattern in different temperature. *Di18S rRNA* was used as a reference gene. I~Ⅳ 28/22 °C, 18/12 °C, 12/8 °C, 8/4 °C.

DiFAD2 from *Davidia involucrate* recognizes two substrates (16:1 and 18:1 fatty acids) in yeast (Fig. 2), which is similar with results of Niu *et al*. (2007), whereas the δ^{12}-FADs from fungi (Sakuradani *et al*. 1999, Sakai and Kajiwara 2005) recognize only one substrate (18:1). The reason is yet unknown, but it may be due to the different transmembrane topologies (Wei *et al*. 2004).

DiFAD2 was expressed abundantly in rapidly expanding tissues such as very young stems (Fig. 3) in agreement with the previous reports (Heppard *et al*. 1996, Martínez-Rivas *et al*. 2001, Li *et al*. 2006). A higher expression pattern of *DiFAD2* in expanding tissue

suggests a correlation between the level of membrane desaturation and cell elongation (Martínez-Rivas *et al*. 2001).

The presence of two seed-specific *FAD2* genes was previously reported for peanut (Jung *et al*. 2000), and one seed-specific and two constitutive *FAD2* genes were also previously reported for cotton (Liu *et al*. 1999, Pirtle *et al*. 2001) and sunflower (Martínez-Rivas *et al*. 2001). In peanut and soybean, both seed specific *FAD2* genes were markedly expressed in developing seeds (Jung *et al.* 2000). The sunflower constitutive *FAD2* genes were weakly and uniformly expressed in all tissues, except for cotyledons after 2 d of germination and for roots, where the expression of one *FAD2* was higher (Martínez-Rivas *et al*. 2001).

The substantial changes of membrane fatty acids were detected at different growth temperatures (Heppard *et al.* 1996). During acclimation to cold temperature, the activity of desaturase increased and the proportion of unsaturated fatty acids rose (Heppard *et al*. 1996, Williams *et al*. 1996). This modification lowered the temperature at which the membrane lipids experience a gradual phase shift from fluid to semi-crystalline (Mariya 2006). We expected to determine the alternation of the membrane fatty acids in response to cold stress and the relationship between the alterations and *DiFAD2* expression. But the transcript levels of *DiFAD2* gene was relatively constant at low temperature treatments (Fig. 4), although low temperature decreased significantly content of the 18:3 fatty acid. These results showed that the altered PUFA content in leaves of *D. involucrata* at low temperature have no direct correlation with the expression of *FAD2* but there may be two or more *FAD2* genes in the *D. involucrata* genome. Another mechanism may be conversion of 18:2 to 18:3 by δ^{15}-FAD (Li *et al*. 2006). Furthermore, *D. involucrate* have more highly unsaturated fatty acids (22:5 and 22:6) in leaves, which is rare in other higher plants. So it is necessary to understand in more detail the fatty acid metabolic pathway of *D. involucrata* in future.

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