Molecular cloning and characterrization of key genes related to lipid biosynthesis from *Descurainia sophia*

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Abstract

Cruciferous species *Descurainia sophia,* for its high linolenic acid content of up to 40% in seeds, may be regarded as special gene resources in molecular breeding and germplasm enhancement of variety improvement targeting to specific oilseed fatty acid constitution in *B*rassica species. Using RACE approach, we identified six different cDNA sequences, named *DsFAD6*、*DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8* and *DsDGAT*, encoding two oleate desaturases, three linoleate desaturases and diacylgcerol acyltransferase from *D. Sophia*. Sequence alignment and phylogenetic tree showed that these sequences from *D. sophia* were more closely related to the respective sequences from *Arabidopsis thaliana* than *Brassica* species. Deduced amino acid sequences of the five fatty acid desaturase genes showed the three histidine boxes characteristic of all membrance-bound desaturase functionality. *DsDGAT* has three specific and conserved domains unique amongst the *DGAT* family members. RT-PCR expression analysis showed that all genes were expressed in examined organs.

Key words: *Descurainia sophia*, Fatty acid desaturases, *DGAT* gene, RT-PCR

In most plant tissues, over 75% of the fatty acids are unsaturated [2]. Plant unsaturated fatty acids mainly including oleic acid (18:1). linoleic acid (18:2) and linolenic acid (18:3), are essential for human diet, are major structural components of membrane lipids. Membrane lipids provide the crucial hydrophobic barrier that prevents water loss, form a protection against pathogens and other environmental stresses and also serve as key precursors for the biosynthesis of messengers in signal transduction mechanisms. In higher plants, the biosysthesis of fatty acids pass through two different pathways [2]. In the chloroplast, the *FAD6* gene product converts 18:1 fatty acids to 18:2 and 16:1(produced from 16:0 by the *FAD5* desaturase) to 16:2. These dienoic acyl chains are then desaturated to 18:3 and 16:3 by the *FAD7* and *FAD8* gene products [3]. In the endoplasmic reticulum, 16:1 and 18:1 fatty acids from the chloroplast are desaturated to 18:2 and 18:3 by the sequential action of the *FAD2* and *FAD3* gene products. In oilseeds, fatty acids are mainly stored as the form of triacylglycerol (TAG) [4]. There are three acyltransferases and a phosphohydrolase involved in the plant storage lipid bioassembly. Diacylglycerol acyltransferase (DGAT) drives the final and only committed acylation step in the synthesis of TAG [5].

Descurainia sophia belongs to *Descurainia* species of *Cruciferae*, is an oil plant of specified function. *D. sophia* rich in oil, up to 40%, has two properties useful. One property is high content of linolenic acid of around 40% in seeds [1], showing characteristics of functional oil; another is that it can tolerate to extreme ecological conditions, specially to extremely low temperature. The advantage of exploring and applying the excellent gene of *D. sophia* is significant to improve the crop's quality and resistance to stress.

Up to now, cDNAs of *FAD6*, *FAD2*, *FAD3*, *FAD7*, *FAD8* and *DGAT* have been isolated from several plant species. Six different cDNA sequences we reported here, designated *DsFAD6*、*DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT*, encoding two oleate desaturases (FAD2 and FAD6)、three linoleate desaturases (FAD3, FAD7 and FAD8) and diacylgcerol acyltransferase (DGAT) have been isolated from *D. sophia* and their mRNA expression were investigated.

1. Materials and methods

1.1 Plant materials

D. sophia was planted in soil under normal conditions. Floral buds, flowers and siliques were harvested from *D. sophia* at different developmental stages. Roots, stems and leaves were harvested from six months after planting. All harvested samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

1.2 RNA extraction and RT reactions

Total RNA was isolated from different *D. sophias* tissues using a RNA extraction kit (Beijing Tianwei Corporation). The first-strand cDNA was synthesized with 2 μg of purified total RNA (pre-treated with DNase I) using the RT-PCR system (Promega) according to the manufacturer's protocol. The oligo $(dT)_{18}$ was used as a primer and the reverse transcription reaction was incubated at 42°C for 1h in a total volume of 20 μL.

1.3 Cloning of target genes and sequence analysis

To amplify conservative cDNA fragment of *DsFAD6*, *DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* primers

were designed based on nucleotide sequences of *FAD6*, *FAD2*, *FAD3*, *FAD7*, *FAD8* and *DGAT* from *Arabidopsis.thaliana* and other orthologues. The first-strand cDNA was synthesized from total RNA from *D. sophia* yong siliques. The PCR products were run on 1% agarose gel and the target band were purified with Gel Extraction Kit (HuaShun, Shanghai) according to the manufacturer's protocol. The purified product were cloned into pGEM-T vector (Promega, USA) and sequenced (SNBC, Shanghai).

For amplification of the unknown 3′- and 5′-ends of the *DsFAD6*、*DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT*, the 3′ and 5′ RACE were performed according to protocols described in GeneRacer^{TM} kit (Invitrogen). Based on sequence information of the conservative cDNA fragment, gene specific primers (GSP) were designed. To amplify the full-length *DsFAD6*、*DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* cDNAs, primers were designed based on the assembled sequence.

The functional domains were analyzed using online server program SMART 4.0 (http://smart.embl hei delberg.de/). The molecular weight was calculated using online server program Protscale (http://au.Exp asy. or g /cgi-bin/protscale.pl). Transmembrane regions were predicted by the TMPRED (http://www.ch.e m bnet.org / software/ TMPRED _form.html) and NetPhosK 1.0 (http://www.cbs.dtu.dk/services/ NetPho s K/). Predictions of subcellular localization were conducted by using the PSORT (http://www.psort.nibb.ac.j p/ form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms. Multiple sequence alignment and phylogenetic tree analysis were performed using ClustalX program[6].

1.4 Semi-quantitative RT-PCR assay

For further understanding the expression of the *DsFAD6*, *DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* gene, we performed semi-quantitative RT-PCR assay using RNA samples from various tissues. PCR amplification were performed using the primers, which amplify the conservative *DsFAD2*、*DsFAD6*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* cDNAs fragment. PCRs were performed using equal amounts of templates and 18S rRNA primers.

2 Results and discussion

2.1 Cloning six genes related to lipid biosynthesis from D. sophia

Based on nucleotide sequences of *FAD6*, *FAD2*, *FAD3*, *FAD7*, *FAD8* and *DGAT* from different plant species, primers were designed, respectively. PCRs employing the primer pairs generated conserved fragments with siliques cDNA of *D. Sophia* as a template. To clone gene full-length cDNA, 3′ - and 5′ - RACE reactions were conducted respectively. Sequence comparisons of the 5′- and 3′-ends with the conservative parts of every genes indicated that the overlapping regions match perfectly. BLAST searches of the deduced amino acid sequences revealed that these sequences represent the missing parts of *DsFAD6*、*DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* gene, respectively. Based on the above sequences data, primers were designed from the 5′- and 3′- UTRs of these genes and the full-length cDNAs were amplified, cloned and sequenced respectively. Basis description of every genes are listed in the table 1.

Table 1 Basis description of six genes from *D. sophia*

Name	Full-length cDNA (bp)	ORF (bp)	Encoded amino acid	Protein molecular mass (kD)	Isoelectric point	Subcellular location
DsFAD2	1343	1152	383	44.10	8.63	ER
DsFAD3	1214	1161	386	44.05	8.22	ER
DsFAD6	1521	1344	477	51.16	9.27	Chloroplast
DsFAD7	1383	1350	449	51.48	8.16	Chloroplast
DsFAD8	1343	1302	433	49.55	8.97	Chloroplast
DsDGAT	1792	1575	524	60.03	8.78	ER

2.2 Sequence analysis of DsFAD6

The *DsFAD6* showed high identity (70–89%) in amino acid sequences with several orthologues in the GenBank through Blast searches. There are three trans-membrane regions at amino acid residues 146-168, 182-201, 276-294 and three histidine-boxes (HXXXH, HXXHH, and HXXHH) with conserved inter-spaces in the *DsFAD6* (Fig.1). The eight histidines arranged into three histidine-boxes in the oleate desaturase sequences have been shown essential for desaturase activity [7]. These histidine boxes were thought to comprise the catalytic centre of the enzyme, since they formed ligands to a diiron cluster in the catalytic site [8]. It was also predict a putative chloroplast transit peptide sequence of 64.amino acids at the N-terminus of *DsFAD6* protein (Fig. 1). This N-terminal sequence also had several characteristrics of plastidial transit peptides, including a high content of hydroxylated residues (Ser, Thr and Tyr), a low content of acidic residues and the conserved N-terminal Met-Ala dipeptide [9]. Deduced amino acid sequences of *DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8* gene also showed the three histidine boxes characteristic of all membrance-bound desaturase functionality. *DsDGAT* has three specific and conserved domains unique amongst the *DGAT* family members.

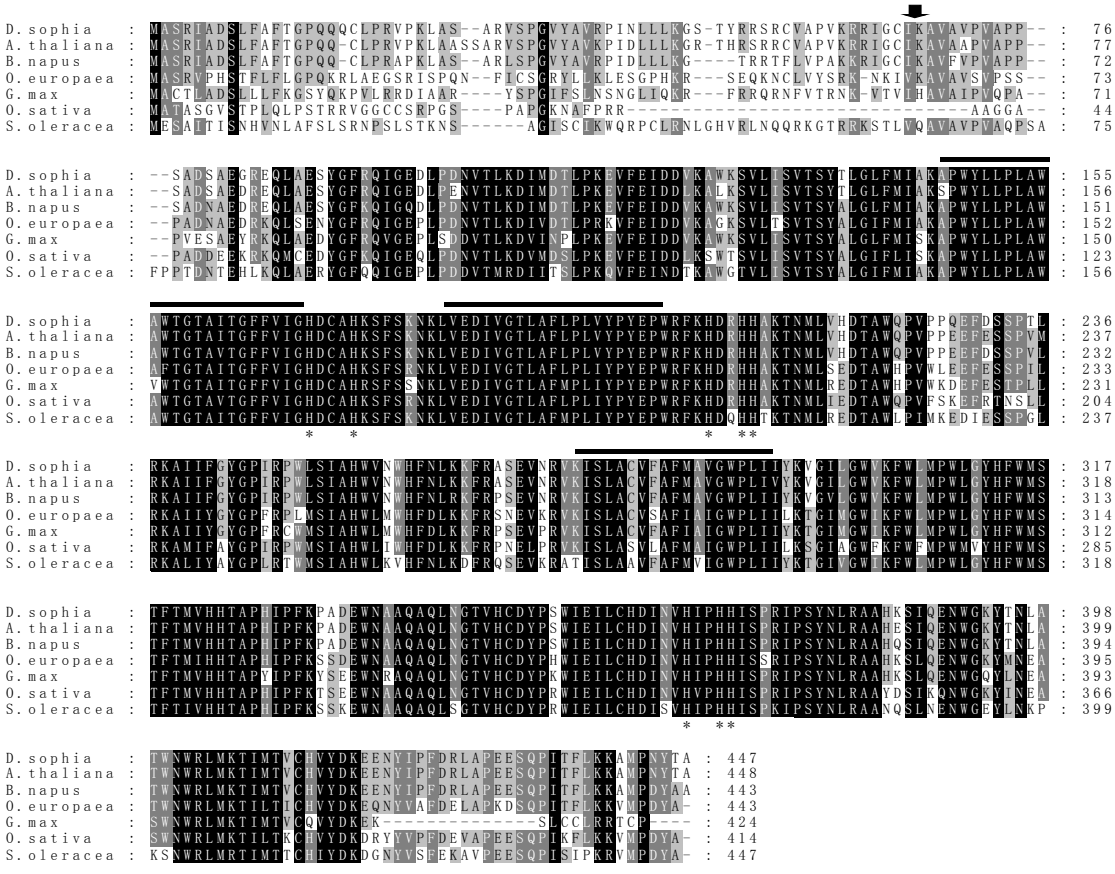

Figure 1 Alignment of amino acid sequences from *DsFAD6* and other plant FAD6.

The fatty acid desaturases were from *Descurainia sophia* (*DsFAD6)*, *Arabidopsis thaliana* (NM-119243), *Olea europaea* (AY772187), *Brassica napus* (L.29214), *Spinacia oleracea* (X78311), *Glycine max* (L29215), *Oryza sativa* (XM482619). Numbers in parentheses refer to GenBank accession No.. Identical or similar amino acids are shaded black or grey respectively. The black arrow indicates a putative signal peptide cleavage site. The eight histidines grouped in three different boxes characteristic for desaturases are indicated by asterisks. The three trans-membrane region are underlined.

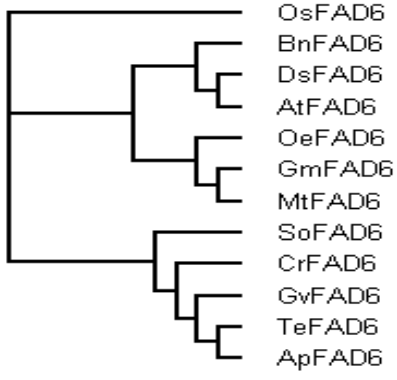

Fig.2. Phylogenetic relationships of oleate desaturases from the plant microsomals and plastidials

The tree was constructed using the Neighbor-Joining algorithm program. The scar bar represents branch distance. The organisms and accession numbers are as follows: *Descurainias ophia* (*DsFAD6)*, *Arabidopsis thaliana* (At-FAD6, NM-119243), *Olea. europaea* (OeFAD6, AY772187), *Brassica napus* (BnFAD6, L29214), *Spinacia oleracea* (SoFAD6, X78311), *Glycine max* (GmFAD6, L29215), *Oryza sativa* (OsFAD6, XM482619), *Medicago truncatula* (MtFAD6, ABE88706), *Chlamydomonas. reinhardtii* (CrFAD6, BAA23881), *Trichodesmium erythraeum* (TeFAD6, ABG49637), *Gloeobacter violaceus* (GvFAD6, AAF61413), *Arthrospira platensis* (ApFAD6, CAA60415).

The neighbor-joining phylogenetic tree (Fig. 2) showed the oleate desaturases was divided into two major groups corresponding to the classical botanical division of plants. *DsFAD6* and *AtFAD6* were clustered into cruciferous species subgroups. Result from sequences analysis were showed that *DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* gene also are more related to these genes from *A. thaliana*.

2.3. Expression analysis of DsFAD6

The expression of DsFAD6 in various tissues by semi-quantitative RT-PCR revealed that DsFAD6 was constitutively expressed in roots, stems, leaves, floral buds, flowers and young siliques. The higher transcript level of DsFAD6 was observed

in green tissues, i.e. leaves, stems and young siliques. The expression of the gene was the lower in roots, floral buds and flowers, and was more accumulated in floral buds, flowers and young siliques (Fig. 3). The result reflected the crucial roles of this enzyme in the fatty acid polyunsaturation pathway to fulfill the flower's developmental needs, such as for the production of alinolenate for pollen development or as precursor for jasmonic acid and oxylipin biosynthesis [10,11]. These results are consistent with former research results in flax and olive[6,12]. Expression analysis of *DsFAD2*、*DsFAD3*、*DsFAD7*、*DsFAD8 and DsDGAT* gene also showed that these genes were expressed in organs examined, but not listed here for shorten length.

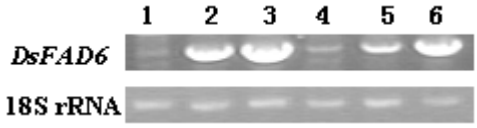

Fig.3. Expression of *DsFAD6* in *D. sophia* tissues

1-6 indicated the results of roots, stems, leaves, floral buds, flowering and young siliques

Acknowledgements

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