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Molecular cloning and characterization of the genes encoding a microsomal oleate Δ^{12} desaturase (*Cs*FAD2) and linoleate Δ^{15} desaturase (*Cs*FAD3) from *Camelina sativa*

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ABSTRACT

Camelina sativa produces oil that is rich in polyunsaturated linoleic (18:2$^{\Delta 9,12}$) and linolenic (18:3$^{\Delta 9,12,15}$) acids. These fatty acids are obtained by the successive desaturation of oleic (18:1$^{\Delta 9}$) acid, which is catalyzed in the endoplasmic reticulum by two different microsomal desaturases: oleate Δ^{12} desaturase (FAD2) and linoleate Δ^{15} desaturase (FAD3). The objective of the present study is to investigate the contribution of these two desaturases to the composition and properties of *C. sativa* seed oil, and look over the effect that temperature exerts on their activity. Three different copies of both these genes were identified, which when analysed contained three histidine rich motifs (HXCGHX, HRXHH and HVXHH) and six highly conserved transmembrane domains. Comparing their sequences, the *Cs*FAD2 copies accommodated four conservative changes (Glu-36-Asp, Arg-48-His, Val-97-Ala and Ala-177-Pro) and two semi-conservative ones (Val-63-Ile and Leu-249-Met), whereas only one semi-conservative change (Ala-327-Ser) was detected in *Cs*FAD3 but with two extra amino acids (His-147 and Gly-148). The *CsFAD2* and *CsFAD3* cDNAs were heterologously expressed in Saccharomyces cerevisiae to confirm that they were active enzymes and their dependence on temperature was investigated by growing the recombinant yeast cells at low (22 °C) and optimal (30 °C) temperatures. The conversion of 18:1$^{\Delta 9}$ to 18:2$^{\Delta 9,12}$ by *Cs*FAD2 was slightly better at 30 °C than at 22 °C, and it was also able to desaturate palmitoleic acid (16:1$^{\Delta 9}$) to hexadecadienoic acid (16:2$^{\Delta 9,12}$). By contrast, the conversion driven by *Cs*FAD3 was enhanced 5-fold at 22 °C compared to 30 °C, although it was not able to desaturate 16:2$^{\Delta 9,12}$. The distribution of these microsomal desaturases was also studied in *C. sativa*, which were expressed most strongly in expanding leaves and developing seeds.

1. Introduction

Camelina sativa (L.) Crtz. is a member of the Brassicaceae family that was cultivated in Europe from the Bronze Age to the twentieth century, being abandoned after World War II (Al-Shehbaz, 1987; Bouby, 1998). There is now interest in resuscitating the use of *C. sativa* as a plant in biotechnology as it is phylogenetically close to the model plant *Arabidopsis thaliana* (Collins-Silva et al., 2011) and it can be readily transformed with *Agrobacterium* by floral dip infiltration under vacuum (Lu and Kang, 2008). Moreover, *C. sativa* presents some agronomic features that give it a competitive advantage over other oilseed crops (Budin et al., 1995). Indeed, it has a rather short vegetative period of about 4 months so that it could be incorporated into double cropping systems during cooler growth periods (Putnam et al., 1993), and it displays very efficient nutrient use that allows it to be grown with reduced nitrogen fertilization in semi-arid regions (Bramm et al., 1990; Zubr, 1997). In addition, *C. sativa* is an allelopathic plant that secretes secondary metabolites and phytoalexins in order to inhibit the development of pathogens, insect pests and neighbouring plants (Lovett and Jackson, 1980), reducing the dependence on pesticides.

The oil accumulated by *C. sativa* seeds is rich in polyunsaturated fatty acids. The most abundant fatty acids in *C. sativa* oil are linoleic (18:2$^{\Delta 9,12}$) and linolenic (18:3$^{\Delta 9,12,15}$) acids, as well as oleic acid (18:1$^{\Delta 9}$), each accounting for about 22%, 29%, and 17% of the oil fatty acids composition, respectively (Rodríguez-Rodríguez et al., 2013). In seeds, *de novo* fatty acid biosynthesis takes place in the

Abbreviations: DAF, days after flowering.

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plastids and it is catalyzed by the fatty acid synthase complex (FAS), producing palmitoyl-ACP (16:0-ACP) and stearoyl-ACP (18:0-ACP) as the final products (Pidkowitch et al., 2007), both of which can be desaturated by the soluble Δ^9 stearoyl-ACP desaturase to yield palmitoyl-ACP (16:1$^{\Delta 9}$-ACP) and oleoyl-ACP (18:1$^{\Delta 9}$-ACP: Shanklin and Cahoon, 1998; Rodríguez-Rodríguez et al., 2015). This pool of acyl-ACPs are hydrolysed by acyl-ACP thioesterases (Voelker et al., 1997; Rodríguez-Rodríguez et al., 2014), and the resulting free fatty acids are exported out of the plastid and esterified with coenzyme A by long-acyl-CoA-synthetases (Aznar-Moreno et al., 2014). These acyl-CoAs can be incorporated into phosphatidylcholine, and oleic acid can be further desaturated by endoplasmic reticulum desaturases to linoleic and linolenic acids (Kang et al., 2011). These desaturated fatty acids are mainly transferred to triacylglycerols (TAG) through different pathways and they accumulate as an energy source in the seeds (Dyer et al., 2002).

Plant fatty acid desaturases can be classified into two main subfamilies: the soluble acyl-acyl carrier protein desaturases found in the stroma of plastids that mainly produce 18:1$^{\Delta 9}$-ACP (Shanklin and Cahoon, 1998); and the acyl-lipid desaturases that desaturate fatty acids esterified to glycerolipids, membrane bound-enzymes associated with the chloroplast (Ohlrogge and Browse, 1995) and endoplasmic reticulum membranes (Totcher et al., 1998) that regulate the linoleic and linolenic acid content in plants. The microsomal Δ^{12} oleate desaturase (1-acyl-2-oleoyl-*sn*-glycero-3-phosphocholine Δ^{12} desaturase or FAD2) desaturates oleic to linoleic acid in the ER, while linolenic acid is produced through the activity of the microsomal Δ15 linoleate desaturase (1-acyl-2-linoleoyl-*sn*-glycero-3-phosphocholine Δ^{15} desaturase or FAD3) that introduces the third double bond in linoleic acid esterified at the *sn*-2 position of phosphatidylcholine (Heinz, 1993). The reactions catalysed by FAD2 and FAD3 involve the concomitant reduction of molecular oxygen to water and they require the presence of NADH, NADH-cytochrome-*b*5 reductase and cytochrome-*b*5 (Smith et al., 1990). In terms of their structure, the FAD2 and FAD3 sequences from Arabidopsis (Arondel et al., 1992), soybean (Bilyeu et al., 2003), rapeseed (Reed et al., 2000), olive (Hernández et al., 2005) or flax (Fofana et al., 2004) have a highly conserved C-terminal amino acid sequence that is involved in sub-cellular microsomal location, and there are three conserved histidine rich motifs or histidine boxes, which coordinate two iron atoms and form the typical di-iron centre present in all desaturases (Shanklin and Cahoon, 1998). Three different *FAD2* genes being expressed during *C. sativa* seed development have been described by Hutcheon et al. (2010) when studding the polyploidy of this species. In a similar way, when doing transcriptome studies in two developmental stages of *C. sativa* seed, Wang et al. (2015) described three *FAD3* genes.

Schulte et al. (2013) describe that fatty acids composition of *C. sativa* oil can be influenced by environment and variety, although the effects detected were smaller than those in other oil crops like sunflower, soybean or canola. Previously, Crowley and Frohlich (1998) found 2% less linolenic acid in *C. sativa* grown during a dry warm year than in the same variety grown during a normal year. More recently, Vollmann and Eynck (2015) when reviewing the current knowledge about *C. sativa*, comment the dependence of linolenic acid content on genotype, agronomic treatment or environmental conditions, pointing the fact that, as described in other oilseeds, linolenic acid content was lower in warmer climates than in cooler ones (Seehuber 1984; Rodríguez-Rodríguez et al., 2013). These changes should be related to the temperature effect on desaturases activities, indicating that FAD2 and FAD3 proteins from *C. sativa* are more stable than the ones from other crops. To verify these hypotheses, we report here the cloning of microsomal oleate Δ^{12} (*Cs*FAD2) and linoleate Δ^{15} (*Cs*FAD3) desaturases from *C. sativa,* their expression in *Saccharomyces cerevisiae* and functional characterization. Furthermore, we identified the *CsFAD3* copies in the *C. sativa* genome and studied the expression of these genes in different plant tissues.

2. Materials and methods

2.1. Biological material and growth conditions

C. sativa plants of the CAS-CS0 cultivar were grown in growth chambers equipped with fertirrigation at 25/15 °C (day/night) with a 16 h photoperiod and at a light intensity of 250 μE m^{-2} s^{-1}. Pots were filled with Presstopf (tray 20/80) as substrate (Gramoflor GmbH & Co. KG, Vechta, Germany) and watered daily. Developing *C. sativa* seeds were collected during the active oil synthesis period (6–30 days after flowering, DAF), frozen in liquid nitrogen and stored at −80 °C.

The *Escherichia coli* XL1-Blue strain (Stratagen) was used as the plasmid host for the cloning of the *CsFAD2* and *CsFAD3* genes. Bacteria were grown in liquid LB media (1% Bacto Tryptone, 0.5% Yeast Extract and 1% NaCl [pH7]) shaken vigorously at 37 °C. Plasmid selection was performed in the presence of ampicillin (100 μg/μl).

Saccharomyces cerevisiae W303-1A strain (MATa, ade2-1, his3-11, leu2-3, 112 trp1-1, ura3-1, can1-100) was used as the plasmid host for the expression of microsomal *C. sativa* desaturase protein. Recombinant yeast was grown in poor, synthetic complete drop out medium (SC medium: 0.5% NH_4SO_4, 0.5% YNB, 0.13% amino acids [pH 5.7]) supplemented with glucose (2% v/v) as the sole carbon source, and the liquid culture was shaken vigorously at low (22 °C) or optimal (30 °C) temperatures.

2.2. mRNA preparation and cDNA synthesis

Developing *C. sativa* seeds (approximately 0.25 g) were ground in liquid nitrogen using a precooled sterile mortar and pestle. Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and the mRNA was isolated from the total RNA using a GenElute mRNA Miniprep Kit (Sigma-Aldrich), resuspending the mRNA pellet in 33 μl RNAase-free TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8]). This mRNA was used as the template to synthesise cDNAs using the Ready-To-Go T- Primed First-Strand Kit (GE Healthcare Life Sciences, Uppsala, Sweden).

2.3. Cloning of genes encoding microsomal fatty acid desaturases *from* C. sativa

The FAD2 and FAD3 protein sequences from public databases were aligned using the ClustalX v1.8 program (Thompson et al., 1997) to identify highly conserved regions that could be used to design primers for PCR amplification. RNA isolated from developing seeds of *C. sativa* was used as template and two different PCR fragments were amplified with degenerate primers designed from the highly conserved regions identified in FAD2 (Deg_FAD2_F and Deg_FAD2_R; see in Table 1) and FAD3 (Deg_FAD3_F and Deg_FAD3_R; see in Table 1). The 5′- and 3′- ends of the corresponding cDNAs were obtained using the SMART-RACE cDNA Amplification Kit (Clontech, France) and two pairs of specific primers for each gene: CsFAD2_F1, CsFAD2_R1, CsFAD2_F2 and CsFAD2_R2 (Table 1) for the *CsFAD2* gene; and CsFAD3_F1, CsFAD3_R1, CsFAD3_F2 and CsFAD3_R2 for *CsFAD3* (Table 1). The PCR fragments were cloned into the pMBL-T vector (Dominion MBL, Spain), sequenced and their identities were confirmed using the BLAST software (Altschul et al., 1990).

To identify different copies of microsomal Δ^{12} oleate desaturase and Δ^{15} linoleate desaturase in *C. sativa*, two specific pairs of primers for each microsomal desaturase were designed: