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Genome-Wide Identification and Expression Analysis of Fatty Acid Desaturase (FAD) Genes in Camelina sativa (L.) Crantz

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Abstract: Camelina sativa (L.) Crantz is an indispensable oilseed crop, and its seeds contain many unsaturated fatty acids. FAD (fatty acid desaturase) regulates the synthesis of unsaturated fatty acids. In this research, we performed CsFAD gene family analysis and identified 24 CsFAD genes in Camelina, which were unevenly distributed on 14 of the 19 total chromosomes. Phylogenetic analysis showed that CsFAD includes four subfamilies, supported by the conserved structures and motifs of CsFAD genes. In addition, we investigated the expression patterns of the FAD family in the different tissues of Camelina. We found that CsFAD family genes were all expressed in the stem, and CsFAD2-2 was highly expressed in the early stage of seed development. Moreover, during low temperature (4 °C) stress, we identified that the expression level of CsFAD2-2 significantly changed. By observing the transient expression of CsFAD2-2 in Arabidopsis protoplasts, we found that CsFAD2-2 was located on the nucleus. Through the detection and analysis of fatty acids, we prove that CsFAD2-2 is involved in the synthesis of linolenic acid (C18:3). In conclusion, we identified CsFAD2-2 through the phylogenetic analysis of the CsFAD gene family and further determined the fatty acid content to find that CsFAD2-2 is involved in fatty acid synthesis in Camelina.

Keywords: Camelina sativa (L.) Crantz; fatty acid desaturase (FAD2-2); gene family; low temperature (4 °C); linolenic acid (C18:3)

1. Introduction

Camelina sativa (L.) Crantz (Camelina), which is an indispensable oilseed crop in the northern regions of the earth, can survive normally at −2 °C [1,2]. In recent years, many studies have shown that Camelina is more suitable for planting in autumn to increase the oil content of the seeds [2,3]. Meanwhile, the oil content of Camelina seeds accounts for 35% of dry weight [4]. Camelina has received more attention because of its good agronomic traits and can provide a large number of high-quality fatty acids [3,5–8]. A growing number of studies have shown that the ω-3 fatty acids, which are an important component of the cytoplasmic membrane, such as hexadecatrienoic acid (C16:3) and α-linolenic acid (C18:3), will accumulate in plants when exposed to low temperature [9–12]. The content of α-linolenic acid in Camelina seeds is close to 40%, which can improve the tolerance of these plants to abiotic stress [13,14]. In conclusion, the content of fatty acid in Camelina is relatively high compared with other species, especially the content of linolenic acid, which accounts for a relatively large proportion of the total fatty acids contained in Camelina seeds.

The intracellular pathway of fatty acid desaturation is generally regulated by FADs (fatty acid desaturases) in plants, including Camelina [9,12,15–18]. Desaturase activity in fatty acid biosynthesis was originally discovered in the cytoplasm of the different tissues of the plant [19]. Although FAD activity in the cytoplasm has been demonstrated, these enzymes are located in different cell compartments [20]. In Arabidopsis, FAD2 and FAD3 are localized to the ER (endoplasmic reticulum), whereas FAD4, FAD7, and FAD8 are only...
found in the plastid [20–24]. It has been shown that FAD2 and FAD4 may be associated with fatty acid synthesis or degradation, and FAD4, FAD7, and FAD8 may be involved in fatty acid metabolism and transport.

Moreover, researchers also found that the protein expression of FAD3 in wheat root tips was significantly upregulated [9], and the accumulation of ω-3 fatty acids (C18:3) was also achieved through the upregulation of FAD3 expression in low temperatures. In addition, the acetylation of the histones of FAD3 also affects fatty acid biosynthesis in seeds [25]. FAD5 is required for hexadecenoic acid (C16:3) synthesis in the chloroplasts of Marchantia polymorpha [15]. Notably, FAD5 regulates autoimmunity in plant cells by catalyzing palmitate acid [12]. The chloroplast-localized FAD8 was more selective for C18:2 and the function of catalyzing the production of C18:3 was non-redundant with FAD7 at 22 °C [20]. Furthermore, previous studies have demonstrated that FAD3 and FAD7 are involved in lipid transport between the ER and chloroplast and also revealed the differential contribution of FADs with different localization to linolenic acid [26]. Increasing the expression of FAD3 and FAD7 increases the C18:3 accumulation in the tomato, which will further promote plants to have stronger resistance to cold stress [27]. In terms of plant response to low-temperature stress, both fad2 and fad6 mutants showed growth defects, and the accumulation of C16:3 and C18:3 was significantly reduced in vivo [28–30]. In plants, the double bonds in the vast majority of fatty acids are associated with membrane lipids with the cis configuration. However, the particularity of FAD4 is that the double bonds of the catalyzed fatty acid have the trans configuration [24]. Meanwhile, the C16:1 catalyzed by FAD4 will participate in the redox reaction of chloroplasts [17]. In summary, most studies have established that FADs are associated with fatty acid metabolism, especially since they catalyze the production of unsaturated fatty acids.

In the FAD gene family, FAD2 has relatively undergone little research. In Arabidopsis, FAD2 catalyzes oleic acid (C18:1) to linolenic acid (C18:2) [31]. FAD2 regulates Arabidopsis response to the endoplasmic reticulum stress by controlling linolenic acid accumulation [16]. The DsFAD2 (Dimorphotheca sinuate) enzyme has a high preference for linolenic acid, indicating that it is closely related to the synthesis of linolenic acid [32]. Linolenic acid (C18:3) can improve plant resistance to low-temperature stress. Moreover, fad2 mutants cannot grow normally at low temperatures [28]. We sought to investigate whether FAD2 could regulate fatty acid synthesis in Camelina. Here, we identified and analyzed the FAD gene family of Camelina at different developmental stages and performed further quantitative analysis of the FAD gene family under low-temperature stress. The analysis of the gene family includes phylogenetic analysis, gene structure analysis, and the analysis of chromosomal locations. Moreover, we also determined that CsFAD2 can improve the resistance of Camelina to low-temperature stress. Meanwhile, subcellular localization was investigated to determine the expression of CsFAD2. Furthermore, we used Pichia pastoris as a receptor for the heterologous expression of CsFAD2 to detect the changes in fatty acid content. We found that CsFAD2-2 can regulate fatty acid synthesis in plants. Meanwhile, we analyzed the expression patterns of the FAD family and found that FAD2-2 has a certain relationship with low-temperature stress.

2. Results
2.1. Genome-Wide Identification and Analysis of the CsFAD Gene Family

Camelina is an allohexaploid plant. All the gene sequence information of the CsFAD family was found in the NCBI database. The sequence analysis of the FAD gene family was carried out using bioinformatic methods. According to the different relationships, the genes of the 24 CsFAD families that may be related to the fatty acid synthesis of Camelina seeds were divided into seven categories, namely CsFAD2, CsFAD3, CsFAD4, CsFAD6, CsFAD7, CsFAD8, and CsSLD, respectively. The shortest gene length is the CsFAD4-5, and the longest is the CsFAD7-1. Subsequently, the amino acid sequence analysis of the CsFAD family showed that the sequence was 408-613 amino acids in full length, and the molecular mass was 38.7–129.8 kDa (Table 1).
2.2. Phylogenetic Distribution of FAD Genes in Camelina, Soybean, and Arabidopsis

To compare the phylogenetic distribution of FAD genes, which is related to fatty acid dehydrogenase in Camelina, soybean, and Arabidopsis, we constructed a phylogenetic tree using MEGA7 (Figure 1). CsFAD2-2 was found to be closely related to CsFAD2-1 and CsFAD2-3 and had different branches and relatives of Arabidopsis AtFAD2, soybean GmFAD2-2B, GmFAD2-2A, and other genera. However, the FAD genes of Camelina were divided into four subfamilies. The DES1 subfamily genes were not identified in Camelina. Therefore, the DES1 genes may have been lost in Camelina during evolution. Omega-3 was the largest of the four subfamilies. FAD4 was the most special subfamily, and no FAD4 was found in soybean [33] (Figure 1, Table S7), suggesting that FAD4 is likely lost in soybean. Furthermore, most of the soybean FAD4 tended to be amplified after the ancestral genes diverged. By contrast, Camelina contained high amounts of FAD4.

Table 1. Sequence analysis of CsFAD gene family.

| Gene ID   | Sequence Number | Chromosome | Gene Location |
|-----------|-----------------|------------|---------------|
| CsFAD2-1  | LOC104776214    | 1          | 4949,343–4952,341(-) |
| CsFAD2-2  | LOC104764975    | 19         | 5522,583–5524,894(-) |
| CsFAD2-3  | LOC104745425    | 15         | 5229,237–5232,157(-) |
| CsFAD3-1  | LOC104700502    | 7          | 6302,189–6305,695(+) |
| CsFAD4-1  | LOC104717530    | 10         | 6575,131–6577,244(-) |
| CsFAD4-2  | LOC104722223    | 11         | 7634,471–7635,739(-) |
| CsFAD4-3  | LOC104714552    | 9          | 36744,852–36746,085(+) |
| CsFAD4-4  | LOC104713733    | 9          | 32860,862–32862,097(-) |
| CsFAD4-5  | LOC104751928    | 16         | 22866,693–22867,739(+) |
| CsFAD4-6  | LOC104730684    | 12         | 7388,093–7389,357(+) |
| CsFAD4-7  | LOC104702825    | 7          | 27024,843–27026,085(+) |
| CsFAD6-1  | LOC104717096    | 10         | 4649,200–4652,284(+) |
| CsFAD6-2  | LOC104730201    | 12         | 4899,503–4902,312(+) |
| CsFAD6-3  | LOC104727652    | 11         | 5222,054–5224,514(+) |
| CsFAD7-1  | LOC104765502    | 1          | 4484,295–4487,748(+) |
| CsFAD7-2  | LOC104745318    | 15         | 4751,636–4754,256(+) |
| CsFAD7-3  | LOC104764868    | 19         | 5028,541–5030,994(+) |
| CsFAD8-1  | LOC104734563    | 13         | 2144,948–2147,363(+) |
| CsFAD8-2  | LOC104708554    | 8          | 25336,842–25339,238(+) |
| CsSLD-1   | LOC104699734    | 7          | 1101,302–1103,202(-) |
| CsSLD-2   | LOC104788453    | 5          | 33807,754–33809,568(+) |
| CsSLD-3   | LOC104783195    | 4          | 29182,382–29184,175(+) |
| CsSLD-4   | LOC104784696    | 5          | 962,282–963,994(+) |
| CsSLD-5   | LOC104793590    | 6          | 25451,740–25453,806(+) |
Figure 1. Phylogenetic analysis of FAD proteins in Camelina sativa, Arabidopsis thaliana, and Glycine max. Neighbor-joining phylogeny of 36 FAD genes in the 3 species was analyzed by using MEGA 7.0 with 2000 times bootstrap replications. Tree scale is 0.1. FAD family was divided into four subfamilies, namely Omega-3, Omega-6, FAD4, and SLD, which are labeled in the outermost layer. Three different colors were selected to represent three species.

2.3. Gene Structure Analysis and Chromosomal Location of FAD Gene Family in Camelina

To characterize the sequence structure of the CsFAD gene family, the coding sequence of the CsFAD gene was analyzed, the amino acid sequence of the CsFAD gene family was used to generate a separate phylogenetic tree, and the exon and intron structures of these genes were compared (Figure 2, Table S4). The results showed that except for CsFAD2-1, CsFAD4 and CsSLD1, CsSLD2, CsSLD3, and CsSLD4, the rest of the genes contained different numbers of introns, among which CsFAD2-2 and CsSLD5 each had one intron. Meanwhile, CsFAD3, CsFAD7, and CsFAD8 each had seven introns, and their conserved genetic structure supported their close evolutionary relationship. CsFAD6-3 had eight introns, while CsFAD6-1 and CsFAD6-2 had nine introns each (Figure 2). The results of protein functional domain analysis showed that, in addition to CsFAD4, the genes that are more closely related are more similar in their protein functional domain structure. It can be seen from the structure of exon–intron that CsFAD2-2 is obviously different from CsFAD2-1 and CsFAD2-3, which may indicate that CsFAD2-2 performs different functions from CsFAD2-1 and CsFAD2-3.

In addition to the exon–intron analysis, conserved motifs may also be important for the various functions of the CsFAD genes. Using TBtools, we analyzed and determined that CsFAD had nine conservative motifs. The length of the motif ranged from 21 to 50 amino acids, and the number of CsFAD motifs ranged from 4 to 6. CsFAD2-1, CsFAD2-2, and CsFAD2-3 all contained six conservative motifs. Overall, the Camelina CsFAD family contained eight motifs (Figure 2). The protein motifs and protein functional domain analysis of the CsFAD gene family showed that the more closely related the genes were, the more similar the protein functional domain structure would be. The CsFAD genes
were distributed on 14 chromosomes (Figure 3), and CsFAD4-7, CsFAD3-1, and CsSLD1 were located on chromosome 7. Chromosomes 1, 5, 9, 10, 11, 12, 15, and 19 contained a significant number of CsFAD genes. According to the results of the gene location on chromosomes, it was revealed that CsSLD and CsFAD4 were randomly distributed on different chromosomes, but on chromosomes 10, 11, and 12, CsFAD4 and CsFAD6 existed at the same time, indicating that there may be some connection between these two subfamilies. At the same time, CsFAD2 and CsFAD7 may also have such a connection, but the specific association analysis needs to be further proved. Meanwhile, using the structural analysis results of the conservative structural domain (Figure S3), we found that CsFAD2 only contained the functional domain of Omega 6 fatty acid dehydrogenase, indicating that CsFAD2 is mainly involved in fatty acid metabolism. CsFAD3, CsFAD6, CsFAD7, and CsFAD8 all contained only the membrane FADS family or superfamily functional domains, which may be involved in the synthesis of membrane fatty acids, while CsSLD contained the cytochrome b5 functional domain, indicating that CsSLD may also be related to the metabolism of cytochrome b5. CsFAD4 is different from the domain of other CsFADs because CsFAD4 catalyzes trans-unsaturated double bonds.

Figure 2. The motifs and structures of FAD gene family from Camelina sativa. MEME was used to analyze the protein sequence, and 10 motifs were predicted in FAD sequence. Conservative domains are represented by different colors, and the black lines are non-conservative domains. Scale bars represent 50 amino acids. Yellow represents CD sequences, black lines represent introns, and blue represents non-coding regions (UTR). Scale bars represent 500 bp.
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gene plays an important role in the fatty acid synthesis process of Camelina. This stage of plant development is an important period for the synthesis of fatty acids in Camelina seeds, so based on this, we can reasonably speculate that the CsFAD2 gene plays an important role in the fatty acid synthesis process of Camelina.

2.4. Tissue- and Organ-Specific FAD Gene Expression in Camelina

We analyzed the expression levels of the CsFAD gene families of Camelina leaves, stems, flowers, and seeds at different developmental stages after flowering (Figure 4, Figure S1, Table S5). The results showed that the expression of CsFAD gene families in Camelina leaves was generally low, and there were different degrees of expression in the stem, among which the relative expression of CsFAD2 genes in seeds was high, especially CsFAD2-2 in seeds 10 days after flowering. It reached 2.4–100 times the expression of genes in other families. This stage of plant development is an important period for the synthesis of fatty acids in Camelina seeds, so based on this, we can reasonably speculate that the CsFAD2-2 gene plays an important role in the fatty acid synthesis process of Camelina.

Figure 3. TBtools was used to draw the maps of chromosome location. Uneven distribution of CsFAD genes on 14 chromosomes. Cluster I represents fatty acid desaturase (FAD) that can catalyze cis-double-bond fatty acids. Cluster II represents fatty acid desaturase (FAD) that can catalyze trans-double-bond fatty acids.

Figure 4. Expression profiles of CsFAD family genes in different Camelina tissues and organs: (A) the normalized expression levels of the hierarchical clustering of 24 CsFAD genes in 5 tissues (leaves, stem, flower, seeds 10 days after flowering (DAF), and seeds 20 days after flowering (DAF)). The transcription level was graded color scale from green to red. Expression data were obtained from the Camelina genome database. The normalized expression level of CsFAD genes is expressed in the form of log2 values; (B) qRT-PCR profiles of CsFAD genes in different Camelina tissues and organs. The expression level of the CsActin gene in Camelina was normalized as “1”. Vertical bars indicated the standard error of the mean. Data are shown as means ± SD of three replicates (n = 3 × 3).
2.5. Expression Analysis of the CsFAD Gene Family under Low-Temperature Stress

The expression of key genes in plant fatty acid anabolism not only controls the synthesis of lipid components but is also closely related to the cold tolerance of plants. The Camelina seedlings were treated at a low temperature of 4 °C, and then the gene expression of Camelina seedlings grown at 22 °C and 4 °C was analyzed, and the expression of 15 genes in the CsFAD gene family was found to decrease to varying degrees, among which the CsFAD2 gene significantly changed during treatment for 3 h, and the expression decreased by 34.6–41.3%. It was also observed that the expression of 11 genes was increased after cryogenic treatment (Figure 5, Figure S2, Table S6). The expression of the FAD gene family was significantly upregulated in the early or late stage of low-temperature stress treatment. After 6 h of low-temperature treatment, the expression of CsFAD2-2 was significantly upregulated. The above results can prove that the CsFAD genes are related to the cold resistance of Camelina, but how these genes specifically affect the cold tolerance of Camelina remains to be investigated. The expression level of CsFAD2-2 in Camelina seeds was very high, and the CsFAD2-2 expression level increased with the increase in low-temperature treatment time.

Figure 5. Expression patterns of CsFAD genes in Camelina during low-temperature stress: (A) normalized expression levels of CsFAD genes in Camelina during low-temperature stress. qRT-PCR was used to investigate the expression levels of the CsFAD genes during low-temperature stress. Log2 fold change for low-temperature treatment was used to present expression change under low-temperature treatment. Expression levels are illustrated by graded color scale from green to red; (B) qRT-PCR profiles of CsFAD genes during low-temperature treatment. The low-temperature (4 °C) treatment lasted 0 h, 3 h, and 6 h. The expression level of CsFADs in control was normalized as “1”. Vertical bars indicate the standard error of the mean. Data are shown as means ± SD of three replicates (n = 3 × 3).

2.6. CsFAD2-2 Subcellular Localization Analysis

The constructed plant fusion expression vector pCAMBIA1302-CsFAD2-2 was transformed into Arabidopsis protoplasts and tobacco, the empty vector pCAMBIA1302 was transformed as a control to observe the distribution of green fluorescence signal under laser confocal microscopy (Figure 6, Figure S5). The subcellular localization results showed that all the GFP signals were observable in Arabidopsis protoplast cells in the control group of the transformed empty vector pCAMBIA1302. In the experimental group of recombinant vector pCAMBIA1302-CsFAD2-2, a strong green fluorescence signal was observed only on the nucleus of Arabidopsis protoplasts (Figure 6). It was found that the protein encoded by the CsFAD2-2 gene was mainly distributed in the nucleus.
The content of myristic acid (C14:1), an unsaturated fatty acid, in recombinant yeast cells was 38.81 mg/kg, the content of C14:1 in empty yeast cells was 31.15 mg/kg, an increase of 24.7% in recombinant yeast cells compared with empty yeast cells (Figure 7, Figure S4). The content of decanoic acid (C10:0) of saturated fatty acid was 29.82 mg/kg, undecanoic acid (C11:0) was 32.91 mg/kg, lauric acid (C12:0) was 39.44 mg/kg, and myristic acid (C14:0) was 39.92 mg/kg. The contents of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) were 39.98 mg/kg and 43.2 mg/kg, respectively. However, in the control group, the content of C10:0 was 23.77 mg/kg, an increase of 25.45%; C11: 0 was 26.39 mg/kg, an increase of 24.59%; C12: 0 was 29.44 mg/kg, an increase of 25.45% respectively. The content of stearic acid (C18:0) did not change significantly.

2.7. Analysis of Fatty Acid in Transgenic Yeast

*Pichia pastoris* has a simple fatty acid structure that lacks an endogenous FAD2 activity that can be used to verify the function of CsFAD2 fatty acid dehydrogenase [34–36]. In *Pichia pastoris* expressing CsFAD2-2, gas chromatography–mass spectrometry was used to compare the accumulation of fatty acids with that in control. Different degrees of fatty acid changes were observed in recombinant yeast cells transformed with the cDNA of CsFAD2-2 (Figure 7, Figure S4). The content of decanoic acid (C10:0) of saturated fatty acid was 29.82 mg/kg, undecanoic acid (C11:0) was 32.91 mg/kg, lauric acid (C12:0) was 39.44 mg/kg, and myristic acid (C14:0) was 39.92 mg/kg. The contents of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) were 39.98 mg/kg and 43.2 mg/kg, respectively. However, in the control group, the content of C10: 0 was 23.77 mg/kg, an increase of 25.45%; C11: 0 was 26.39 mg/kg, an increase of 24.7%; C12: 0 was 29.44 mg/kg, an increase of 25.45% respectively. But the content of stearic acid (C18:0) did not change significantly.

![Figure 6](image)

**Figure 6.** Subcellular localization of CsFAD2-2. Subcellular localization of CsFAD2-2 in Arabidopsis protoplasts. CsFAD2-2 was fused with CaMV35S: GFP to obtain the GFP-CsFAD2-2 fusion protein. The upper row shows the fluorescent signal from transforming CaMV35S: GFP vector. The bottom row shows the fluorescent signal from transforming CaMV35S: CsFAD2-2–GFP vector. Scale bars, 2 μm.

![Figure 7](image)

**Figure 7.** Determination of fatty acid in *Pichia pastoris*: Determination of fatty acid content in *Pichia pastoris*. Data are shown as means ± SD of three replicates (n = 3 × 3); Statistical significance analysis was carried out by performing Student’s t-test (**p < 0.01 and ***p < 0.001).
increase of 24.59%, and the content of cis-10-pentanoic acid (C15:1) was 38.13 mg/kg. Although the content of cis-13, 16-docosadienoic acid (C22:2), cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6), and erucic acid (C22:1) accumulated more in transgenic yeast cells than in controls, the overall level was still low. It is worth noting that the content of linolenic acid (C18:3) increased by 47.7%, from 41.72 mg/kg to 61.6 mg/kg, and the content of oleic acid (C18:1) decreased from 294 mg/kg in the control group to 35.97 mg/kg in recombinant yeast cells, with a decrease of 87.8%. This indicates that the main function of the CsFAD2-2 gene is to increase the unsaturated degree of fatty acids and further catalyze the dehydrogenation of oleic acid (C18:1) to linolenic acid (C18:3).

3. Discussion

In plants, fatty acids are a major factor in determining the value of vegetable oils. Thus, optimizing the content of high-quality fatty acids in seeds is one of the important tasks in oil crop breeding. Although there are fatty acids with different carbon chains in natural plants, most commercial vegetable oils are abundant in C16 and C18 and contain up to three unsaturated double bonds [37]. The fatty acids’ desaturating enzymes encoded by the FAD genes in plants catalyze and generate unsaturated fatty acids containing 2–3 double bonds, such as hexadecatrienoic acid (C16:3) and α-linolenic acid (C18:3) [38]. Recently, some studies have demonstrated that FAD genes can increase the oil content of oil crops [39,40]. FAD genes not only promote the synthesis of unsaturated fatty acids in seeds but also play a crucial role in plant resistance to abiotic stress, especially low-temperature stress [41]. There are few studies on the response of FAD genes to low-temperature stress, among which AtFAD7 and AtFAD8 have been shown to respond to low temperatures [20,27]. In Arabidopsis and cotton, FAD2 has been shown to promote fatty acid accumulation and improve seedling tolerance to cold stress [42,43]. Nevertheless, there are relatively few studies on the function of FAD2 in Camelina, especially its resistance to low temperatures.

In this research, we identified and compared 24 FAD family members in Camelina. The phylogenetic tree results showed that CsFAD2 was relatively close to AtFAD2 (Figure 1). This suggests that they may have similar evolutionary events and have approximately the same functions. It is worth noting that, compared with Arabidopsis thaliana and Glycine max, the specific FAD4 subfamily in Camelina sativa may be related to fatty acid synthesis, but clear experimental evidence is required to prove its correlation. Gene structure analysis showed that the structures of introns and exons of the FAD gene family were relatively conserved (Figure 2). The results of chromosome mapping showed that the members of the Camelina FAD gene family were distributed on different chromosomes (Figure 3). This indicates that FADs are involved in the regulation of many plant functions.

We analyzed the expression patterns of the FAD gene family in the different tissues of Camelina (Figure 4). The CsFAD4 gene had the highest expression level in the stem, and FAD4 was found to be the only fatty acid desaturase in the FAD gene family that catalyzes the trans double bond [17], indicating that trans fatty acid in the sporophyte may be mainly distributed in the stem or transported through the stem. Both CsFAD7 and CsFAD8 were expressed in flowers and stems, and in previous studies, FAD7/8 was found to promote plant resistance to low temperatures [20,27]. Therefore, we speculate that, under low-temperature conditions, CsFAD7/8 may regulate the flowering and development of plants. The expression of CsSLDs was relatively high in the stems and seeds of 10 DAF, indicating that CsSLDs may be involved in the desaturation of fatty acids in the early stage of seed formation. Although CsFAD2 was expressed in stems, its expression level changed most obviously in seeds; CsFAD2-2, in particular, was highly expressed in the early formation of seeds. The seeds synthesized unsaturated fatty acids during the formation process, indicating that CsFAD2 may be involved in the synthesis of plant unsaturated fatty acids. Subsequently, using transgenic yeast, we verified this conjecture that CsFAD2-2 promotes the accumulation of linolenic acid (C18:3). Meanwhile, the Camelina FAD family genes were found to respond to low temperatures (Figure 5).
The expressions of CsFAD2, CsFAD7, and CsFAD8 were downregulated at 4 °C for 3 h (Figure 5) but were significantly upregulated at 4 °C for 6 h. In contrast, the CsSLD was upregulated at 4 °C for 3 h. Such results suggest that the response of CsFAD2 to low temperatures may be after CsSLD; in other words, CsFAD2 is the operative gene in a low-temperature environment in Camelina.

Interestingly, AtFAD2 was located on the endoplasmic reticulum, but CsFAD2 was expressed in the nucleus (Figure 6). The reason may be that AtFAD2 is located on chromosome 1, but CsFAD2-2 is located on chromosome 19 and is located close to AtFAD7. Moreover, AtFAD7 is localized on the plastid [22], which also provides good evidence for the subcellular localization of CsFAD2-2. In this study, the fatty acid contents of transgenic CsFAD2-2 yeast cells and control yeast cells were compared and analyzed. The results showed that the contents of various fatty acids in transgenic yeast changed to varying degrees; for instance, the content of oleic acid decreased by 87.8%, but the content of linolenic acid increased by 47.7%, which proves that the main function of CsFAD2-2 is to catalyze the dehydrogenation of oleic acid to linolenic acid. Notably, in the course of the fatty acid analysis of the CsFAD2-2 group, we found that linoleic acid (C18:2) almost disappeared, in addition to the substantial reduction in oleic acid (C18:1) content. This result fully demonstrates that CsFAD2-2 can catalyze C18:2 to C18:3. In summary, CsFAD2-2 can catalyze linoleic acid (C18:2) to C18:3, thereby enhancing the resistance of Camelina to low temperatures.

4. Materials and Methods

4.1. Identification and Analysis of FAD Gene Family

The genome and protein sequences of FAD in soybean, Arabidopsis, and Camelina were downloaded from the NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome-next.jgi.doe.gov/) [44] databases. The HMM profiles of the FAD domain (PF00487) were downloaded from the Pfam protein database (https://pfam.xfam.org/) for soybean, Arabidopsis, and Camelina [45]. The FAD protein sequences were submitted to the SMART database (http://smart.embl.de/) [46] for further domain analysis. Molecular Evolutionary Genetics Analysis (MEGA7) was used for multiple sequence alignment of FAD proteins in the three species [47]. The structural diversity values of intron and exon organizations were obtained from genome annotation [11]. The conserved domains and motifs of these proteins were annotated using the MEME (https://meme-suite.org/) database [48]. The final structure diagram was performed using TBtools (v1.0692) [49]. The syntenic analysis of the CsFAD gene family was performed using MCScanX [50]. Subsequently, TBtools was used to draw a schematic diagram of the chromosomal location distribution of the CsFAD gene family.

4.2. Plant Materials

The growth and treatment of plants were analyzed following [51], with slight modifications. Camelina sativa (L.) Crantz was grown at 22 °C, 50% relative humidity, and a photoperiod of 16 h light/8 h dark. The plants were regularly watered with a 1/2 Hoagland solution. The 2-week-old seedlings were treated with cold stress (4 °C). The seedling leaves of the control (22 °C) and treatment (4 °C) were taken out. Subsequently, they were frozen with liquid nitrogen (N2) and then stored at −80 °C. For the identification of the expression levels of the FAD gene family in different tissues, the samples included leaves, flowers, stems, seeds after 10 days of flowering (DAF), and seeds after 20 days of flowering (DAF); these samples were put into N2 for freezing treatment and then transferred to −80 °C for storage.

4.3. RNA Extraction and qRT-PCR Analysis

The extraction and purification of the total RNA following the product instructions of a TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa). The detection concentration of RNA was expressed as ng/µL. The reverse transcription of RNA followed the manual of
a PrimeScript™ RT Reagent Kit (TaKaRa). TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa) was used for the qRT-PCR reaction. The standard procedure for two-step PCR amplification was first 95 °C for 30 s, then 40 cycles of 95 °C for 5 s, and 60 °C for 30 s [11]. The primers required for qRT-PCR are summarized in Supplementary Table S1.

4.4. Subcellular Localization Assay

To demonstrate the intracellular organelle-specific presence of CsFAD2-2, CsFAD2-2 was inserted into the pCAMBIA1302 vector using the polyclonal site EcoR I/Kpn I. The protoplasts were extracted from Arabidopsis leaves as previously described [52]. Additionally, the transient expression of CsFAD2-2–GFP fusion protein was analyzed in Arabidopsis mesophyll protoplasts. Fluorescence signals were monitored using a Leica TCS SP8 laser scanning confocal microscope (Germany). The primers required for the subcellular localization assay are summarized in Supplementary Table S2.

4.5. Determination of Fatty Acid (FA) Content

To determine the FA content in yeast (Pichia pastoris), the full-length CsFAD2-2 coding sequence was cloned into the pPICZalphaA vector with the polyclonal site EcoR I/Kpn I. The recombinant vector was transformed into Pichia pastoris X33. A YPD medium containing Zeocin was used to grow transgenic yeasts. The transgenic yeasts were grown at 22 °C, 250 rpm for 3 d. The extraction of the total lipids from yeast cells followed the method of [53], with minor modifications. Subsequently, the samples were developed in methanol: CH₂Cl₂ (v1: v2 = 1:3) for 10 min at 30 °C. 6% The KOH in the methanol solution was added into the reaction system for 12 h. Finally, hexane: diethyl ether (v1: v2 = 80:20) was added to the reaction system. The extracted FA methyl ester was dried under nitrogen gas (N2). The analysis of FA methyl ester was performed according to the previous studies of gas chromatography–mass spectrometry methods [54]. The primers required for pPICZalphaA–CsFAD2-2 are summarized in Supplementary Table S3.

5. Conclusions

We performed a phylogenetic analysis of the CsFAD gene family and identified the expression patterns of the CsFAD gene family under low-temperature (4 °C) stress. Meanwhile, we identified CsFAD2-2 to be localized in the nucleus and found that CsFAD2-2 can catalyze the linolenic acid (C18:3) biosynthesis.

Statistical Analysis

All data were statistically analyzed by performing Student’s t-test (* p < 0.05, ** p < 0.01, and *** p < 0.001). The data are expressed as mean ± standard deviation (SD). The physiological phenotypic data were also compared using analysis of variance (ANOVA).

Supplementary Materials: The supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232314550/s1. Reference [33] is cited in the supplementary materials.

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