Received: 8 June 2020 | Accepted: 29 September 2020

DOI: 10.1002/csc2.20362

Crop Science

ORIGINAL RESEARCH ARTICLE

Crop Breeding & Genetics

Genome-wide identification of *FAD* gene family and functional analysis of *MsFAD3.1* involved in the accumulation of α-linolenic acid in alfalfa

Zhengshe Zhang[1,2,3,4,5] | **Xiaoyu Jin**[1,2,3,4,5] | **Zhipeng Liu**[1,2,3,4,5] | **Jiyu Zhang**[1,2,3,4,5] | **Wenxian Liu**[1,2,3,4,5]

[1] State Key Laboratory of Grassland Agro-ecosystems, Lanzhou University

[2] Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs

[3] Western China Technology Innovation Centre for Grassland Industry, Gansu Province, China

[4] Engineering Research Center of Grassland Industry, Ministry of Education

[5] College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730000, People's Republic of China

Correspondence
Wenxian Liu, State Key Laboratory of Grassland Agro-ecosystems, Lanzhou University; Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs; Western China Technology Innovation Centre for Grassland Industry, Gansu Province, China; Engineering Research Center of Grassland Industry, Ministry of Education; College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, 730000, People's Republic of China.
Email: liuwx@lzu.edu.cn

Assigned to Associate Editor Randy Dinkins.

Abstract

As an important forage legume in the world, alfalfa (*Medicago sativa* L.) has high adaptability to various unfavorable climatic conditions and high biomass, and have been playing critical roles in animal husbandry and industrial applications. As α-linolenic acid cannot be synthesized by animals, and most must be obtained from plants, the increasing of α-linolenic acid content in alfalfa will greatly contribute to improve quality of livestock. However, the molecular mechanisms for α-linolenic acid synthesis and accumulation in alfalfa are still limited. In this study, the importance of ω-3 fatty acid desaturase (FAD) was demonstrated by analyzing α-linolenic acid metabolic pathways, combined with the dynamics of accumulation of unsaturated fatty acids in alfalfa. Moreover, the FAD3.1 identified in alfalfa was located in the endoplasmic reticulum, and its expression level was consistent with the accumulation patterns of α-linolenic acid in leaves. Heterologous expression in yeast cells proves that *MsFAD3.1* was involved in the synthesis of α-linolenic acid, and the α-linolenic acid content in *MsFAD3.1*-overexpression transgenic alfalfa lines was significantly increased. These results indicate that new alfalfa germplasm with high α-linolenic acid content can be successfully created through biotechnology, providing a theoretical basis for further improving the quality of alfalfa and the nutritional value of dairy products.

1 | INTRODUCTION

As one of the important industrial products, α-linolenic acid (ALA, C18:3$\triangle^{9,12,15}$) belongs to the ω-3 series of polyunsaturated fatty acids and is the synthetic precursor of known as "brain gold" eicosapentaenoic acid (EPA) and

Abbreviations: ALA, α-linolenic acid; cDNA, complementary DNA; CDS, coding sequence; ER, endoplasmic reticulum; GFP, green fluorescent protein; LA, linoleic acid; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

docosahexaenoic acid (DHA) (Hageman et al., 2012; Han et al., 2020). Besides, ALA is an essential fatty acid for the human body that cannot be synthesized by animals and must be obtained from plants or seafood (Liu, Yin, Xiao, Xu, & Qu, 2012; Saeki et al., 2004). In addition, the ALA synthetic precursor linoleic acid (LA, C18:2$\triangle^{9,12}$) belongs to the ω-6 series of polyunsaturated fatty acids. The ratio of intake of fatty acids in ω-3 series and ω-6 series is often affected by the diet structure and easily leads to the frequent occurrence of cardiovascular diseases, senile dementia, and diabetes, decreased immunity, and obesity (Nguemeni, Gouix, Bourourou, Heurteaux, & Blondeau, 2013). Research has shown that dairy products are one of the important sources for humans to obtain essential fatty acids (Dewhurst, Shingfield, Lee, & Scollan, 2006; Nguyen, Malau-Aduli, Cavalieri, Nichols, & Malau-Aduli, 2019). The content of ALA in dairy products can be used as an important indicator to evaluate the quality of livestock products. However, the ALA content in milk is extremely low, only accounting for 0.4–2.5% of total fatty acids (Chilliard, Ferlay, & Doreau, 2001), so the improvement of ALA in dairy products is of great significance for improving the dairy product quality and dairy farmer income.

The previous studies have shown that the content of ALA in milk is positively correlated with the content of ALA in the pasture. For example, the content of ALA in the fresh Italian ryegrass (*Lolium multiflorum* Lam., 40.1%) is higher than that of Italian ryegrass hay (6.7%) and Italian ryegrass haylage (36.4%). Along with the increased feeding time, the content of ALA in the milk of cows offered fresh grass (1.97%) is significantly higher than from those offered hay (1.46%) and haylage (1.71%) only (Aii, Takahashi, Kurihara, & Kume, 1988; Dewhurst, Scollan, Lee, Ougham, & Humphreys, 2003). In addition, the grazing of fresh pasture increased the content of ALA in beef by six times, correspondingly making the ratio of ω-6/ω-3 fatty acids less than 5:1, reaching a level beneficial to human health (Lorenz et al., 2002). Therefore, increasing the content of ALA in forage, especially in alfalfa (*Medicago sativa* L.), can fully guarantee the acquisition of livestock products with high content of ALA.

In the plant kingdom, the synthesis of polyunsaturated fatty acids is the result of the catalytic actions of a series of desaturases and elongases with saturated fatty acids as substrates (Napier, 2007). Desaturase is a key enzyme in the synthesis of polyunsaturated fatty acids, which controls the degree of unsaturation of polyunsaturated fatty acids. Among these unsaturated fatty acids, the conversion of linoleic acid to ALA is catalyzed by Δ15 (ω-3) fatty acid desaturase, which is a transmembrane protein encoded by multiple genes (Yin et al., 2014). Because of the large differences in expression patterns and biological functions among gene family members, the ALA biosynthesis pathway is accompanied by a complex regulatory network (Liu et al., 2012). For example, the subcellular localization of members of the Δ15 fatty acid desaturase family is different. The FAD3 is located in the endoplasmic reticulum (ER) membrane, whereas FAD7 and FAD8 are located in the chloroplast membrane. In addition, the expression patterns of different genes in response to temperature are also different. The normal expression of *FAD3* and *FAD7* is not regulated by temperature, whereas *FAD8* gene has low temperature induction (Gibson, Arondel, Iba, & Somerville, 1994; Iba et al., 1993). For example, *OsFAD3* and *OsFAD7* are highly expressed in rice (*Oryza sativa* L.) roots and leaves, respectively, whereas *OsFAD8* is mainly expressed in leaves after low temperature induction (Kodama, Akagi, Kusumi, Fujimura, & Iba, 1997; Yara et al., 2007). Under low temperature stress, the expression level of the *GmFAD7-2* gene in soybean [*Glycine max* (L.) Merr.] returns to normal levels after a short period of upregulation, indicating that the *GmFAD7-2* gene has a specific response to low temperature stress (Roman et al., 2012). The expression of the *FAD7/8-1* genes in cotton (*Gossypium hirsutum* L.) also increases significantly at low temperatures. In addition, these genes also respond to drought stress (Yurchenko et al., 2014). In *Carthamus tinctorius* L., the expression level of the *CtFAD7* and *CtFAD8* genes in leaves is the highest, whereas the expression level of these genes in flowers and seeds is extremely low. However, the *CtFAD3* gene is expressed to different levels in all tissues, with higher expression levels in leaves and flowers (Guan et al., 2014). Under low temperatures and mechanical injury stress, the linolenic acid content in *Portulaca oleracea* L. leaves increased to different levels. The expression of the *PoleFAD8* gene increased 3.5-fold under low temperature stress, and the expression level slightly increased under mechanical injury conditions (Teixeira, Carvalho, & Brodelius, 2010).

It is generally believed that *FAD3* catalyzes ALA biosynthesis in nonphotosynthetic tissues, whereas *FAD7* and *FAD8* play roles mainly in plant leaf development (Wang et al., 2006; Yu et al., 2009). For example, the linolenic acid content of the seeds in the *LuFAD3A* and *LuFAD3B* double-mutated *Linum usitatissimum* L. is <2%, whereas the linolenic acid content of the wild-type seeds is as high as 52%

Core Ideas

- *MsFAD3.1* encodes a functional fatty acid desaturase 3 enzyme.
- *MsFAD3.1* can convert linoleic acid to linolenic acid in yeast.
- Overexpression of *MsFAD3.1* in alfalfa increased the C18:3 content of leaves.

(Vrinten, Hu, Munchinsky, Rowland, & Qiu, 2005). When the *GmFAD3* gene family was silenced in soybeans, the linolenic acid content in soybean seeds decreased from 7.1 to 1.0% (Flores et al., 2008). In *Arabidopsis thaliana* (L.) Heynh., the overexpression of *AtFAD3* leads to the enhancement of the seed linolenic acid content from 19 to 40% (Puttick, Dauk, Lozinsky, & Smith, 2009). After overexpression of the ER type *OsFAD3* in the endosperm of rice seeds, the ALA content in the seeds increased 27.9-fold, but overexpression of chloroplast type *OsFAD7* and *OsFAD8* caused no obvious increase in ALA content (Liu et al., 2012). *BnFAD3* and *StFAD7* encode ER type and chloroplast type Δ15 fatty acid desaturase in *Brassica napus* L. and *Solanum tuberosum* L., respectively. After co-expression of these two genes in tomato (*Solanum lycopersicum* L.), the ALA content in the leaves and fruits increased by 6.1 and 123.4%, respectively, compared with the control, whereas the C18:3/C18:2 ratio also increased significantly, 6.8- and 11.3-fold, respectively, compared with the control (Dominguez et al., 2010). These research findings indicate that the Δ15 fatty acid desaturase gene has diverse functions in the ALA biosynthesis pathway in plants. Therefore, according to different requirements, different genes are used to increase the content of ALA.

As the king of forage legumes, alfalfa is the most widely planted legume forage in the world, with a global planting area of about 30 million ha (Mouttet et al., 2014). It not only has great feeding value for livestock but also plays an important role in solving the energy crisis. The crude protein in alfalfa leaves is approximately 30% dry matter, making it good for animal feed (Dale, 1983). Alfalfa also has potential as a bioenergy plant due to its high fiber content (Zhou & Runge, 2015; Zhou, Yang, & Runge, 2015). However, considering the extremely low ALA content in alfalfa, which only accounts for 0.36% of the fresh weight (Dal et al., 2014) and 2.2% of the dry weight (Goossen, Bosworth, Darby, & Kraft, 2018), the increase of the ALA content in alfalfa has great potential and application value. Although predecessors have studied more about the *FAD3* gene in different species (Hernandez, Sicardo, & Martinez-Rivas, 2016; Lee et al., 2016; Zhang, Bi, Liu, & Shan, 2009), the role of FAD3 in the accumulation of ALA in alfalfa remains unclear. In this study, the key genes responsible for the high content of ALA in alfalfa were identified. Moreover, we identified a *MsFAD3.1* gene in the *M. sativa* genome and analyzed the expression pattern and subcellular localization of the gene. Functional analyses in the alfalfa indicated that MsFAD3.1 was ER specific in the synthesis of ALA. The results of this study described above would help to understand the molecular mechanism by which high amounts of ALA accumulate in alfalfa.

2 | MATERIALS AND METHODS

2.1 | Sequence retrieval and alignment analysis

The genome data of *M. sativa* were downloaded from the alfalfa breeder's toolbox (https://www.alfalfatoolbox.org/) identified *MsFAD* genes. A total of 62 identified FAD protein sequences from nine plants (Supplemental Table S1) were used as a query, and the local BLAST search was performed with the *E* value set to 1×10^{-5}. The gene and protein sequences were obtained, the CD-HIT Suite (http://weizhongli-lab.org/cd-hit/) online tool was used for redundant analysis, and the protein sequence was further analyzed by Pfam and SMART tools. The molecular weights (MWs), grand average hydropathicity (GRAVY), and isoelectric points (p*I*s) of the candidate MsFAD proteins were detected using ExPASy (http://web.expasy.org/protparam/).

2.2 | Construction of phylogenetic tree of *MsFAD* gene

The ClustalX program was used to perform multiple sequence alignments on the amino acid sequence of ω-3 fatty acid desaturase, and the results were displayed by DNAMAN software. Neighbor-join (NJ) used MEGA 7 to build a phylogenetic tree, and bootstrap tests were performed using 1,000 replicates to support statistical reliability.

2.3 | Cloning of the full-length *MsFAD3* cDNA

After growing in the greenhouse for 8 wk, the healthy alfalfa leaves of cultivar Regen SY4D were collected for total RNA extraction. First-strand complementary DNA (cDNA) was synthesized with an M-MuLV first-strand cDNA synthesis kit (Sangon Biotech). We used gene-specific primers to amplify full-length coding sequences (CDS) of *MsFAD3.1* and cloned the polymerase chain reaction (PCR) products into the pGEM-T vector (Promega) and sequenced them (Supplemental Table S2).

2.4 | qRT-PCR for sequence validation and gene expression analysis

Total RNA from alfalfa leaves was extracted using the UNIQ-10 Column TRIzol total RNA isolation kit (Sangon Biotech), and the RNA quality and concentration were detected with

a NanoDrop 2000 ultraviolet spectrophotometer. According to the manufacturer's protocol, the first-strand cDNA was synthesized using the M-MuLV first-strand cDNA synthesis kit. After the concentration was measured, the cDNA was uniformly diluted to 100 ng μl^{-1} for use as the quantitative reverse transcription PCR (qRT-PCR) reaction template. The primers were designed by PerlPrimer version 1.1.21 software (Marshall, 2004; Supplemental Table S2). The qRT-PCR was carried out with 2 × SG Fast qPCR Master Mix (Low Rox) for PCR on an Applied Biosystems 7500 real-time PCR system. The PCR conditions were as follows: 95 ˚C for 10 min, 40 cycles of 95 ˚C for 15 s, and 60 ˚C for 1 min.

2.5 | Subcellular localization of the MsFAD3.1 protein

The MsFAD3.1 protein coding sequence was amplified and inserted into the pBI121-EGFP vector (Miaoling Plasmid) to generate an N-terminal green fluorescent protein (GFP) fusion protein driven by the CaMV 35S promoter. The transient expression vector was transformed into tobacco (*Nicotiana tabacum* L.) leaves according to a method previously described. The GFP-MsFAD3.1 fusion proteins and ER markers (ER-RK) were co-expressed (Liu et al., 2012). Three days after infiltration, the transfected leaves were examined using a FluoView FV1000MPE confocal microscope equipped with a 488-nm argon laser (Olympus) to detect green fluorescence signals. A 543-nm laser was used for mCherry excitation, and a 488-nm laser was used for GFP fusion excitation, and a 40 × lens was used to capture a fluorescent image.

2.6 | Function of the MsFAD3.1 protein in yeast cells

The *MsFAD3.1* gene was expressed in *Saccharomyces cerevisiae* strain INVSc1 (Invitrogen) and subsequently produced ALA. According to the product operating instructions, the yeast was transformed with pYES2-*MsFAD3.1* and pYES2. Yeast culturing and the induction of *MsFAD3.1* were performed as follows: culture of yeast cells containing pYES2-*MsFAD3.1* in uracil-deficient medium containing 2% glucose and 1% Tergitol NP-40 at 30 ˚C. At an overall optical density (OD_{600}) of the yeast solution equal to 0.5–0.6, LA (0.5 mM) and 2% galactose as an inducer were added to the culture, which was then incubated for 3 d at 20 ˚C.

To test for low temperature and salt tolerance, the yeast cells were resuspended in SC-Ura medium and placed in a refrigerator set to −20 ˚C for 24 h. In addition, the same amount of yeast culture sample was resuspended in 5 M NaCl for 36 h. For generating the controls, yeast cells were resuspended in equal amounts in 200 µl of sterile water and incubated at 30 ˚C for 36 h. The treated yeast liquid was diluted to 1:10 and cultured on SC-Ura medium for 3 d.

2.7 | Expression vector construction and transformation of alfalfa

To overexpress in alfalfa, the full-length coding region of *MsFAD3.1* cDNA between the *BamH*I and *Sac*I restriction sites in the *pBI121* binary vector containing the CaMV *35S* promoter and *NOS* terminator was introduced into *Agrobacterium tumefaciens* strain GV3101, and Regen SY4D was transfected according to the sonication-assisted *Agrobacterium*-mediated transformation method (Jiang, Fu, & Wang, 2019). The obtained transgenic plants were used to analyze fatty acid content.

2.8 | Fatty acid composition analysis of alfalfa leaves

In order to analyze fatty acids in transgenic alfalfa leaves, fatty acid extraction and fatty acid methyl ester (FAME) preparation were performed using the protocol described above (Miquel, James, Dooner, & Browse, 1993). An Agilent Technologies 6890N 5975C gas chromatography/mass spectrometry (GC/MS) system was used with a capillary column (Agilent DB-FFAP; 30 m × 0.25 mm × 0.5 µm). Helium was used as the carrier gas at a linear rate of 1 ml min^{-1} and a split ratio of 100:1. The injector and detector temperatures were 250 ˚C, and the column temperature was set to rise from 70 to 230 ˚C (12-min holding) at a rate of 5 ˚C min^{-1}. The voltage of the ionization source was 1,788 V, and the solvent delay was 3 min. The quadrupole, ion source, and interface temperatures were 150, 230, and 250 ˚C, respectively.

2.9 | Statistical analysis

Using the SPSS 22.0 software for the statistical data analysis. The LSD (Duncan) test was used to test the significance, and the significance threshold was 5%. The samples included in the analysis were created to produce three biological replicates.

3 | RESULTS

3.1 | Identification and phylogenetic analysis of the *MsFAD* gene family

To understand the number and evolution of *MsFAD* genes in the *M. sativa* genome, 62 known FAD proteins from nine species (Supplemental Table S1) were used as query to blast

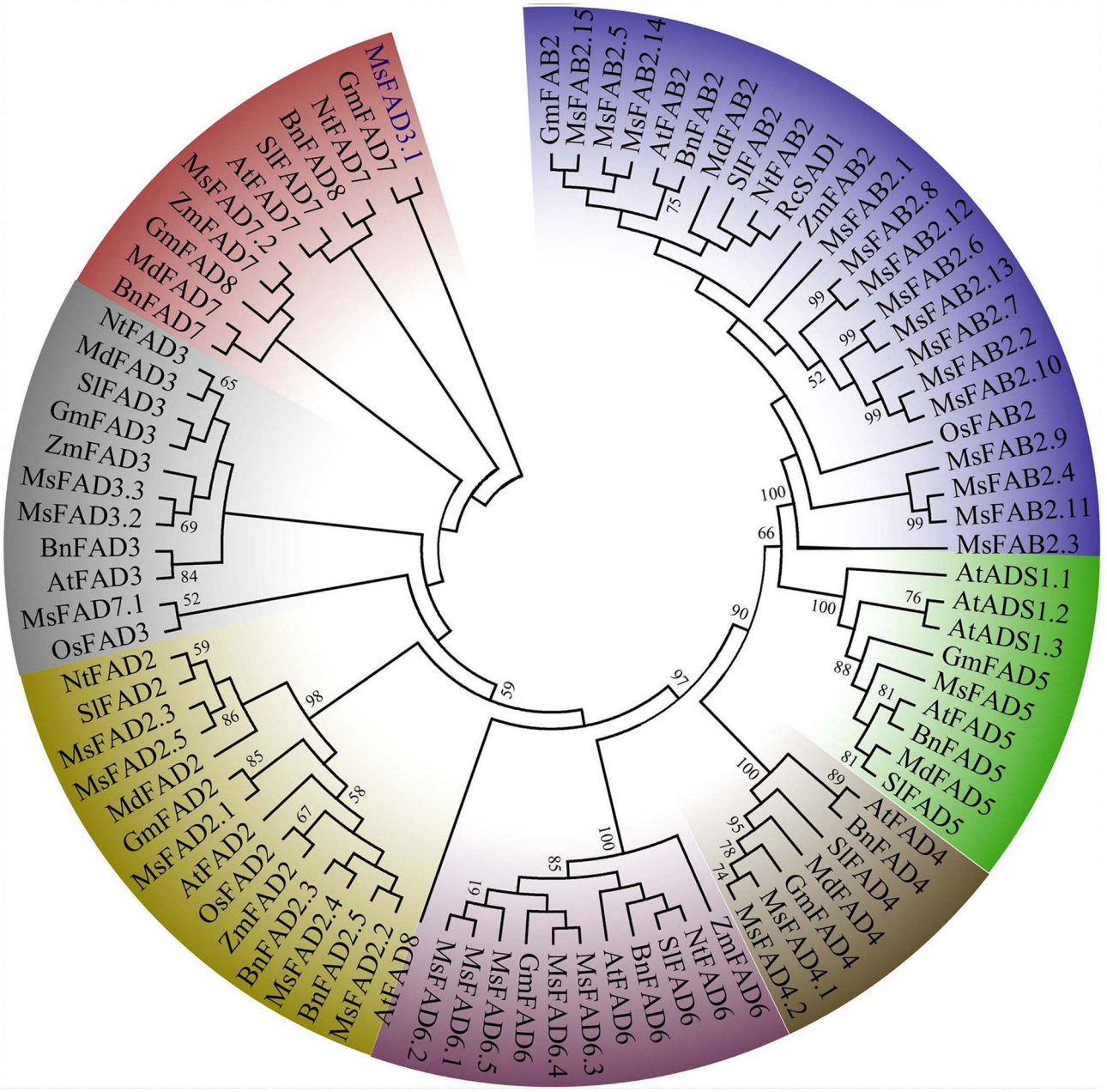

FIGURE 1 Phylogenetic relationships of *FAD* genes from *Medicago sativa*, *Arabidopsis*, *Brassica napus*, *Glycine max*, *Malus domestica*, *Nicotiana tabacum*, *Solanum lycopersicum*, *Oryza sativa*, *Zea mays*, and *Ricinus communis*. Using the MEGA7 program and a bootstrap test (1,000 replicates), a phylogenetic tree of all detected *FAD* genes was constructed by the neighbor-joining (NJ) method. Each colored branch indicates a different subfamily

the *M. sativa* genome database. A total of 33 nonredundant genes were identified as *MsFAD* genes (Figure 1). These *MsFAD* genes included 15 *MsFAB2* genes, one *MsFAD5* gene, three *MsFAD3* genes, five each of *MsFAD2* and *MsFAD6* genes, two *MsFAD4* genes, and two *MsFAD7* genes. The length of the protein sequence encoded by the *MsFAD* genes ranges from 89 amino acids of MsFAB2.9 to 798 amino acids of MsFAB2.4, with the average length of 374 amino acids. The predicted theoretical isoelectric points (p*I*s) ranged from 5.09 to 9.36, the molecular weights (MWs) of the MsFAD proteins ranged from 10.20 to 91.10 kDa, and the average hydrophilicity ranged from −0.448 to 0.047, indicating that MsFADs are all primarily hydrophilic proteins (Supplemental Table S3).

To investigate the evolutionary relationships among the *FAD* gene families in *M. sativa* (33), the FAD protein sequences from *Arabidopsis* (16), *Brassica napus* (9), *Glycine max* (8), *Solanum lycopersicum* (7), *Malus domestica* auct. non Borkh. (6), *Oryza sativa* (5), *Zea mays* L. (5), *Nicotiana tabacum* (5), and *Ricinus communis* L. (1) were used to

generate a phylogenetic tree (Figure 1). According to the phylogenetic tree, the MsFAD proteins could be separated into seven subfamilies. In plants, FAB2 proteins, also known as SADs, can introduce a double bond at the △-9 position of stearoyl-ACP (C18:0-ACP) (Wallis & Browse, 2002). According to the phylogenetic tree, the *MsFAB2.5*, *MsFAB2.14*, *MsFAB2.15*, and *GmFAB2* genes are clustered in the same branch, and the remaining *MsFAB2* genes do not extensively cluster with the *FAB2* genes of other species (Figure 1). FAD5 can introduce a double bond at the △-3 or △-7 position of the saturated acyl chain (Wallis & Browse, 2002). However, only one MsFAD5 protein is included in the FAD5 protein subfamily (Figure 1).

The other two subfamilies encode the FAD2 and FAD6 proteins, which are often located in the ER and plastid (Wallis & Browse, 2002). In this study, the two proteins were categorized into different subfamilies (Figure 1). The *MsFAD2.1* and *GmFAD2* genes are clustered on the same branch, and the *MsFAD2.2*, *MsFAD2.4*, *BnFAD2.3*, and *BnFAD2.5* genes are clustered on the same branch. The *MsFAD2.3*, *MsFAD2.5*, *NtFAD2*, and *SlFAD2* genes are clustered in the same branch. The five *MsFAD6* genes are all clustered into the same branch as the *GmFAD6* gene.

Similar to the FAD2 and FAD6 subfamilies, the two subfamilies that encode the FAD3 and FAD7/FAD8 proteins are also located in the ER and plastid (Wallis & Browse, 2002). In this study, only two MsFAD7 proteins (FAD7/FAD8 subfamily) were included (Figure 1). However, it was difficult to distinguish *FAD7* from *FAD8* based on their phylogenetic relationships, owing to their high sequence similarity (Liu et al., 2015; Yurchenko et al., 2014). The *MsFAD3.2*, *MsFAD3.3*, and *ZmFAD3* genes are clustered on the same branch, but the *MsFAD3.1* is located with the *FAD7/8* subfamily, the *MsFAD7.1* and *OsFAD3* are clustered together, and the *MsFAD7.2* and *ZmFAD7* genes are clustered on the same branch. The *MsFAD4.1*, *MsFAD4.2*, and *GmFAD4* genes are clustered on the same branch.

In plants, two distinct pathways involved in the biosynthesis of polyunsaturated fatty acids are found in the chloroplast and the ER (Sato & Moriyama, 2007). Furthermore, the desaturases are classified into two main types (soluble and membrane bound), and FAB2 is a unique type that belongs to soluble FAD (Shanklin & Cahoon, 1998). To further understand the catalytic activities of MsFAB2 isoforms in *M. sativa*, multiple sequence alignment was performed with AtFAB2, BnFAB2, GmFAB2, MdFAB2, NtFAB2, SlFAB2, OsFAB2, ZmFAB2, RcSAD1, and 15 MsFAB2 proteins identified in this study. As indicated in Supplemental Figure S1, these FAB2 proteins had ultrahigh identity with each other except MsFAB2.2, MsFAB2.3, MsFAD2.6, MsFAD2.9, and MsFAB2.12. Similar to the FAB2 proteins in other plants, these proteins have a chloroplast signal peptide region in the presumptive N-terminal. By the crystalline structure of RcSAD1, 10 MsFAB2 proteins were composed of 11 highly conserved α-helices and two β-sheets (Supplemental Figure S1).

In addition, the 10 MsFAB2 isomers also have a conserved active center of divalent iron atoms that interact with the same catalytic substrate, and these proteins have six amino acid residues bound to the iron atoms (marked in red boxes in Supplemental Figure S1). Additionally, residues marked in the blue boxes in Supplemental Figure S1 were proven to be crucially important in RcSAD1, which is a △-9 C18:0-ACP desaturase, and substitutes of these residues converted RcSAD1 into an enzyme that could function as a △-6 C18:0-ACP desaturase (Heilmann, Mekhedov, King, Browse, & Shanklin, 2004). Similarly, 13 MsFAB2 proteins had the same residues at these positions as nine protein sequences of other plants.

The other 18 *M. sativa* FAD proteins were identified as membrane-bound desaturase family members in this study. The sequences were aligned with other plant homologous sequences to find the conserved motifs in the FAD proteins (Supplemental Figures S2–S7). All these *M. sativa* FAD proteins had three histidine boxes (His-boxes, Supplemental Figures S2–S7), which participate in the formation of the diiron center (Los & Murata, 1998). Similar results were also reported in *Gossypium raimondii* Ulbr. (Liu et al., 2015). The amino acids in these His-boxes were found to be highly conserved, particularly among the MsFAD proteins of the same subfamily (Table 1), as well among different plants (Supplemental Figures S2–S7). MsFAD6.4 lacks the first and second histidine boxes, and a histidine in the third histidine box is mutated to an aspartate, causing this histidine box to change from HIPHH to DIPHH. In addition to MsFAD4, 55 other amino acids were found between the first and second histidine boxes among the members of this family protein, and 31 amino acids were found between the first and second histidine boxes for the other transmembrane FAD proteins. The number of amino acids between the second histidine box and the third histidine box is not fixed. For example, five MsFAD2 proteins have 169 or 170 amino acid residues, whereas the MsFAD3 and MsFAD7 protein each has 162 amino acid residues. The MsFAD4 protein has 24 amino acid residues, the MsFAD5 protein has 127 amino acid residues, most of the MsFAD6 proteins have 155 amino acid residues, and the MsFAD6.1 and MsFAD6.5 proteins have 150 and 169 amino acid residues, respectively (Table 1).

The synthesis of unsaturated fatty acids is performed through an alternative eukaryotic pathway within the ER (Shanklin & Cahoon, 1998). FAD2 and FAD3 are considered ER-localized FADs based on the potential locations of their metabolic pathways (Gao et al., 2014). Both FAD2 and FAD3 have an ER retention signal. For FAD2, the ER retention signal is embedded within Φ-X-X-K-Φ (where Φ is a hydrophobic amino acid residue) on the C-terminus, and

TABLE 1 The conserved histidine boxes of the membrane bound FAD proteins in *M. sativa*

		His-box 1		His-box 2		His-box 3	
Type	Protein	Sequence	Position amino acids	Sequence	Position amino acids	Sequence	Position amino acids
ω-6/△-12	MsFAD2.1	HECGH	98–102	HRRHH	134–138	HVAHH	308–312
	MsFAD2.2	HECGH	112–116	HRRHH	148–152	HVAHH	323–327
	MsFAD2.3	HECGH	106–110	HRRHH	142–146	HVVHH	316–320
	MsFAD2.4	HECGH	105–109	HRRHH	141–145	HVAHH	316–320
	MsFAD2.5	HECGH	115–119	HRRHH	151–155	HVVHH	325–329
	MsFAD6.1	HDCAH	163–167	HDKHH	199–203	HIPHH	354–358
	MsFAD6.2	HDCAH	170–174	HDKHH	206–210	HIPHH	366–370
	MsFAD6.3	HDCAH	172–176	HDRHH	208–212	HIPHH	368–372
	MsFAD6.4	–	–	–	–	DIPHH	77–81
	MsFAD6.5	HDCAH	170–174	HDKHH	206–210	HIPHH	380–384
ω-3/△-15	MsFAD3.1	HDCGH	104–108	HRTHH	140–144	HVIHH	307–311
	MsFAD3.2	HDCGH	109–113	HKTHH	145–149	HVVHH	312–316
	MsFAD3.3	TIGH	99–102	HKTHH	134–138	HVVHH	301–305
	MsFAD7.1	HDCGH	163–167	HRTHH	199–203	HVIHH	366–370
	MsFAD7.2	HDCGH	157–161	HRTHH	193–197	HVIHH	360–364
△-3	MsFAD4.1	QGHH	158–161	HAWAH	217–221	HGAHH	246–250
	MsFAD4.2	QGHH	160–163	HAWAH	219–223	HGAHH	248–252
△-7	MsFAD5	HRNLSH	164–169	HRYHH	201–205	HNNHH	333–337

this signal sequence is "YNNKL" in AtFAD2 (Supplemental Figure S2; McCartney et al., 2004). In addition to the deletion of the carboxy terminus of MsFAD3.3, the ER retention signal sequences in MsFAD2 and MsFAD3 were identified in the respective C-terminus and marked with a red box (Supplemental Figures S2–S3). The protein sequence alignment revealed chloroplast signal peptides at the amino terminus of the MsFAB2, MsFAD4, MsFAD5, MsFAD6, and MsFAD7 protein sequences (red dashed box, Supplemental Figures S1 and S4–S7).

3.2 | Isolation and analysis of *MsFAD3*

For improving the ALA content of alfalfa, a key gene in the ALA biosynthesis metabolic pathway of alfalfa, *MsFAD3.1*, was cloned based on the identification and analysis of the alfalfa fatty acid dehydrogenase gene family. We cloned the full-length CDS of the *MsFAD3.1* gene sequence, which was a 1,164-bp sequence encoding 387 amino acids, with a predicted molecular weight of 45.28 kDa and a p*I* value of 8.65. MsFAD3.1 contains three histidine boxes (H1-H3, Figure 2a) and four conserved transmembrane domains (TMDs, Figure 2a), and is considered highly conserved among all membrane-bound FADs (Shi, Yue, & An, 2018). The sequence analysis data showed that *MsFAD3.1* gene has high identity with other microsomal ω-3 *FAD* genes, such as *AtFAD3* (64.32%), *BnFAD3* (65.09%), *NtFAD3* (65.96%), *SlFAD3* (68.17%), *GmFAD3* (79.21%), *MdFAD3* (70.00%), and *OsFAD3* (63.20%). The phylogenetic tree showed that *MsFAD3.1* was closest to *GmFAD3* and *MdFAD3* (Figure 2c). These analyses indicated that *MsFAD3.1* is a microsomal ω-3 *FAD* gene.

In order to investigate whether the MsFAD3.1 protein targets to the ER, subcellular localization of the MsFAD3.1 protein was observed by transiently co-expressing N-terminal GFP fusion constructs into tobacco epidermis. The fluorescence signal of GFP-MsFAD3.1 fusion protein was merged to the ER marker (ER-mCherry) in epidermal cells, suggesting that the MsFAD3.1 protein is indeed localized to the ER (Figure 2b). For phylogenetic analysis of the *MsFAD3.1* gene, a phylogenetic tree was constructed using the amino acid sequence predicted for alfalfa and the ω-3 fatty acid desaturase gene of other known plants (Figure 3). It can be seen from the phylogenetic tree that the *MsFAD3.1* gene and *MtFAD3.1* gene are clustered together, indicating that they are differentiated from the same ancestor.

To analyze the expression of *MsFAD3.1* in *M. sativa*, qRT-PCR was performed. The results showed that the expression level of *MsFAD3.1* was highest in leaves, followed by roots and stems, and lowest in flowers (Figure 4). This expression pattern is similar to that of *CbFAD3* in *Chorispora bungeana* Fisch. et Mey. (Shi et al., 2018).

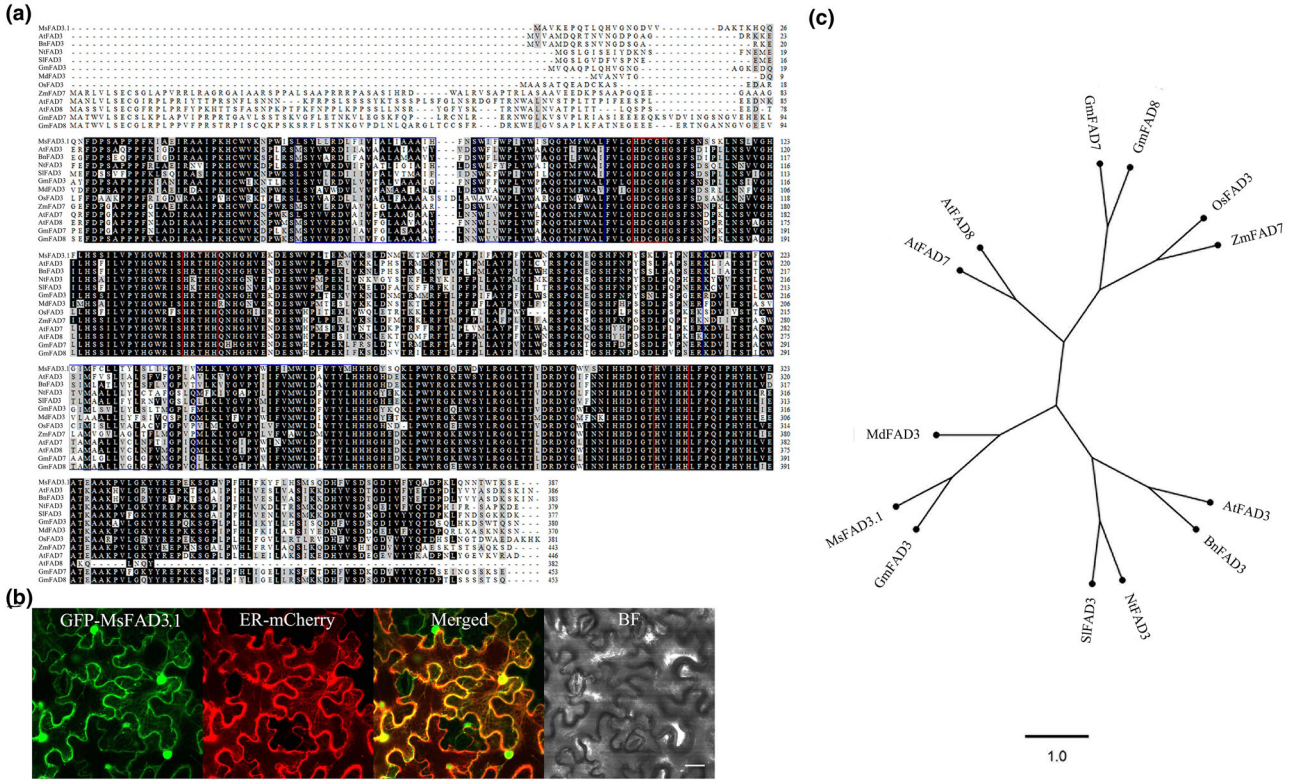

FIGURE 2 Analysis of the sequence and subcellular localization of MsFAD3.1 and ω-3 FADs in various plant species. (a) According to the crystal structure of other ω-3 FADs, the secondary protein structure of MsFAD3.1 protein was compared, deduced, and annotated. Sequence alignment was performed using DNAMAN. Three conserved histidine boxes are marked with red boxes, and four transmembrane domains are marked with blue boxes. (b) ER-mCherry, endoplasmic reticulum marker protein; BF, brightfield; bars = 20 μm. (c) Phylogenetic tree analysis of MsFAD3.1 and other FAD family members from select plant species

3.3 | Heterologous expression of *MsFAD3.1* in yeast can promote the transformation of C18:2 to C18:3

Studies have shown that the function of *MsFAD3.1* gene can be studied by heterologous expression of the ω-3 *FAD* gene in yeast cells (Dyer, Chapital, Cary, & Pepperman, 2001). To further clarify the function of *MsFAD3.1*, the gene was constructed into pYES2 vector and transformed into yeast, with galactose added to the culture medium to induce pYES2 expression in the yeast cells. The fatty acid analysis of the entire yeast cell clearly showed that MsFAD3.1 can induce the conversion of C18:2 to C18:3 in yeast cells transformed with *MsFAD3.1*, whereas C18:3 was not detected in the control (Supplemental Figure S8).

Previous studies have shown that the plant Δ15 fatty acid desaturase gene is involved in the regulation of various abiotic stress responses (Roman et al., 2012; Yurchenko et al., 2014). In this study, the *MsFAD3.1* gene was transformed into the INVSc1 yeast cells to verify its function response to cold, salt, and high-temperature abiotic stress. The experimental results showed that the growth of the transgenic and control yeast containing the empty vector under nonstress conditions (YPD plate, 30 ˚C) was consistent. Under a condition of low temperature at −20 ˚C and 5 M NaCl, the growth of the transgenic yeast was greater than that of the control yeast (Figure 5).

3.4 | Overexpression of *MsFAD3.1* increased alfalfa leaves C18:3 content

To determine the biological functions of *MsFADs* in alfalfa, especially in the accumulation of ALA, we further overexpressed this gene in alfalfa, and nine *MsFAD3.1* transgenic plant lines with high expression level were obtained. To illustrate the effect of overexpressing *MsFAD3.1* on the proportion of fatty acid profiles, total fatty acids were extracted from the leaves, and the palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) were separated and measured for their contents. The content of C16:0, C16:1, C18:0, C18:1, C18:2, and C18:3 accounted for 17.7, 1.6, 2.2, 1.4, 15.1, and 62.0% of total fatty acids, respectively, in control alfalfa leaves. In the leaves of transgenic lines, the proportion of the six classes of fatty acid was slightly lower than that of control plant except for the contents of ALA, which was

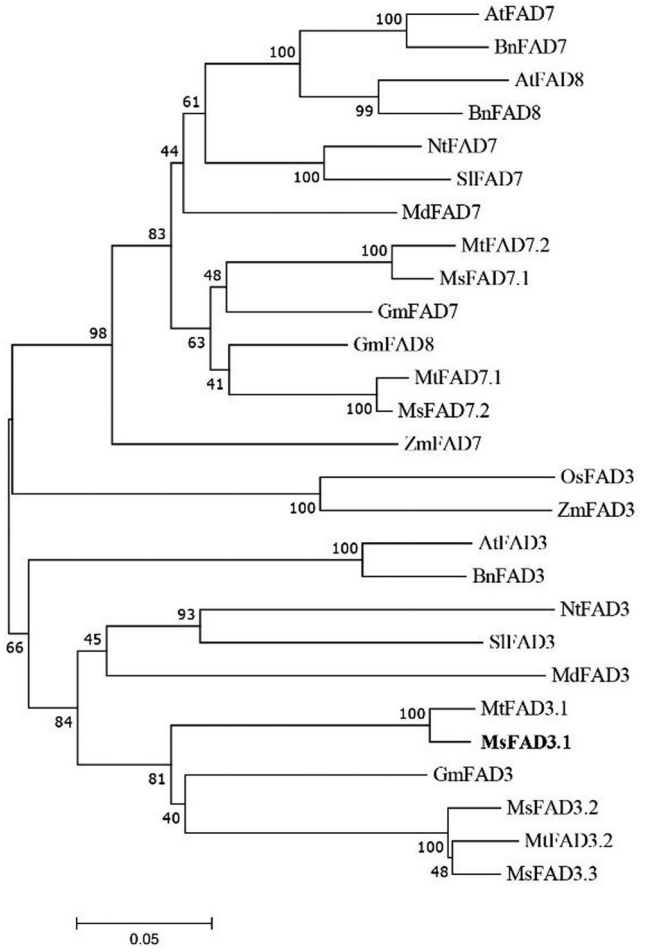

FIGURE 3 Phylogenetic relationship and gene structure of the *FAD3* genes in *M. sativa*.
Scale bars represent 0.05 amino acid substitutions per site. At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Gm, *Glycine max*; Os, *Oryza sativa*; Mt, *Medicago truncatula*; Ms, *Medicago sativa*; Md, *Malus domestica*; Nt, *Nicotiana tabacum*; Sl, *Solanum lycopersicum*; Zm, *Zea mays*. The bold MsFAD3.1 in the figure is the cloned gene in this study

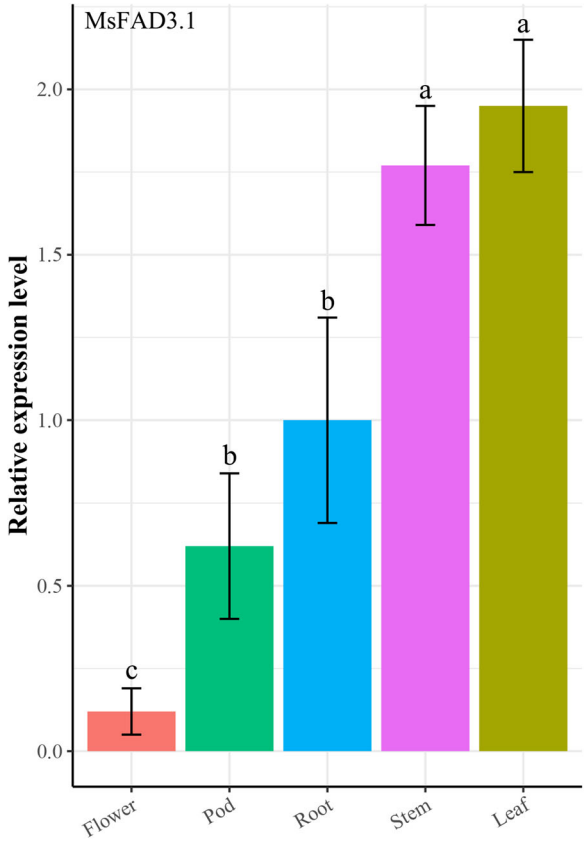

FIGURE 4 Relative expression levels of *MsFAD3.1* in different tissues. The relative expression levels of the *FAD3.1* gene in different tissues of *M. sativa* were determined by quantitative polymerase chain reaction (qPCR). The data represent the means of three independent replicates ± SE. Samples not sharing a letter are significantly different according to ANOVA

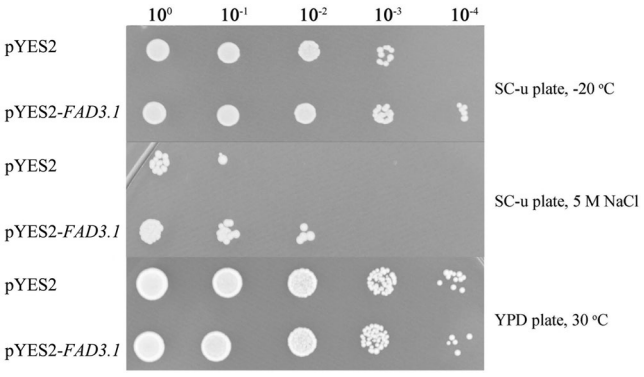

FIGURE 5 Growth of yeast cells transformed with the pYES2-*FAD3.1* and with the empty vector pYES2 under cold and salt stress. To test the low temperature and salt tolerance, yeast cells were resuspended in SC-Ura medium and placed in a refrigerator at −20 ˚C for 24 h. In addition, the same amount of yeast culture samples was resuspended in 5 M NaCl for 36 h. An equal amount of yeast cells was resuspended in 200 µl of sterile water and incubated at 30 ˚C for 36 h to serve as a control. The treated yeast liquid was diluted 1:10 and cultured on SC-Ura medium for 3 d

1.3-fold higher than that of control plant (Supplemental Table S4). This may be caused by the conversion of other fatty acids in the transgenic lines to linolenic acid. The lowest ALA level reached to 76.7% in the F3-S-70 line, and the highest level reached to 84.0% in F3-S-55 with the mean level of 79.7% of the total fatty acids in the transgenic lines, as compared with 62.0% in the control plant. The ALA content of the transgenic plants was increased by 23.76–35.48% (Figure 6), indicating a critical function of MsFAD3.1 in the accumulation of ALA.

4 | DISCUSSION

Unsaturated fatty acids have high value in edibles and for industrial use, and the modification of plants through genetic

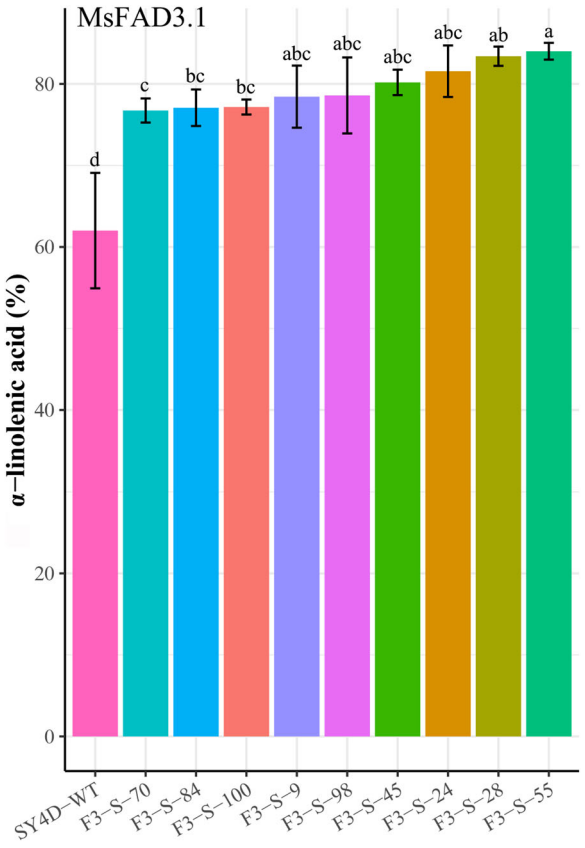

FIGURE 6 Analysis of α-linolenic acid content in transgenic alfalfa leaves. Values are means ± SE of three biological replicates for each experiment. Samples not sharing a letter are significantly different according to ANOVA

engineering to obtain a large number of unsaturated fatty acid products has good application prospects (Chen et al., 2015). Therefore, FAD, a key enzyme regulating the biosynthesis of polyunsaturated fatty acids, has become a research hotspot. In this study, a *MsFAD3* gene was cloned from alfalfa, and the hydrophilicity and hydrophobicity of the amino acids were predicted by ProtScale software. The results showed that the hydrophilic amino acid of the protein was evenly distributed throughout the peptide. Basically, two desaturases, FAD2 and FAD3, are located to the ER and play important roles in the polyunsaturated fatty acid synthesis pathway. Like FAD2, FAD3 are generally hydrophilic proteins and can use the phosphatidylcholine (PC)-18:2 (products of FAD2) as the substrates to produce PC-18:3 (Guo et al., 2018; Kim et al., 2016). The results of phylogenetic tree construction based on multiple sequence alignments indicated that the *MsFAD* gene has high homology with the *MtFAD* gene (Figure 3). Similar to the FAD3 protein of other plants, the MsFAD3 contains highly conserved histidine boxes (H-boxes: HXXHH and HXXXH; Alonso, Garcia-Maroto, Rodriguez-Ruiz, Garrido, & Vilches, 2003), which are required for desaturase activity and are responsible for the formation of the diiron–oxygen complex used in biochemical catalysis (Shanklin & Cahoon, 1998). According to the analysis results and similar to other plant ω-3 fatty acid desaturases, MsFAD3 is predicted to have four transmembrane domains (TMDs) similar to double-pass membrane domains (Figure 2a), and the result is the same as other plant ω-3 fatty acid desaturases (Lee et al., 2016; Yin et al., 2018). In addition, MsFAD3 has no N-terminal chloroplast transit peptide (cTP) in the N-terminus, which is a characteristic of chloroplast-localized ω-3 fatty acid desaturases (Lee et al., 2016).

Previous studies have shown that FAD3 dehydrogenase catalyzes the conversion of linoleic acid (C18:2) to ALA (C18:3) (Zhang, Maximova, & Guiltinan, 2015). *FAD3A* appears to be the key gene responsible for C18:3 accumulation in olive (*Olea europaea* L.) seeds, whereas *FAD7* contributes more in mesocarp (Hernandez et al., 2016). In this study, the *MsFAD3.1* was expressed to different degrees in flowers, pods, roots, stems, and leaves, especially in leaves (Figure 4). The AhFAD3 gene of peanut (*Arachis hypogaea* L.) was also expressed to different degrees in different tissues, and the expression level was highest in seeds (Peng et al., 2020). The fluorescence signal of GFP-MsFAD3.1 was merged to the ER marker in tobacco epidermal cells, indicating that MsFAD3 is localized to the ER (Figure 2b). However, the MsFAD3.3 protein lacks a -KDEL or -KXKXX retrieval motif, which is an ER retrieval motif in the C-terminal region (McCartney et al., 2004). This phenomenon has been previously observed in olive (Hernandez et al., 2016), soybean (Anai, Yamada, Kinoshita, Rahman, & Takagi, 2005), and tree peony (*Paeonia suffruticosa* Andr.; Yin et al., 2018) FAD3 proteins, suggesting that they may contain unknown ER retrieval motifs in the C-terminal region.

The previous study has shown that overexpression of *SsSAD* gene from *Sapium sebiferum* (L.) Roxb. could effectively increase the C18:3 content in rapeseed by 35–40% (Peng et al., 2018). Besides, the constitutive expression of the peanut *AhFAD3* gene in *Arabidopsis thaliana* successfully increased the total fatty acid content of the seed by between 24.8 and 33.3%, especially that of C18:3 (Peng et al., 2020). In this study, overexpression of *MsFAD3* gene can effectively increase the content of ALA in alfalfa by 23.76–35.48% (Figure 6). The results indicated that the use of genetic engineering technology to manipulate the regulatory genes in the plant ALA synthesis pathway can effectively increase the content of ALA in alfalfa.

Previous studies indicated that overexpression of the *FAD3* gene can both increase the C18:3 content and enhance the response to various stresses in plants (Torres-Franklin et al., 2009; Wang et al., 2014; Yu et al., 2009; Zhang et al., 2005). In addition, the yeast system has been widely used to confirm the predicted activity of fatty acid dehydrogenases in several plants, such as in the study of *Arabidopsis*, *Phaeodactylum tricornutum* Bohlin, *Gossypium hirsutum*, and *Brassica*

napus (Covello & Reed, 1996; Domergue et al., 2003; Zafar et al., 2020; Zhang, Pirtle, et al., 2009). Similarly, heterologous expression of *MsFAD3* in yeast cells in our study also improved the freeze resistance and salt tolerance of yeast cells. Similarly, overexpression of the *AtFAD7* gene in vivo increased the ALA content while also enhancing ability in response to wounding (Angel et al., 2019). This result indicates that the ω-3 *FAD* gene not only promotes the increase in ALA content but also contributes to the response of plants to abiotic stress. The specific principle of this mechanism is based on a change in the fluidity of the membrane under the action of fatty acid desaturase and the prevention of damage to the biofilm caused by stress, which effectively maintains the structural and functional integrity of the cell membrane and thus enhances cellular resistance (Los & Murata, 2004; Mikami & Murata, 2003; Upchurch, 2008).

5 | CONCLUSIONS

In this study, the function of *MsFAD3*, the key gene in the alfalfa ALA synthesis pathway, was examined. On the basis of the successful cloning of the *MsFAD3.1* gene, the subcellular localization of the protein encoded by the *MsFAD3* gene was located in the ER. The results of the fatty acid determination in transgenic plants showed that the overexpression of *MsFAD3* in alfalfa significantly increased the content of ALA in alfalfa leaves by 23.76–35.48%. The increased ALA content in alfalfa can improve alfalfa quality and the nutritional value of dairy products.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (31502000), the Fundamental Research Funds for the Central Universities (lzujbky-2020-18), the Special Funding for Open and Shared Large-Scale Instruments and Equipments of Lanzhou University (LZU-GXJJ-2019C041), and the Program for Changjiang Scholars and Innovative ResearchTeam in University (IRT_17R50).

CONFLICT OF INTEREST

There is no conflict of interest among authors.

ORCID

Zhipeng Liu https://orcid.org/0000-0002-2460-076X
Jiyu Zhang https://orcid.org/0000-0002-3642-373X

REFERENCES

Aii, T., Takahashi, S., Kurihara, M., & Kume, S. (1988). The effects of Italian ryegrass hay, haylage and fresh Italian ryegrass on the fatty acid composition of cows' milk. *Japanese Journal of Zootechnical Science*, *59*, 718–724. https://doi.org/10.2508/chikusan.59.718

Alonso, D. L., Garcia-Maroto, F., Rodriguez-Ruiz, J., Garrido, J. A., & Vilches, M. A. (2003). Evolution of the membrane-bound fatty acid desaturases. *Biochemical Systematics and Ecology*, *31*, 1111–1124. https://doi.org/10.1016/S0305-1978(03)00041-3

Angel, S. G., Maria, C. R., Beatriz, L., Sara, L. G., Maria de los, A. L., Raúl, D. G., … Miguel, A. (2019). Tissue distribution and specific contribution of *Arabidopsis* FAD7 and FAD8 plastid desaturases to the JA- and ABA-mediated cold stress or defense responses. *Plant and Cell Physiology*, *60*, 1025–1040. https://doi.org/10.1093/pcp/pcz017

Anai, T., Yamada, T., Kinoshita, T., Rahman, S. M., & Takagi, Y. (2005). Identification of corresponding genes for three low-α-linolenic acid mutants and elucidation of their contribution to fatty acid biosynthesis in soybean seed. *Plant Science*, *168*, 1615–1623. https://doi.org/10.1016/j.plantsci.2005.02.016

Covello, P. S., & Reed, D. W. (1996). Functional expression of the extraplastidial *Arabidopsis thaliana* oleate desaturase gene (*FAD2*) in *Saccharomyces cerevisiae*. *Plant Physiology*, *111*, 223–226. https://doi.org/10.1104/pp.111.1.223

Chen, Y. C., Cui, Q. Q., Xu, Y. J., Yang, S. S., Gao, M., & Wang, Y. D. (2015). Effects of tung oilseed *FAD2* and *DGAT2* genes on unsaturated fatty acid accumulation in *Rhodotorula glutinis* and *Arabidopsis thaliana*. *Molecular Genetics and Genomics*, *290*, 1605–1613. https://doi.org/10.1007/s00438-015-1011-0

Chilliard, Y., Ferlay, A., & Doreau, M. (2001). Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livestock Production Science*, *70*, 31–48. https://doi.org/10.1016/S0301-6226(01)00196-8

Dal, B. A., Mugnai, C., Roscini, V., Mattioli, S., Ruggeri, S., & Castellini, C. (2014). Effect of dietary alfalfa on the fatty acid composition and indexes of lipid metabolism of rabbit meat. *Meat Science*, *96*, 606–609. https://doi.org/10.1016/j.meatsci.2013.08.027

Dale, B. E. (1983). Biomass refining: Protein and ethanol from alfalfa. *Industrial & Engineering Chemistry Research*, *22*, 466–472. https://doi.org/10.1021/i300011a016

Dewhurst, R. J., Scollan, N. D., Lee, M. R., Ougham, H. J., & Humphreys, M. O. (2003). Forage breeding and management to increase the beneficial fatty acid content of ruminant products. *Proceedings of the Nutrition Society*, *62*, 329–336. https://doi.org/10.1079/pns2003241

Dewhurst, R. J., Shingfield, K. J., Lee, M. R. F., & Scollan, N. D. (2006). Increasing the concentrations of beneficial polyunsaturated fatty acids in milk produced by dairy cows in high-forage systems. *Animal Feed Science and Technology*, *131*, 168–206. https://doi.org/10.1016/j.anifeedsci.2006.04.016

Domergue, F., Spiekermann, P., Lerchl, J., Beckmann, C., Kilian, O., Kroth, P. G., … Heinz, E. (2003). New insight into *Phaeodactylum tricornutum* fatty acid metabolism. Cloning and functional characterization of plastidial and microsomal Δ12-fatty acid desaturases. *Plant Physiology*, *131*, 1648–1660. https://doi.org/10.1104/pp.102.018317

Dominguez, T., Hernandez, M. L., Pennycooke, J. C., Jimenez, P., Martinez-Rivas, J. M., Sanz, C., … Sanmartin, M. (2010). Increasing ω-3 desaturase expression in tomato results in altered aroma profile and enhanced resistance to cold stress. *Plant Physiology*, *153*, 655–665. https://doi.org/10.1104/pp.110.154815

Dyer, J. M., Chapital, D. C., Cary, J. W., & Pepperman, A. B. (2001). Chilling-sensitive, post-transcriptional regulation of a plant fatty acid desaturase expressed in yeast. *Biochemical and Biophysical*

Research Communications, *282*, 1019–1025. https://doi.org/10.1006/bbrc.2001.4667

Flores, T., Karpova, O., Su, X., Zeng, P., Bilyeu, K., Sleper, D. A., … Zhang, Z. J. (2008). Silencing of *GmFAD3* gene by siRNA leads to low α-linolenic acids (18:3) of *fad3*-mutant phenotype in soybean [*Glycine max* (Merr.)]. *Transgenic Research*, *17*, 839–850. https://doi.org/10.1007/s11248-008-9167-6

Gao, C. J., Cai, Y., Wang, Y. J., Kang, B. H., Aniento, F., Robinson, D. G., & Jiang, L. W. (2014). Retention mechanisms for ER and Golgi membrane proteins. *Trends in Plant Science*, *19*, 508–515. https://doi.org/10.1016/j.tplants.2014.04.004

Gibson, S., Arondel, V., Iba, K., & Somerville, C. (1994). Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from *Arabidopsis-thaliana*. *Plant Physiology*, *106*, 1615–1621. https://doi.org/10.1104/pp.106.4.1615

Goossen, C. P., Bosworth, S. C., Darby, H. M., & Kraft, J. (2018). Microwave pretreatment allows accurate fatty acid analysis of small fresh weight (100 g) dried alfalfa, ryegrass, and winter rye samples. *Animal Feed Science and Technology*, *239*, 74–84. https://doi.org/10.1016/j.anifeedsci.2018.02.014

Guan, L. L., Wu, W., Hu, B., Li, D., Chen, J. W., Hou, K., & Wang, L. (2014). Developmental and growth temperature regulation of omega-3 fatty acid desaturase genes in safflower (*Carthamus tinctorius* L.). *Genetics and Molecular Research*, *13*, 6623–6637. https://doi.org/10.4238/2014.August.28.7

Guo, C. Y., Wang, Z. L., Wei, P. F., Su, Y., Li, F. M., & Chen, Q. H. (2018). Cloning and expression analysis of *FAD2* gene in *Toxicodendron vernicifluum*. *Molecular Plant Breeding*, *16*, 6282–6291. https://doi.org/10.13271/j.mpb.016.006282

Hageman, J. H. J., Hooyenga, P., Diersen-Schade, D. A., Scalabrin, D. M. F., Wichers, H. J., & Birch, E. E. (2012). The impact of dietary long-chain polyunsaturated fatty acids on respiratory illness in infants and children. *Current Allergy and Asthma Reports*, *12*, 564–573. https://doi.org/10.1007/s11882-012-0304-1

Han, L. H., Usher, S., Sandgrind, S., Hassall, K., Sayanova, O., Michaelson, L. V., … Napier, J. A. (2020). High level accumulation of EPA and DHA in field-grown transgenic camelina: A multi-territory evaluation of TAG accumulation and heterogeneity. *Plant Biotechnology Journal*. https://doi.org/10.1111/pbi.13385 (in press).

Heilmann, I., Mekhedov, S., King, B., Browse, J., & Shanklin, J. (2004). Identification of the *Arabidopsis* palmitoyl-monogalactosyldiacylglycerol Δ7-desaturase gene *FAD5*, and Effects of Plastidial Retargeting of *Arabidopsis* Desaturases on the *fad5* Mutant Phenotype. *Plant Physiology*, *136*(4), 4237–4245. https://doi.org/10.1104/pp.104.052951

Hernandez, M. L., Sicardo, M. D., & Martinez-Rivas, J. M. (2016). Differential contribution of endoplasmic reticulum and chloroplast ω-3 fatty acid desaturase genes to the linolenic acid content of olive (*Olea europaea*) fruit. *Plant and Cell Physiology*, *57*, 138–151. https://doi.org/10.1093/pcp/pcv159

Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V., … Somerville, C. (1993). A gene encoding a chloroplast omega-3-fatty-acid desaturase complements alterations in fatty-acid desaturation and chloroplast copy number of the *fad7* mutant of *Arabidopsis-thaliana*. *Journal of Biological Chemistry*, *268*, 24099–24105. https://doi.org/10.1016/0014-5793(93)81721-B

Jiang, Q. Z., Fu, C. X., & Wang, Z. Y. (2019). A unified agrobacterium-mediated transformation protocol for alfalfa (*Medicago sativa* L.) and *Medicago truncatula*. *Methods in Molecular Biology*, *1864*, 153–163. https://doi.org/10.1007/978-1-4939-8778-8_11

Kim, H. U., Lee, K., Shim, D., Lee, J. H., Chen, G. Q., & Hwang, S. (2016). Transcriptome analysis and identification of genes associated with ω-3 fatty acid biosynthesis in *Perilla frutescens* (L.) var. *frutescens*. *BMC Genomics*, *17*, 474. https://doi.org/10.1186/s12864-016-2805-0

Kodama, H., Akagi, H., Kusumi, K., Fujimura, T., & Iba, K. (1997). Structure, chromosomal location and expression of a rice gene encoding the microsome ω-3 fatty acid desaturase. *Plant Molecular Biology*, *33*, 493–502. https://doi.org/10.1023/A:1005726210977

Lee, K. R., Lee, Y. J., Kim, E. H., Lee, S. B., Roh, K. H., Kim, J. B., … Kim, H. U. (2016). Functional identification of oleate 12-desaturase and ω-3 fatty acid desaturase genes from *Perilla frutescens* var. *frutescens*. *Plant Cell Reports*, *35*, 2523–2537. https://doi.org/10.1007/s00299-016-2053-4

Liu, H. L., Yin, Z. J., Xiao, L., Xu, Y. N., & Qu, L. Q. (2012). Identification and evaluation of ω-3 fatty acid desaturase genes for hyperfortifying α-linolenic acid in transgenic rice seed. *Journal of Experimental Botany*, *63*, 3279–3287. https://doi.org/10.1093/jxb/ers051

Liu, W., Li, W., He, Q. L., Daud, M. K., Chen, J. H., & Zhu, S. J. (2015). Characterization of 19 genes encoding membrane-bound fatty acid desaturases and their expression profiles in *Gossypium raimondii* under low temperature. *PLOS ONE*, *10*(4). https://doi.org/10.1371/journal.pone.0123281

Lorenz, S., Buettner, A., Ender, K., Nurnberg, G., Papstein, H. J., Schieberle, P., & Nurnberg, K. (2002). Influence of keeping system on the fatty acid composition in the longissimus muscle of bulls and odorants formed after pressure-cooking. *European Food Research and Technology*, *214*, 112–118. https://doi.org/10.1371/10.1007/s00217-001-0427-4

Los, D. A., & Murata, N. (1998). Structure and expression of fatty acid desaturases. *Biochimica et Biophysica Acta-Lipids and Lipid Metabolism*, *1394*, 3–15. https://doi.org/10.1016/S0005-2760(98)00091-5

Los, D. A., & Murata, N. (2004). Membrane fluidity and its roles in the perception of environmental signals. *Biochimica et Biophysica Acta-Biomembranes*, *1666*, 142–157. https://doi.org/10.1016/j.bbamem.2004.08.002

Marshall, O. J. (2004). PerlPrimer: Cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics*, *20*, 2471–2472. https://doi.org/10.1093/bioinformatics/bth254

McCartney, A. W., Dyer, J. M., Dhanoa, P. K., Kim, P. K., Andrews, D. W., McNew, J. A., & Mullen, R. T. (2004). Membrane-bound fatty acid desaturases are inserted co-translationally into the ER and contain different ER retrieval motifs at their carboxy termini. *Plant Journal*, *37*, 156–173. https://doi.org/10.1046/j.1365-313X.2003.01949.x

Mikami, K., & Murata, N. (2003). Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Progress in Lipid Research*, *42*, 527–543. https://doi.org/10.1016/S0163-7827(03)00036-5

Miquel, M., James, D., Dooner, H., & Browse, J. (1993). *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 6208–6212. https://doi.org/10.1073/pnas.90.13.6208

Mouttet, R., Escobar-Gutiérrez, A., Esquibet, M., Gentzbittel, L., Mugniéry, D., Reignault, P., … Castagnone-Sereno, P. (2014). Banning of methyl bromide for seed treatment: Could *Ditylenchus dipsaci* again

become a major threat to alfalfa production in Europe? *Pest Management Science*, *70*, 1017–1022. https://doi.org/10.1002/ps.3745

Napier, J. A. (2007). The production of unusual fatty acids in transgenic plants. *Annual Review of Plant Biology*, *58*, 295–319. https://doi.org/10.1146/annurev.arplant.58.032806.103811

Nguemeni, C., Gouix, E., Bourourou, M., Heurteaux, C., & Blondeau, N. (2013). Alpha-linolenic acid: A promising nutraceutical for the prevention of stroke. *PharmaNutrition*, *1*, 1–8. https://doi.org/10.1016/j.phanu.2012.12.002

Nguyen, Q. V., Malau-Aduli, B. S., Cavalieri, J., Nichols, P. D., & Malau-Aduli, A. E. O. (2019). Enhancing omega-3 long-chain polyunsaturated fatty acid content of dairy-derived foods for human consumption. *Nutrients*, *11*(4). https://doi.org/10.3390/nu11040743

Peng, Z. Y., Ruan, J., Tian, H. Y., Shan, L., Meng, J. J., Guo, F., … Li, X. G. (2020). The family of peanut fatty acid desaturase genes and a functional analysis of four ω-3 *AhFAD3* members. *Plant Molecular Biology Reporter*, *38*, 209–221. https://doi.org/10.1007/s11105-019-01191-0

Peng, D., Zhou, B., Jiang, Y. Q., Tan, X. F., Yuan, D. Y., & Zhang, L. (2018). Enhancing freezing tolerance of *Brassica napus* L. by overexpression of a stearoyl-acyl carrier protein desaturase gene (*SAD*) from *Sapium sebiferum* (L.) Roxb. *Plant Science*, *272*, 32–41. https://doi.org/10.1016/j.plantsci.2018.03.028

Puttick, D., Dauk, M., Lozinsky, S., & Smith, M. A. (2009). Overexpression of a FAD3 desaturase increases synthesis of a polymethylene-interrupted dienoic fatty acid in seeds of *Arabidopsis thaliana* L. *Lipids*, *44*, 753–757. https://doi.org/10.1007/s11745-009-3315-5

Roman, A., Andreu, V., Luisa Hernandez, M., Lagunas, B., Picorel, R., Manuel Martinez-Rivas, J., & Alfonso, M. (2012). Contribution of the different omega-3 fatty acid desaturase genes to the cold response in soybean. *Journal of Experimental Botany*, *63*, 4973–4982. https://doi.org/10.1093/jxb/ers174

Saeki, K., Matsumoto, K., Kinoshita, M., Suzuki, I., Tasaka, Y., Kano, K., … Iritani, A. (2004). Functional expression of a Δ12 fatty acid desaturase gene from spinach in transgenic pigs. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 6361–6366. https://doi.org/10.1073/pnas.0308111101

Sato, N., & Moriyama, T. (2007). Genomic and biochemical analysis of lipid biosynthesis in the unicellular rhodophyte *Cyanidioschyzon merolae*: Lack of a plastidic desaturation pathway results in the coupled pathway of galactolipid synthesis. *Eukaryotic Cell*, *6*, 1006–1017. https://doi.org/10.1128/EC.00393-06

Shanklin, J., & Cahoon, E. B. (1998). Desaturation and related modifications of fatty acids. *Annual Review of Plant Physiology and Plant Molecular Biology*, *49*, 611–641. https://doi.org/10.1146/annurev.arplant.49.1.611

Shi, Y. L., Yue, X. L., & An, L. Z. (2018). Integrated regulation triggered by a cryophyte omega-3 desaturase gene confers multiple-stress tolerance in tobacco. *Journal of Experimental Botany*, *69*, 2131–2148. https://doi.org/10.1093/jxb/ery050

Teixeira, M. C., Carvalho, I. S., & Brodelius, M. (2010). ω-3 fatty acid desaturase genes isolated from purslane (*Portulaca oleracea* L.): Expression in different tissues and response to cold and wound stress. *Journal of Agricultural and Food Chemistry*, *58*, 1870–1877. https://doi.org/10.1021/jf902684v

Torres-Franklin, M. L., Repellin, A., Huynh, V. B., D Arcy-Lameta, A., Zuily-Fodil, Y., & Pham-Thi, A. (2009). Omega-3 fatty acid desaturase (*FAD3*, *FAD7*, *FAD8*) gene expression and linolenic acid content in cowpea leaves submitted to drought and after rehydration. *Environmental and Experimental Botany*, *65*, 162–169. https://doi.org/10.1016/j.envexpbot.2008.12.010

Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*, *30*, 967–977. https://doi.org/10.1007/s10529-008-9639-z

Vrinten, P., Hu, Z. Y., Munchinsky, M. A., Rowland, G., & Qiu, X. (2005). Two *FAD3* desaturase genes control the level of linolenic acid in flax seed. *Plant Physiology*, *139*, 79–87. https://doi.org/10.1104/pp.105.064451

Wallis, J. G., & Browse, J. (2002). Mutants of *Arabidopsis* reveal many roles for membrane lipids. *Progress in Lipid Research*, *41*, 254–278. https://doi.org/10.1016/S0163-7827(01)00027-3

Wang, H. S., Yu, C., Tang, X. F., Zhu, Z. J., Ma, N. N., & Meng, Q. W. (2014). A tomato endoplasmic reticulum (ER)-type omega-3 fatty acid desaturase (LeFAD3) functions in early seedling tolerance to salinity stress. *Plant Cell Reports*, *33*, 131–142. https://doi.org/10.1007/s00299-013-1517-z

Wang, J. W., Ming, F., Pittman, J., Han, Y. Y., Hu, J., Guo, B., & Shen, D. L. (2006). Characterization of a rice (*Oryza sativa* L.) gene encoding a temperature-dependent chloroplast ω-3 fatty acid desaturase. *Biochemical and Biophysical Research Communications*, *340*, 1209–1216. https://doi.org/10.1016/j.bbrc.2005.12.126

Yara, A., Yaeno, T., Hasegawa, M., Seto, H., Montillet, J. L., Kusumi, K., … Iba, K. (2007). Disease resistance against *Magnaporthe grisea* is enhanced in transgenic rice with suppression of ω-3 fatty acid desaturases. *Plant and Cell Physiology*, *48*, 1263–1274. https://doi.org/10.1093/pcp/pcm107

Yin, Z. J., Liu, H. L., Dong, X. B., Tian, L. H., Xiao, L., Xu, Y. N., & Qu, L. Q. (2014). Increasing α-linolenic acid content in rice bran by embryo-specific expression of ω3/Δ15-desaturase gene. *Molecular Breeding*, *33*, 987–996. https://doi.org/10.1007/s11032-013-0014-y

Yin, D. D., Xu, W. Z., Shu, Q. Y., Li, S. S., Wu, Q., Feng, C. Y., … Wang, L. S. (2018). Fatty acid desaturase 3 (*PsFAD3*) from *Paeonia suffruticosa* reveals high α-linolenic acid accumulation. *Plant Science*, *274*, 212–222. https://doi.org/10.1016/j.plantsci.2018.05.027

Yu, C., Wang, H. S., Yang, S., Tang, X. F., Duan, M., & Meng, Q. W. (2009). Overexpression of endoplasmic reticulum omega-3 fatty acid desaturase gene improves chilling tolerance in tomato. *Plant Physiology and Biochemistry*, *47*, 1102–1112. https://doi.org/10.1016/j.plaphy.2009.07.008

Yurchenko, O. P., Park, S., Ilut, D. C., Inmon, J. J., Millhollon, J. C., Liechty, Z., … Dyer, J. M. (2014). Genome-wide analysis of the omega-3 fatty acid desaturase gene family in *Gossypium*. *BMC Plant Biology*, *14*. https://doi.org/10.1186/s12870-014-0312-5

Zafar, S., Tang, M. Q., Wang, Y. K., Sarwar, R., Liu, S. Y., & Tan, X. L. (2020). Candidate genes-association study to identify loci related to oleic acid in *Brassica napus* using SNP markers and their heterologous expression in yeast. *Plant Physiology and Biochemistry*, *146*, 294–302. https://doi.org/10.1016/j.plaphy.2019.11.026

Zhang, D. Y., Pirtle, I. L., Park, S. J., Nampaisansuk, M., Neogi, P., Wanjie, S. W., … Chapman, K. D. (2009). Identification and expression of a new delta-12 fatty acid desaturase (*FAD2-4*) gene in upland cotton and its functional expression in yeast and *Arabidopsis thaliana* plants. *Plant Physiology and Biochemistry*, *47*, 462–471. https://doi.org/10.1016/j.plaphy.2008.12.024

Zhang, H. T., Bi, Y. P., Liu, Z. J., & Shan, L. (2009). Heterologous expression of two *Glycine max* omega-3 fatty acid desaturases in *Saccharomyces cerevisiae*. *Russian Journal of Plant Physiology*, *56*, 569–574. https://doi.org/10.1134/S1021443709040189

Zhang, M., Barg, R., Yin, M., Gueta-Dahan, Y., Leikin-Frenkel, A., Salts, Y., … Ben-Hayyim, G. (2005). Modulated fatty acid desaturation via overexpression of two distinct ω-3 desaturases differentially alters tolerance to various abiotic stresses in transgenic tobacco cells and plants. *The Plant Journal*, *44*, 361–371. https://doi.org/10.1111/j.1365-313X.2005.02536.x

Zhang, Y. F., Maximova, S. N., & Guiltinan, M. J. (2015). Characterization of a stearoyl-acyl carrier protein desaturase gene family from chocolate tree, *Theobroma cacao* L. *Frontiers in Plant Science*, *6*. https://doi.org/10.3389/fpls.2015.00239

Zhou, S. F., & Runge, T. M. (2015). Mechanism of improved cellulosic bio-ethanol production from alfalfa stems *via* ambient-temperature acid pretreatment. *Bioresource Technology*, *193*, 288–296. https://doi.org/10.1016/j.biortech.2015.06.096

Zhou, S. F., Yang, Q., & Runge, T. M. (2015). Ambient-temperature sulfuric acid pretreatment to alter structure and improve enzymatic digestibility of alfalfa stems. *Industrial Crops and Products*, *70*, 410–416. https://doi.org/10.1016/j.indcrop.2015.03.068

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Zhang Z, Jin X, Liu Z, Zhang J, Liu W. Genome-wide identification of *FAD* gene family and functional analysis of *MsFAD3.1* involved in the accumulation of α-linolenic acid in alfalfa. *Crop Science*. 2020;1–14. https://doi.org/10.1002/csc2.20362