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ARTICLE

ω-3 Fatty Acid Desaturase Genes Isolated from Purslane (*Portulaca oleracea* L.): Expression in Different Tissues and Response to Cold and Wound Stress

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Two full-length cDNA clones *PoleFAD7* and *PoleFAD8*, encoding plastidial ω-3 fatty acid desaturases were isolated from purslane (*Portulaca oleracea*). The encoded enzymes convert linoleic to α-linolenic acid (C18:3*n*-3). Three histidine clusters characteristic of fatty acid desaturases, a putative chloroplast transit peptide in the N-terminal, and three putative transmembrane domains were identified in the sequence. Both genes were expressed in all analyzed tissues showing different levels of expression. *PoleFAD7* was up-regulated by wounding but not by low temperature. *PoleFAD8* was up-regulated by cold stress but not by wounding. Total fatty acid and linolenic acid content were higher both, in wounded and intact leaves of plants exposed to low temperature.

KEYWORDS: *Portulaca oleracea*; omega-3 fatty acid desaturase; *FAD7*; *FAD8*; cold stress; wounding

INTRODUCTION

Purslane has a long history of use for human food, animal feed, and medicinal purposes. A nutritive characterization of purslane accessions conducted by Ezekwe et al. (1999) showed that, in spite of its genetic diversity, purslane remains one of the most abundant terrestrial vegetable sources of ω-3 fatty acids and other essential nutrients potentially beneficial for humans and animals (*1*). The presence and concentration of α-linolenic acid in purslane may vary with the cultivar, geographic distribution, developmental stage, and environmental factors (*1*−*4*). Total fatty acid content (TFA) differs among purslane tissues; it was found to be higher in seeds and leaves (*2*, *5*, *6*) and lower in stems (about one-third). Purslane is an excellent vegetable source of linolenic acid (ALA), which accounts for 60% of TFA in leaves, 30−40% in seeds, and 10−25% in stems.

Unsaturated fatty acids are essential components required for normal cellular function, being involved in roles ranging from membrane fluidity to acting as signal molecules (*7*−*10*). Trienoic acids also serve as precursors for several fatty acid-derivate signal molecules, such as traumatic acid and jasmonic acid (*11*, *12*). In particular, the class of fatty acids known as the polyunsaturated fatty acids (PUFAs) has attracted considerable interest as pharmaceutical and nutraceutical compounds (*9*, *13*). Linoleic and α-linolenic acids are essential fatty acids synthesized in plant tissues from oleic acid by the introduction of double bonds between the existing double bond and the terminal methyl group (**Figure 1**). These double bonds are inserted by specific fatty acid desaturase enzymes, which have been the subject of intense research in recent years (*14*, *15*).

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ω-3 fatty acid desaturases catalyze the insertion of a third double bond into linoleic acid precursors to produce linolenic acid. Three distinct desaturases have been characterized that can insert the Δ15 double bond, i.e. two ω-3 desaturase plastidial enzymes (codified by *FAD7* and *FAD8* genes) that use the terminal methyl group as a reference point and one extraplastidial oleate Δ15 desaturase (codified by *FAD3* gene) (*7*). Many ω-3 FAD genes have been isolated and characterized from a diverse pool of plant species. Genes encoding two plastidial ω-3 desaturases (*FAD7*/*FAD8*) were isolated from *Glycine max* (*16*), *Arabidopsis thaliana* (*17*, *18*), and *Zea mays* (*19*). Here we report on the cloning and expression analysis of two purslane (*Portulaca oleracea* L.) cDNAs encoding two plastidial ω-3 desaturases, *PoleFAD7* and *PoleFAD8*. Fatty acid desaturases in all organisms are subjected to several types of regulation, depending on their localization or function, and there may be a requirement for different or specific desaturase activities during certain development processes in various tissues (*20*). Here we investigate the expression patterns of plastidial ω-3 fatty acid desaturases in purslane by measuring the transcript accumulation levels of these genes in different tissues of the purslane plant.

Plants often encounter the abiotic stresses of low or elevated temperature, exposure to salt, drought, and, less commonly, heavy metals, as well as biotic pathogen and insect attack, sometimes simultaneously. Even with the best available land and agricultural practices, the impacts of these stresses can significantly reduce the productivity of food and fiber crops (*21*). Thus, the current situation of diminishing farm land worldwide and the potential heightened effects of global climate change on environmental, pathogen, and insect stresses provide increased impetus to understand stress resistance in crop plants. More complete knowledge of fatty acid unsaturation, mobilization, and regulation processes may significantly aid the development of

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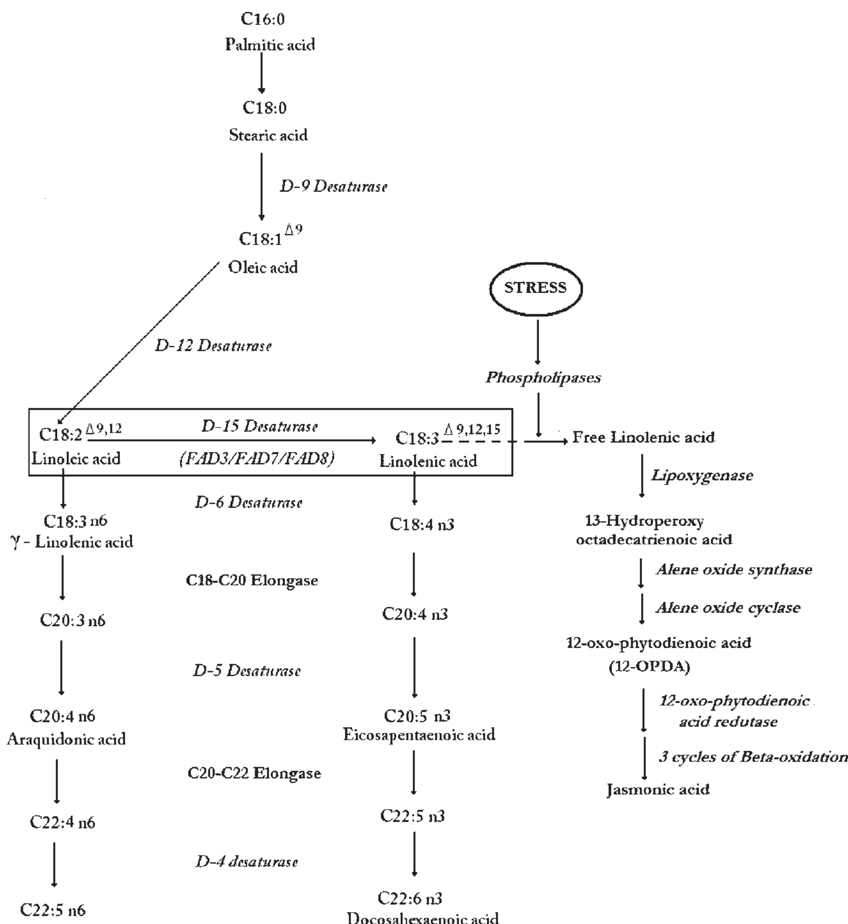

Figure 1. Outline of the pathway of polyunsaturated fatty acids and jasmonic acid biosynthesis. The enzymes involved in the bioprocesses are represented in italic. The genes that code for the desaturases referenced in this article are in parentheses (FAD3, endoplasmatic reticulum; FAD7, and FAD8, chloroplast). Interrupted line indicates an alternative pathway (octadecanoic pathway), whereas linolenic acid is converted into jasmonic acid, activated in response to stress.

effective strategies for managing abiotic and biotic stresses for these plants (*21*). Fatty acid desaturases (FAD), such as oleic and linoleic desaturases, play multiple roles in plants. FAD can modulate the membrane fluidity by modifying the degree of lipid unsaturation to respond to temperature changes. Experiments using seed and leaf tissues of soybean and wheat showed that the levels of α-linolenic (C18:3*n*-3) and linoleic (C18:2*n*-2) gradually increased as the temperature decreased (*22*, *23*). In order to elucidate the effect of chilling and wounding stresses on the expression patterns of *FAD7* and *FAD8* genes in purslane leaves, transcript accumulation levels of these genes and linolenic acid accumulation were investigated in intact and wounded leaves of purslane plants exposed to standard (25 °C) and low (5 °C) growth temperatures.

MATERIAL AND METHODS

Plant Materials. Young expanding leaves, mature leaves, young stems, mature stems, shoot tips, roots, and seeds were harvested from 1 month old seedlings of purslane (*Portulaca oleracea* L.), grown in a growth chamber at 25 °C under a 16 h photoperiod. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. For expression analysis under cold stress, 1 month old *P. oleracea* plants were placed at 5 °C for 24 h. The wounding method applied was based on the procedures described by Nishiuchi et al. (1997) (*24*). Two 1 cm long fissures were performed into leaves of 1 month old *P. oleracea* plants across the midrib with a sterile razor blade and then incubated for 24 h under normal and cold growth conditions. All samples, taken from wounded or intact leaves of control or cold-stressed plants, were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. These samples were used for total RNA extraction and fatty acid analysis in a GC−MS system. We took and analyzed samples at 2, 12, and 24 h of exposure, and we observed that the highest gene expression levels were observed at 24 h, so this result was chosen to be presented.

PCR Amplification and Cloning of Two ω-3 FADs cDNA Fragments. Primers to amplify cDNA fragments corresponding to plastidial ω-3 desaturase genes in *Portulaca oleracea* were designed on the basis of conserved nucleotide sequences of these homologous genes from *Arabidopsis thaliana* and other orthologues. The primers *FAD3*-1fw (5′-CTC/TGGG/A/C/TCAC/TGATCTGC/TGGG/A/C/TCATGG-3′) and *FAD3*-1rv (5′-TGA/GTAA/GTGA/C/TGGA/GATC/TTGG/A/C/TGGA/GAA-3′) were used to amplify the central region of *PoleFAD7* and *PoleFAD8* clones. Total RNA was isolated from different *P. oleracea* tissues using a plant RNA purification reagent kit (Invitrogen, Carlsbad CA). By use of the RT-PCR system (Fermentas), the first strand cDNA was synthesized with 2 μg of purified total RNA (pretreated with DNase I) according to the manufacture's protocol. The oligo(dT)$_{18}$ was used as a primer. PCR was performed in a 25 μL reaction mixture using PuReTaqTM Ready-To-Go PCR beads (PuReTaq polymerase (2.5 units), GE Healthcare) together with 1 μL of 10 mM of each primer and 2 μL of RT products. The reaction mixture was subjected to an initial denaturation for 3 min at 94 °C and subsequently 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90s at 72 °C followed by one cycle of 7 min at 72 °C. The PCR products were run on 1% agarose gel, and the target bands were purified, inserted into pGEM-t easy vector (Promega) cloned into NovaBlue *E. coli* strain, and sequenced (Eurofins MWG GmbH, Germany).

Rapid Amplification of cDNA Ends (RACE). For amplification of unknown 3′- and 5′-ends of the above conservative cDNA fragments, the 3′- and 5′-RACE were performed according to protocols described in the GeneRacer Kit (Invitrogen). On the basis of sequence information of the conservative cDNA fragments, gene specific primers (GSP) were

designed: 5′*FAD7* rv1 (5′-GACCAACAAGCTGTGGAAGTTA-3′), 5′*FAD7* rv2 (5′-GGGTTGAATAAATCACTGTCTGGGTGATA-3′), 5′*FAD7* rv3 (5′-GCCTGACTTTCCTGGACTTCTTTTCCACAA-3′), 5′*FAD7* rv4 (5′-AAACCCCTCCGGCGACCCATTGACC-3′), 5′*FAD7* rv5 (5′-AATCCTCCGCCGCCGTAGACGGC-3′) 5′*FAD7* rv6 (5′-AATGGGGCGGCGACATTCACCTCCC-3′) for *PoleFAD7*, and *FAD8_3R* GSP (5′-CCACGACATCGGAACTCACGTCATT-3′), 5′*FAD8* rv1 (5′-CCTTGTGGTTTCAGGCACACTTCTAT-3′), 5′*FAD8* rv2 (5′-GGCCACGGATGCCATGACTCGTCGTT-3′), 5′*FAD8* rv3 (5′-CCATGGTTTGCATGATGAGTCCTGTGACTTA-3′), 5′*FAD8* rv4 (5′-GCAGCCATGGCCATGAATGCAGCAA-3′), 5′*FAD8* rv5 (5′-GGTGGTGGTGCCCCATGATCAAACCCTAAT-3′), 5′*FAD8* rv6 (5′-GGTGAGCATTTGTGGGGTGCTAGGGTTA-3′) for *PoleFAD8*. To amplify the full-length *PoleFAD7* and *PoleFAD8* cDNAs, primers were designed on the basis of the assembled sequences: *FAD7* flfw (5′-ATAGGATCCGGAATGGCGAGTTGGGTACTCTC-3′)/*FAD7* flrv (5′-TGCGAATTCCAAAAGCTCAGGGTCTG-3′); *FAD8* flfw (5′-ATAGGATCCGGAATGGCGAGTTGGGTACTCTCA-3′)/*FAD8* flrv (5′-TCATGTTCTTTGTCCATTGAGTTTTGGAT-3′).

The full length cDNAs of *PoleFAD7* and *PoleFAD8* were inserted into pGem-t easy cloning vector, cloned into NovaBlue *E. coli* strain and sequenced (Eurofins MWG GmbH, Germany) to confirm identity and verify if clones were in frame.

Semiquantitative RT-PCR Assay. The steady state levels of *PoleFAD7* and *PoleFAD8* transcripts were estimated in various purslane tissues and in seed. The FAD7 RT-PCRfw (5′-GAGGTTCCCCTGTTTCCCCGGAT-3′) and FAD7 RT-PCRrv (5′-AATGCGGTGGTCAATGGGTCGCC-3′) primers were used for real-time semiquantitative PCR analysis of the *PoleFAD7* gene, designated as mentioned above, and for the *PoleFAD8* gene the FAD8 RT-PCRfw (5′-CTCCCATCTTCTCTCATTTTCATT-3′) and FAD8 RT-PCRrv (5′-TTAGGGTTTGATCATGGGGCACC-3′) primers, both designed from the coding region of the correspondent genes. We tested three control genes for transcript analysis. Elongation factor 1 (EF1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and ubiquinin (UBI). We determined that GAPDH should be used as control for spatial expression of the genes in purslane tissues, since it showed steady state expression in all analyzed tissues. UBI was used as control in the stress assay because GAPDH showed some expression variation in the stress samples. So a part of the coding region of GAPDH and UBI genes were amplified with the specific pairs of primers GAPDHfw (5′-CACGGCCACTGGAAGCA-3′)/GAPDHrv (5′-TCCTCAGGGTTCCTGATGCC-3′), and UBIfw (5′-GGCATGCAGATCTTTGTGAAGAC-3′) and UBIrv (5′-GGGATACCCTCCTTGTCCTG-3′), respectively. PCRs were performed using equal amounts of templates (25 ng of cDNA) and gene specific or GAPDH primers and carried out for different number of cycles in order to optimize reproducibility and ensure that reactions remained in the log−linear range. RT-qPCR results were analyzed with the sequence detection software SDS, version 1.1 (Applied Biosystems). A standard fluorescence threshold was set to a ΔRn of 0.5 on the log fluorescence scale to determine the fractional cycle number (Ct value). Relative expression of target genes in purslane tissues was calculated using the efficiency calibrated model (*25*). In this model, for each sample, target gene and a reference gene for internal control are included in the PCR amplification from serially diluted samples. The relative expression is given by a ratio between the target gene and the reference gene, calculated according to eq 1 (*25*).

$$\text{relative expression (RE)} = (E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}}/(E_{\text{reference}})^{\Delta\text{Ct}_{\text{reference}}} \quad (1)$$

Assays of transcript levels for each target gene in the stress assay were normalized by ubiquinin (UBI) gene expression for ΔCt value calculation. Target gene expression level in plants exposed to the treatments was also normalized by the expression level of each corresponding gene in the leaves of control plants. We considered an up-regulation of the gene for RE ≥ 1.5 and a down-regulation for RE ≤ 0.5. The RT-qPCR protocol was optimized by determining the optimal primer concentration and primer efficiency. The RT-qPCR products were separated by 1% agarose gel electrophoresis. The RT-qPCR fragments were inserted into pGem-t easy cloning vector, cloned in NovaBlue *E. coli* strain, and sequenced by Eurofins MWG GmbH (Germany), confirming the identity of the amplified products.

Sequence Analysis. Nucleotide sequences from cDNA clones and deduced amino acid sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Predictions of open reading frames (ORFs) were made using the DNALC Bioinformatics software (http://www.dnalc.org/bioinformatics/2003/2003_dnalc_nucleotide_analyzer.htm). Theoretical molecular weights of deduced polypeptides and hydropathy analysis were made by using the peptide properties calculator software (http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp), and transmembrane regions were predicted by the HMMTOP server, version 2.0 (http://www.enzim.hu/hmmtop/html/submit.html).

Predictions of subcellular localization of the deduced polypeptides were conducted by using PSORT (http://www.psort.nibb.ac.jp./form.html) and TargetP (http://www.cbs.dtu.dk/services/TargetP/) algorithms. Amino acid multiple alignments were made with the ClustalW program under default parameters. A phylogenetic tree was constructed using the neighbor-joining algorithm included in the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and visualized using Tree View program (*26*).

Fatty Acid Analysis. Fatty acid methyl esters (FAME) were prepared using 3 N methanolic HCL as described by Vriten et al. (2005) (*27*). FAME were analyzed on a Hewlett-Packard 5890 series II gas chromatography (GC) instrument equipped with a SGE 25QC2/BPX-70 (25 m × 220 μm × 0.30 μm) Supelco column connected to a mass detector Agilent 5973 network. The samples were injected with an automatic injector Agilent 7683 series in the splitless mode at 250 °C. The oven was programmed to hold the temperature at 60 °C for 2 min, increase to 170 °C at 30 °C/min and hold for 5 min, increase to 200 at 4 °C/min and hold for 5 min, and increase to 250 at 50 °C/min and hold for 5 min. Helium was used as a carrier gas. Temperatures of ionization source and quadrupole were 230 and 150 °C, respectively. FAME were identified by comparative analysis of retention times to standards and % FAME was calculated on the basis of peak area counts in relation to a internal control (heneicosanoic acid, C21:0). All lipid analysis experiments were performed independently at least three times. Values are reported as average and standard deviation.

Statistical Analysis. The expression levels of *PoleFAD7* and *PoleFAD8* genes and fatty acid levels in purslane leaves were subjected to analysis of variance (ANOVA) using the general linear model procedure of the SAS statistical software. Differences in cold and wounding treatments were determined with ANOVA using a split-plot model with replicates (leaves) as the blocks, plants as the main plot factor, and treatments as the subplot factor. No transformation to data was required for any of the ANOVAs. Significant differences between mean values were determined by least significant difference (Student's *t* test), with a significance level of 0.05. Three individual pots containing one plant were used for each determination and three isolated intact and wounded leaves were taken from each plant, so the results of nine independent measurements were used in the statistical analysis.

RESULTS

Cloning and Sequence Analysis of Purslane ω-3 Desaturases Full-Length cDNAs. On the basis of the nucleotide sequences of *FAD7* and *FAD8* from different plant species, two pairs of primers were designed. By use of these primer pairs, two conserved cDNA fragments were obtained by PCR using cDNA aliquots of *P. oleracea* L. leaves. To clone the full-length cDNA, 3′- and 5′-RACE reactions were conducted. The 3′-RACE-PCRs generated a fragment of 481 bp for the *PoleFAD7* and 493 bp for the *PoleFAD8* gene. The 5′RACE reactions generated fragments of 395 and 500 bp for *PoleFAD7* and *PoleFAD8*, respectively. Sequence comparisons of the 3′- and 5′-ends with the conservative fragments of these genes previously amplified showed overlapping regions that matched perfectly. Blast searches of the deduced amino acid sequences revealed that these sequences represented the missing parts of *PoleFAD7* and *PoleFAD8* genes. On the basis of the above sequences data, primers were designed for the 3′- and 5′-UTRs (untranslated regions) of these genes, and the full-length cDNA was amplified, cloned, and sequenced, respectively.

Table 1. Description of Three Full-Length cDNAs Encoding Plastidial ω-3 Fatty Acid Desaturases from *Portulaca oleracea* L.

name	full-length cDNA (bp)	ORF (bp)	encoded amino acid	protein molecular mass (kDa)	isoelectric point	predicted subcellular location
PoleFAD7	1523	1338	446	50.5	7.5	chloroplast
PoleFAD8	1606	1362	453	52.4	8.2	chloroplast

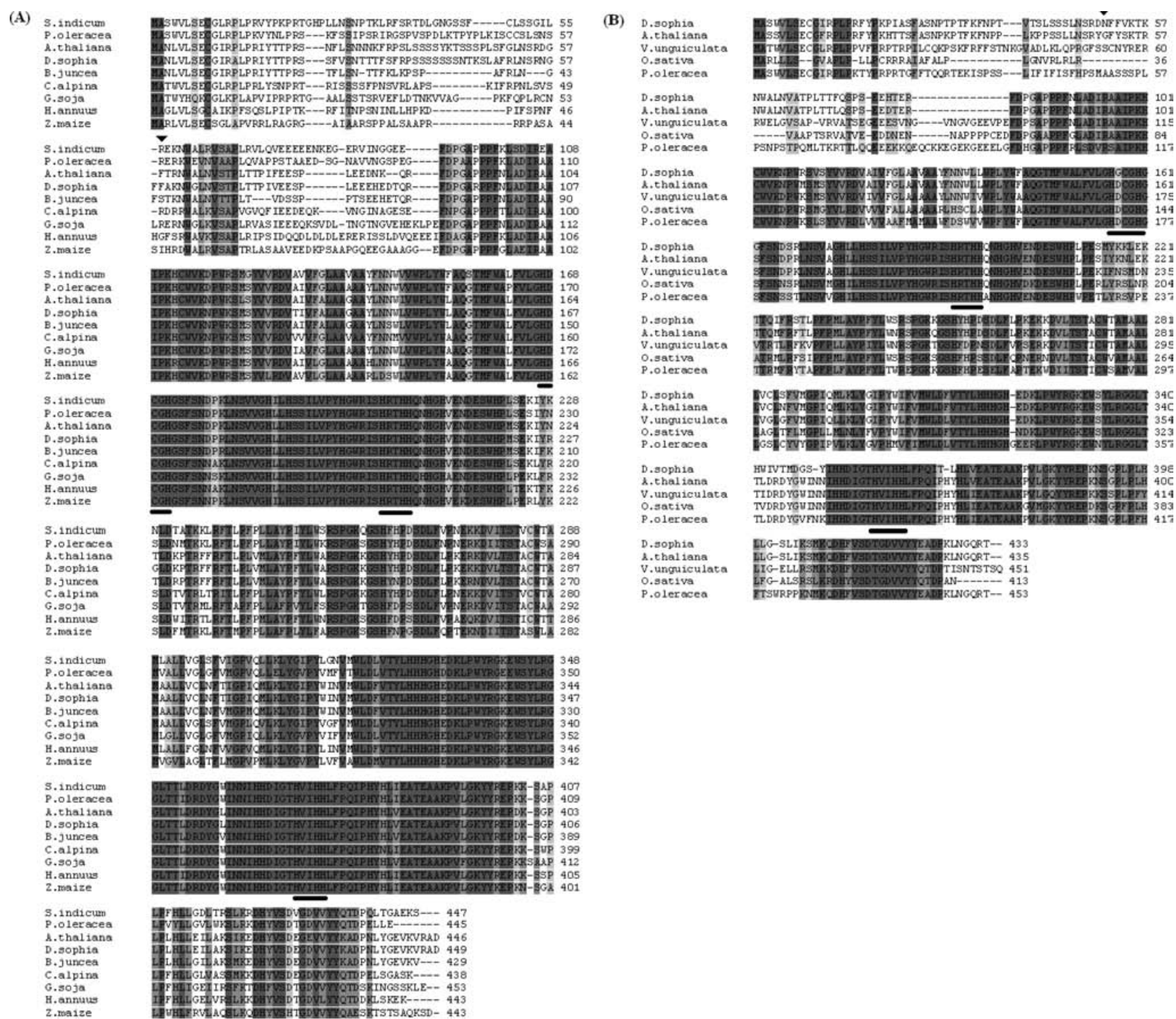

Figure 2. (**A**) Alignment of the deduced amino acid sequences determined for *PoleFAD7* (ABL61262) and previously published plastidial ω-3 fatty acid desaturases from *Arabidopsis thaliana* (BAA03106), *Brassica juncea* (CAB85467), *Sesamum indicum* (AAA70334), *Helianthus annuus* (AAP78965), *Zea mays* (BAA22441), *Crespis alpina* (ABA55807), *Brassica napus* (AAA61774), *Glycine soja* (AAA61776), and *Descurainia sophia* (ABK91880). (**B**) Alignment of the deduced amino acid sequences determined for *PoleFAD8* (EU872255) and previously published plastidial ω-3 fatty acid desaturases from *Arabidopsis thaliana* (AAA65621), *Descurainia sophia* (ABK91881), *Vigna unguiculata* (ABY60737), and *Oryza sativa* japonica (AAW32557). The arrows indicate a putative signal peptide cleavage site. Numbers in parentheses refer to GenBank accession numbers. Identical or similar amino acids are shaded black or gray, respectively. The three characteristic histidine boxes encountered in *FAD7* or *FAD8* subfamilies are underlined. Numbers to the right indicate the position of amino acids.

Descriptions of the properties of the two distinct *P. oleracea* complete cDNAs are listed in **Table 1**.

Inspection of both sequences revealed the presence of eight highly conserved histidine residues in three separate clusters corresponding to the amino acid residues 169−173, 205−209, 372−376 for PoleFAD7 and to residues 172−176, 208−212, 376−380 for *PoleFAD8*. The residues are necessary for the function of all membrane-bound fatty acid desaturases (*14*). These invariant residues are arranged in three histidine boxes (HXXXH, HXXHH, and HXXHH) with conserved spaces between them (**Figure 2**) that participate in the formation of active sites with iron and are conserved in all ω-3 type FA desaturases (*10*). The putative chloroplast transit peptide sequence has, respectively, 52 and 74 amino acids at the N-termini of *PoleFAD7* and *PoleFAD8* (**Figure 2**). The N-terminal sequences also had several characteristics of plastidial transit peptides, including a high content of hydroxylated residues (Ser, Thr), a low content of acidic residues, and the conserved N-terminal Met-Ala dipeptide (*28*).

Multiple sequence alignment revealed high similarities of the two distinct ω-3 FAD amino acid sequences to other orthologous

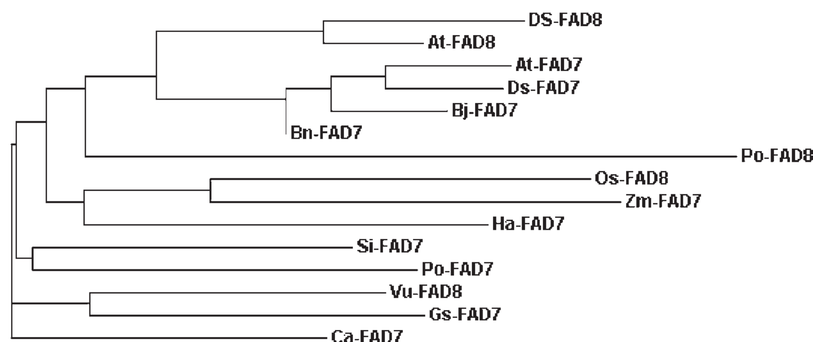

Figure 3. Phylogenetic relationships between deduced amino acid sequences from *PoleFAD7* and *PoleFAD8* cDNAs and other plant microsomal plastidial oleate desaturases: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Bj, *Brassica juncea*; Os, *Oryza sativa japonica*; Ds, *Descurainia sophia*; Ha, *Helianthus annuus*; Si, *Sesamum indicum*; Vu, *Vigna unguiculata*; Ca, *Crespis alpina*; Gs, *Glycine soja*; Po, *Portulaca oleracea*. The GenBank accession numbers of the above sequences are shown in **Figure 1**. The tree was constructed by using the Neighbor-Joining algorithm.

desaturase sequences available in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html). *PoleFAD7* and *PoleFAD8* exhibited 68−76% and 60−67% of identity to other *FAD7* and *FAD8* proteins from different plant species, respectively. *PoleFAD7* exhibited 76% of identity with the *Sesamum indicum FAD7* gene and 71% of identity to *Arabidopsis thaliana* and *Helianthus annuus FAD7* genes. *PoleFAD8* exhibited 67% of identity with the *Arabidopsis thaliana* FAD8 *gene*, 65% of identity to *Descurainia sophia*, 62% *Oryza sativa*, and 60% *Vigna unguiculata*. The sequence identity between *PoleFAD7* and *PoleFAD8* was 63%.

Hydropathy analysis of *PoleFAD7* showed that the encoded protein contained three putative membrane spanning domains at amino acid residues 133−152, 283−305, and 312−330. *PoleFAD8* also contained three putative membrane-spanning domains at amino acid residues 134−153, 284−306, and 313−331. Three to four putative transmembrane domains were also present in *FAD7* and *FAD8* translated sequences of other plant ortologues such as *A. thaliana*, *S. indicum*, and *B. napus*, in similar positions. The prediction of three putative transmembrane regions for *PoleFAD7* and *PoleFAD8* polypeptides supported the notion that the two desaturases are membrane-bound.

The neighbor-joining phylogenic tree (**Figure 3**) showed that *PoleFAD7* and *PoleFAD8* are distributed in the same group. The presence of a chloroplastidial signal peptide in the N-terminal on both gene sequences indicates that they may share common characteristics, like subcellular location. We can also see that *PoleFAD8* sequence is very divergent in relation to *PoleFAD7* and other plastidial *FAD*, being placed in an isolated branch, closer to *A. thaliana* and *D. sophia FAD7* and *FAD8* genes. *PoleFAD7* showed a closer relation to *S. indicum FAD7*.

Spatial Analysis of *Portulaca FAD7* and *FAD8* Expression in Purslane Tissues. There were similar expression profiles among the two distinct ω-3 desaturases in *P. oleracea* tissues (**Figure 4**). A dominant but not restricted expression of *PoleFAD7* was observed in photosynthetic tissues particularly in the young developing leaves and stems. Expression of *PoleFAD7* was also detected in root and seeds. A low expression level of *PoleFAD8* gene was detected in all purslane tissues, with slightly higher levels in stems and root tips (**Figure 4**).

In *A. thaliana* this gene has been characterized as temperature-dependent, being expressed when the plants are exposed to low temperatures and undetectable at optimal growth temperature (*17*). If *PoleFAD8* shares the same characteristic, it could explain the low but detectable levels of expression observed, since the plants were grown at 25 °C, which is 5 °C below the optimal growth temperature, because of the greenhouse room restriction in terms of heating system.

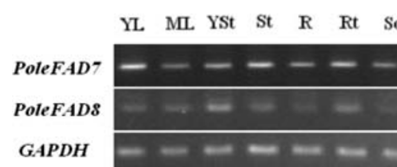

Figure 4. Real-time PCR spatial analysis of *PoleFAD7* and *PoleFAD8* genes. First strand cDNAs were synthesized from total RNA extracted from young expanding leaves (YL), full-expanded mature leaves (ML), young stems tips (YSt), stems (St), roots (R), root tips (Rt), and seeds (Sd). To ensure equal amounts of template, GAPDH was used as a reference gene.

Effect of Chilling and Wounding on Linolenic Acid Accumulation and ω-3 Desaturase Gene Expression on Purslane Leaves. The chloroplast membranes of higher plants have unusually high concentrations of trienoic fatty acids, with linolenic (C18:3) or a combination of linolenic and hexadecatrienoic (C16:3) acids making up more than 80% of the fatty acids found in this organelle (*7*, *29*). Although the processes of acclimatization are considered to include complex and various biochemical processes, Kodama et al. (2005) showed that an increase in total fatty acid content during the acclimation process was one of the important factors in chilling tolerance in tobacco leaves (*30*). An increase in trienoic acids, particularly C16:3, was also detected in leaves of plants grown at low temperature (15 °C). In our work we observed an increase in total fatty acid (TFA) content in purslane leaves of chilled and wounded plants, particularly for linoleic (LA, C18:2) and linolenic (ALA, C18:3) acids (**Table 2**). TFA, LA, and LNA mean values from treated plants were significantly different ($p < 0.05$, 24) from those of control plants, and higher values were obtained in wounded chilled plants (**Table 2**). No significant differences ($p > 0.05$, 24) in ALA/LA ratio between the control and treated plants were observed.

An increase on linolenic acid accumulation in wounded purslane leaves was observed, which may be associated with the activation of octadecanoic pathway to produce jasmonic acid (JA), since linolenic acid is a known precursor for this stress-signaling molecule (**Figure 1**). The highest level of linolenic acid was detected in leaves of plants subjected to both low temperature and wounding, but no cumulative effect was noticed (**Table 2**).

To determine *PoleFAD7* or *PoleFAD8* gene transcript levels in leaves of purslane plants submitted to chilling and wounding stresses, semiquantitative real-time PCR analysis was carried out. cDNA was synthesized from total RNA extracted from intact and wounded leaves of plants grown at 25 °C and plants exposed to chilling temperature, 5 °C, for 24 h. In purslane plants grown at

Table 2. ω-3 Plastidial Desaturase Gene Expression, Total Fatty Acid Content, and Linoleic (C18:2) and Linolenic (C18:3) Acids Content in Purslane Leaves of Plants Exposed to Chilling and/or Wounding Stresses[a]

	gene relative expression[b]		fatty acids (mg g^{-1} FW)[b]			
treatment	FAD7	FAD8	TFA	LA	ALA	ALA/LA
C	1.00 ± 0.00 a,b	1.00 ± 0.00 c	3.41 ± 0.29 c	0.51 ± 0.08 c	1.99 ± 0.24 c	3.89 ± 0.74 a
T	0.91 ± 0.02) b	3.27 ± 0.38 a	4.64 ± 0.29 b	0.67 ± 0.06 b	2.74 ± 0.28 b	4.11 ± 0.66 a
W	1.21 ± 0.03 a	0.86 ± 0.05 c	4.30 ± 0.30 b	0.62 ± 0.10 b	2.68 ± 0.18 b	4.32 ± 0.82 a
T + W	1.20 ± 0.03 a	2.03 ± 0.06 b	6.10 ± 0.58 a	0.80 ± 0.07 a	3.63 ± 0.36 a	4.55 ± 0.73 a
LSD[c] (p = 0.05, 24)	0.2283	0.4037	0.7032	0.0758	0.2860	0.6867

[a] C, intact leaves from plants kept at 25 °C; T, intact leaves from plants exposed to 5 °C for 24 h; W, wounded leaves from plants kept at 25 °C; T + W, wounded leaves from plants exposed to 5 °C for 24 h; TFA, total fatty acids; LA, linoleic acid; ALA, α-linolenic acid. Different on-line letters mean significant differences between mean values. [b] Mean values of nine individual measurements and corresponding standard deviation values. [c] Differences between treatment mean values were tested using the least significant difference (LSD) Student's *t* test (p = 0.05, 24).

25 °C, *PoleFAD7* and *PoleFAD8* transcripts were detected in intact leaves, although *PoleFAD8* was expressed at a very low level (**Figure 4**). Statistical analysis revealed that *PoleFAD7* and *PoleFAD8* expression levels were significantly affected by the applied treatments ($p < 0.05$). In response to low temperature, a slight decrease in expression level was detected for the *PoleFAD7* gene (**Table 2**). On the other hand, *PoleFAD8* transcripts level increased significantly after exposure to 5 °C and became dominantly expressed in relation to *PoleFAD7* (**Table 2**). A 3.5-fold increase in the *PoleFAD8* expression level was detected in leaves of plants exposed to chilling temperatures. With respect to wounding, *PoleFAD7* was dominantly expressed, although a slight increase in *PoleFAD8* was also present (**Table 2**). No cumulative effect was noticeable when the two treatments, low temperature and wounding, were applied simultaneously, but 3-fold increase on *PoleFAD8* was detected (**Table 2**).

DISCUSSION

Unsaturated fatty acids have several important roles in both plant development and defense response (*31*, *32*) and alternatively possess a crucial role to polyunsaturated acid content of vegetable oils, which is an important factor for oil quality. In the plant fatty acid desaturation pathway, the first step is catalyzed by stearoyl-ACP desaturase to convert stearic acid into oleic acid (**Figure 1**). In the second desaturation step oleic acid is converted to linoleic acid by the action of ω-6 fatty acid desaturases (*7*). Further desaturation of linoleic acid to produce trienoic fatty acids is catalyzed by ω-3 fatty acid desaturases, codified by one microsomal (*FAD3*) and two plastidial (*FAD7* and *FAD8*) genes (**Figure 1**) (*7*).

In the present study, using a PCR-based approach, we have cloned two distinct plastidial ω-3 fatty acid desaturase cDNAs from purslane, designated as *PoleFAD7* and *PoleFAD8*, which show a relatively high homology to other plant ω-3 fatty acid desaturases that have putative plastidial transit peptides. The *FAD7* and *FAD8* genes of *Portulaca oleracea* L. have been isolated and characterized by genetic criteria as encoding chloroplast-localized ω-3 desaturases. The expression pattern of these two genes in purslane tissues was analyzed by real time PCR analysis, and we detected similar expression profiles among the two distinct ω-3 desaturases (**Figure 4**). *PoleFAD7* showed a dominant, but not restricted, expression in photosynthetic tissues, such as leaves and stems (**Figure 4**), also being detected in roots and seeds. The transcript of the *FAD7* gene was observed only in the chlorophyllous tissue of *Arabidopsis thaliana* (*33*) and *Nicotiana tabacum* (*34*) plants; however, other reports have indicated that *FAD7* gene expression can also be detected in nonphotosynthetic tissues, including developing embryos of linseed (*35*), in maturing pollen grains of *B. napus* (*36*), and in the flower apparatus of *Olea europaea* (*37*). In *Descuraina sophia* plants, *FAD8* gene expression was restricted to photosynthetic tissues such as leaves, stems, and young siliques (*38*); however, we observed *PoleFAD8* gene expression in all tested tissues, although at low levels, with slightly higher levels in stems and root tips (**Figure 4**).

Much of the interest in fatty acid desaturation is the result of a proposed correlation between the high degree of membrane lipid polyunsaturation and tolerance to low and freezing temperatures. Evidence of the importance of trienoic fatty acid composition in conditioning cold tolerance was demonstrated in transgenic tobacco plants (*N. tabacum*) that have increased levels of trienoic acids (*39*) when exposed to low temperatures. The deleterious effects of low temperature, observed on mutants of *Arabidopsis* with reduced levels of polyunsaturation, are direct evidence that fatty acid composition is a component of low temperature fitness (*39*, *40*). We also detected an increase in total fatty acid content, particularly linolenic acid, in wounded and intact leaves of chilled purslane plants (**Table 2**). A cumulative effect of the two treatments in the total fatty acid content or linolenic acid content was not observed, but higher values were obtained when both treatments were applied simultaneously (**Table 2**).

The effect of chilling and wounding in the transcript level of ω-3 desaturase genes, *PoleFAD7* and *PoleFAD8*, was also analyzed. Total RNA isolated from purslane plants grown at 25 °C contained detectable levels of *FAD7* and *FAD8* genes. A slight reduction of *PoleFAD7* transcript accumulation level was detected in plants exposed to chilling temperatures (**Table 2**), while *FAD8* mRNA had an increased in transcript levels, becoming dominantly expressed in relation to *PoleFAD7*. These differences in steady-state mRNA levels could be the result of alterations either in the rate of *FAD8* transcription or in the stability of the *FAD8* mRNA. A similar result was reported by Tang et al. (2007), who observed a down-regulation of *Descuraina sophia FAD3* and *FAD7* genes in leaves induced by cold stress, together with an up-regulation of the *FAD8* gene (*38*). Gibson et al. (1994) also reported an increase in *FAD8* mRNA transcript level in *Arabidopsis* plants grown at low temperature (20 °C), compared to plants grown at 30 °C. It was also stated that the enhanced expression of *Arabidopsis FAD8* gene at low temperatures could indicate the existence of a mechanism that responds to temperature rather than to the physical characteristics of the membrane (*17*). *PoleFAD8* gene expression was not significantly ($p > 0.05$, 24) affected when only wounding stress was applied (**Table 2**). It is possible that this gene is regulated by a temperature-responsive transcriptional activation rather than a wound-responsive one. Therefore, the function of the *Portulaca FAD8* desaturase might be to provide a rapid increase in chloroplast ω-3 desaturase activity after a sudden cold exposure, particularly in rapid growing tissues where rates of fatty acid desaturation may not match rates of fatty acid synthesis.

Trienoic acids (C16:3 + C18:3) are the starting material for the biosynthesis of fatty acid-derivate signaling molecules such as jasmonic acid (JA) (*9*, *42*−*44*). Wounding activates the octadecanoic pathway in which linolenic acid is converted to JA, resulting in a significant accumulation of this hormone (**Figure 1**). JA is one of several candidate molecules for wound signaling and is thought to play a pivotal role in the transcriptional activation of wound-inducible genes (*9*, *42*, *45*).

We observed an up-regulation of purslane *FAD7* gene in wounded leaves; this is consistent with findings in *N. tabaccum*, *A. thaliana*, and *D. sophia* where ω-3 FAD genes expression was up-regulated after wounding (*33*, *34*, *38*). Wounding treatments increased the amount of *FAD7* mRNA but did not affect *FAD3* mRNA in tobacco (*34*). In *A. thaliana*, the *FAD7* gene was similarly activated in wounded leaves and roots (*33*). The up-regulation of PoleFAD7 in response to wounding may indicate that the *FAD7* gene plays a role in the wound response in purslane by supplying a precursor for JA biosynthesis (**Figure 1**), although jasmonic acid levels were not determined in this work.

The up-regulation of these genes in this plant may play a role in purslane resistance to stresses such as temperature and mechanical injury. This raises the possibility of using these genes, by recombinant technology, to improve resistance to stress in economical important crops. Such an approach was already successfully applied in tobacco plants (*41*). The precise molecular mechanism by which this regulation takes place remains unclear and needs further experimental research.

ABBREVIATIONS USED

FAD, fatty acid desaturase; PUFA, polyunsaturated fatty acid; TFA, total fatty acids; LA, linoleic acid; ALA, α-linolenic acid; DA, dienoic acid; TA, trienoic acid; JA, jasmonic acid.

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LITERATURE CITED

(1) Ezekwe, M. O.; Omara-Alwala, R.; Membrahtu, T. Nutritive characterization of purslane accessions as influenced by planting date. *Plant Foods Hum. Nutr.* **1999**, *54*, 183–191.
(2) Liu, L.; Howe, P.; Zhou, Y.-F.; Xu, Z.-Q.; Hocart, C.; Zhang, R. Fatty acids and β-carotene in Australian purslane (*Portulaca oleracea*) varieties. *J. Chromatogr., A* **2000**, *893*, 207–213.
(3) Liu, L.; Howe, P.; Zhou, Y.-F.; Xu, Z.-Q.; Hocart, C.; Zhang, R. Fatty acids profiles of leaves of nine edible wild plants: an Australian study. *J. Food Lipids* **2002**, *9*, 65–71.
(4) Palaniswamy, U. R.; McAvoy, R. J.; Bible, B. B. Stage of harvest and polyunsaturated fatty acid concentrations in purslane (*Portulaca oleracea*) leaves. *J. Agric. Food Chem.* **2001**, *49*, 3490–3493.
(5) Guil, J. L.; Torija, M.; Gimenez, J.; Rodriguez, I. Identification of fatty acids in edible wild plants by gas chromatography. *J. Chromatogr., A* **1996**, *719*, 229–235.
(6) Simopoulos, A. P.; Norman, H. A.; Gillapsy, J. E.; Duke, J. A. Common purslane: a source of omega-3 fatty acids and antioxidants. *J. Am. Coll. Nutr.* **1992**, *11*, 374–382.
(7) Ohlrogge, J.; Browse, J. Lipid biosynthesis. *Plant Cell* **1995**, *7*, 957–970.
(8) Mikami, K.; Murata, N. Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Prog. Lipid Res.* **2003**, *42*, 527–543.
(9) Zheng, H.; Rowland, O.; Kunst, L. Disruptions of the *Arabidopsis* enoyl-CoA reductase gene reveal an essential role for very-long-chain fatty acid synthesis in cell expansion during plant morphogenesis. *Plant Cell* **2005**, *17*, 1467–1481.
(10) Los, D. A.; Murata, N. Structure and expression of fatty acid desaturases. *Biochim. Biophys. Acta* **1998**, *1394*, 3–15.
(11) Farmer, E. E. Fatty acid signaling in plants and their associated microorganisms. *Plant Mol. Biol.* **1994**, *26*, 1423–37.
(12) León, J.; Rojo, E.; Sánchez-Serrano, J. J. Wound signaling in plants. *J. Exp. Bot.* **2001**, *354*, 1–9.
(13) Horrobin, D. F. Essential omega-6 and omega-3 fatty acids in medicine: a practical guide. *J. Adv. Med.* **1990**, *3*, 151–161.
(14) Shanklin, J.; Cahoon, E. B. Desaturation and related modifications of fatty acids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 611–641.
(15) Napier, J. A.; Sayanova, O.; Sperling, P.; Heinz, E. A growing family of cytochrome b_5-domain fusion proteins. *Trends Plant Sci.* **1999**, *4*, 2–4.
(16) Van de Loo, F.; Somerville, C. R. A plastid ω-3 fatty acid desaturase cDNA from *Ricinus communis*. *Plant Physiol.* **1994**, *105*, 443–444.
(17) Gibson, S.; Arondel, V.; Iba, K.; Somerville, C. Cloning of a temperature-regulated gene encoding for a cloroplast ω-3 desaturase from *Arabidopsis thaliana*. *Plant Physiol.* **1994**, *106*, 1615–1621.
(18) Iba, K.; Gibson, S.; Nishiuchi, T.; Fuse, T.; Nishimura, M.; Arondel, V.; Hugly, S.; Somerville, C. A gene encoding a chloroplast ω-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the *fad7* mutant of *Arabidopsis thaliana*. *J. Biol. Chem.* **1993**, *268*, 24099–24105.
(19) Berberich, T.; Harada, M.; Sugawara, K.; Kodama, H.; Iba, K.; Kusano, T. Two maize genes encoding ω-3 fatty acid desaturase and their differential expression to temperature. *Plant Mol. Biol.* **1998**, *36*, 297–306.
(20) Murphy, D. J.; Piffanelli, P. Fatty Acid Desaturases: Structure Mechanism and Regulation. In *Plant Lipid Biosynthesis*; Harwood, J. L., Ed.; Cambridge University Press: Cambridge, U.K., 1998; pp 95−130.
(21) Upchurch, R. G. Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnol. Lett.* **2008**, *30*, 967–977.
(22) Heppard, E. P.; Kinney, A. J.; Stecca, K. L.; Miao, G. H. Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybean. *Plant Physiol.* **1996**, *110*, 311–319.
(23) Horiguchi, G.; Fuse, T.; Kawakami, N.; Kodama, H.; Iba, K. Temperature-dependent translational regulation of the ER omega-3 fatty acid desaturase gene in wheat root tips. *Plant J.* **2000**, *24*, 805–813.
(24) Nishiuchi, T.; Hamada, T.; Kodama, H.; Iba, K. Wounding changes the special expression pattern of the *Arabidopsis* plastid ω-3 fatty acid desaturase gene (FAD7) through differential signal transduction pathways. *Plant Cell* **1997**, *9*, 1701–12.
(25) Yuan, J. S.; Reed, A.; Chen, F.; Stewart, C. N., Jr. Statistical analysis of real-time PCR data. *BMC Bioinf.* **2006**, *7*, 85–97.
(26) Page, R. D. M. TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **1996**, *12*, 357–358.
(27) Vrinten, P.; Hu1, Z.; Munchinsky, M.-A.; Rowland, G.; Qiu, X. Two FAD3 desaturase genes control the level of linolenic acid in flax seed. *Plant Physiol.* **2005**, *139*, 79–87.
(28) Yadav, N. S.; Wiertzbicki, A.; Aegerter, M.; Caster, C. S.; perez-Grau, L.; Kinney, A. J.; Hitz, W. D.; Booth, J. R.; Schweiger, B.; Stecca, K. L. Cloning of higher plant omega-3 fatty acid desaturases. *Plant Physiol.* **1993**, *103*, 467–476.
(29) Harwood, J. Fatty acid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 101–138.
(30) Kodama, H.; Horiguchi, G.; Nishiuchi, T.; Nishimura, M.; Iba, K. Fatty acid desaturation during chilling acclimation is one of the factors involved in conferring low-temperature tolerance to young tobacco leaves. *Plant Physiol.* **1995**, *107*, 1177–1185.
(31) McConn, M.; Browse, J. The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. *Plant Cell* **1996**, *8*, 403–416.
(32) McConn, M.; Creelman, R. A.; Bell, E.; Mullet, J. E.; Browse, J. Jasmonate is essential for insect defense in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 5473–5477.

(33) Hamada, T.; Nishiuchi, T.; Kodama, H.; Nishimura, M.; Iba, K. cDNA cloning of a wounding-inducible gene encoding for a plastid ω-3 fatty acid desaturase from tobacco. *Plant Cell Physiol.* **1996**, *37*, 606–11.

(34) Lay, C. L.; Dybing, C. D. Lindseed. In *Oil Crops of the World*; Robbelen, G., Downey, R. K., Ashir, A., Eds; McGraw-Hill: New York, 1989; pp 416−430.

(35) Piffanelli, P.; Ross, J. H. E.; Murphy, D. J. Intra- and extra-cellular lipid composition and associated gene expression patterns during pollen development in *Brassica napus*. *Plant J*. **1997**, *11*, 549–562.

(36) Poghosyan, Z. P.; Haralampidis, K.; Anna, I.; Martsinkovskaya, A. I.; Murphy, D. J.; Hatzopoulos, P. Developmental regulation and spatial expression of a plastidial fatty acid desaturase from *Olea europaea*. *Plant Physiol. Biochem*. **1999**, *37*, 109–119.

(37) Tang, S.; Guan, R.; Zhang, H.; Huang, J. Cloning and expression analysis of three cDNAs encoding omega-3 fatty acid desaturases from *Descurainia sophia*. *Biotechnol. Lett*. **2007**, *29*, 1417–1424.

(38) Hugly, S.; Somerville, C. R. A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. *Plant Physiol*. **1992**, *99*, 197–202.

(39) Miquel, M.; James, D. J.; Dooner, H.; Browse, J. *Arabidopsis* requires polyunsaturated lipids for low-temperature survival. *Proc. Natl. Acad. Sci. U.S.A*. **1995**, *90*, 6208–6212.

(40) Kodama, H.; Hamada, T.; Horiguchi, G.; Nishimura, M.; Iba, K. Genetical enhancement of cold tolerance by expression of a gene for chloroplast omega-3 fatty acid desaturase in trangenic tobacco. *Plant Physiol*. **1994**, *105*, 601–605.

(41) Farmer, E. E.; Weber, H.; Vollenweider, S. Fatty acid signaling in *Arabidopsis*. *Planta* **1998**, *206*, 167–174.

(42) Weber, H. Fatty acid-derived signals in plants. *Trends Plant Sci*. **2002**, *7*, 217–224.

(43) Farmer, E. E.; Ryan, C. A. Octadecanoic precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* **1992**, *4*, 129–134.

(44) Nishiuchi, T.; Iba, K. Roles of plastid ω-3 fatty acid desaturases in defense response of higher plants. *J. Plant Res*. **1998**, *111*, 481–86.

(45) Williams, J. P.; Imperial, V.; Khan, M. U.; Hodson, J. N. The role of phosphatidylcholine in fatty acid exchange and desaturation in *Brassica napus* L. leaves. *Biochem. J*. **2000**, *349*, 127–133.

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