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cDNA Cloning of Chloroplast ω-3 Fatty Acid Desaturase from *Capsicum annuum* and Its Expression upon Wounding

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A clone for a plastid ω -3 fatty acid desaturase (FAD) was isolated from a leaf cDNA library of hot pepper (*Capsicum annuum* L.). The nucleotide sequence of a 1,317 bp open reading frame in the *CachFAD* showed 80.9% homology with that of tobacco plant. It codes for a polypeptide of 438 amino acids with molecular mass of 50.5 kDa and a pI of 8.14. The CachFAD had a putative transit peptide for targeting the chloroplast. Genomic Southern hybridization indicated that it exists as small gene family. Northern hybridization revealed that its mRNA was present in leaves, but not in roots. Transcript levels in the leaves upon wounding increased rapidly to reach the first peak between 1–3 h, decreased thereafter and slightly increased at 24 h after wounding. The levels of linolenic acid (18:3) in wounded leaves also reached the first peak at 6 h, decreased thereafter and reached the second peak at 18 h. These results indicated that wounding not only enhanced the accumulation of the *CachFAD* mRNA but also increased the conversion of linoleic acid (18:2) to linolenic acid (18:3) in leaf lipids of hot pepper.

Keywords: ω-3 Fatty Acid Desaturase (FAD); cDNA; Hot Pepper; Linolenic Acid; Wounding.

Introduction

The membrane lipids of higher plants are characterized by a high proportion of polyunsaturated fatty acids. In particular, about 70–80% of the fatty acids present in the galactolipids of chloroplast membranes are trienoic fatty acids, namely linolenic acids (18:3) and hexadecatrienoic acids (16:3) (Somerville and Browse, 1991). The conversion of dienoic fatty acids to trienoic fatty acids is catalyzed by ω-3 fatty acid desaturase (FAD) (Browse and Somervile, 1991). In *Arabidopsis* three genes are responsible for the production of trienoic acids (McConn and Browse, 1996). The FAD3 enzyme is localized in the microsome, while the FAD7 and FAD8 enzymes are localized in the plastid membrane (Gibson *et al*., 1994; Hamada *et al*., 1994).

Trienoic fatty acids appear to have at least two important physiological roles. Firstly, they are believed to be important for low temperature fitness in higher plants (Kodama *et al*., 1994; 1995). Secondly, 18:2 and 18:3 fatty acids serve as precursors for several fatty acid-derived signaling molecules, such as traumatic acid and jasmonic acid (Farmer, 1994). Linolenic acid is converted to jasmonic acid in a lipoxygenase-dependent octadecanoid pathway (Farmer and Ryan, 1992). These facts imply that regulation of trienoic fatty acids level is involved in the defensive response of higher plants to environmental stresses.

Effects of phytohormones and environmental stimuli such as light, temperature and wounding on the expression of the ω-3 *FAD* gene have been reported in several recent studies. The transcript levels of the *FAD7* gene from *Arabidopsis* (Nishiuchi *et al*., 1995) and wheat (Horiguchi *et al*., 1996) rapidly increased when dark-adapted plants were transferred to white light. The *FAD8* gene from *Arabidopsis* and maize was expressed substantially at moderately low temperatures below 20°C (Berberich *et al*., 1998; Gibson *et al*., 1994). The transcript level of the microsome ω-3 *FAD* gene from rapeseed was upregulated by abscisic acid treatment (Zou *et al*., 1995).

The transcripts of the mung bean microsome ω-3 *FAD* gene, which was isolated initially as an auxin-inducible gene, rapidly accumulated in hypocotyls after they were wounded (Yamamoto *et al*., 1992). In tobacco leaves, mRNA levels of the plastid ω-3 *FAD* gene also increased

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Abbreviations: FAD, ω-3 fatty acid desaturase; PCR, polymerase chain reaction; pfu, plaque forming unit; pI, isoelectric point; SOD, superoxide dismutase; SSC, standard saline citrate.

after wounding treatment, and this increase was accompanied by an increase in trienoic acid in the major polar lipids (Hamada *et al*., 1996). The mRNA levels of the *Arabidopsis FAD7* gene rose rapidly after local wounding treatment in rosette leaves and roots. The wound-responsive expression in roots is thought to be mediated via an octadecanoid pathway, whereas jasmonate-independent wound signals in leaves may induce the activation of the *FAD7* gene (Nishiuchi *et al*., 1997).

In an effort to elucidate hot pepper's response to diverse stresses, we have characterized cDNAs for *Cu/ZnSOD* (Kim *et al*., 1977) and *MnSOD* (Kwon and An, 1999). In this paper we report a plastid ω-3 *FAD* cDNA sequence and its expression patterns in different organs, the effects of wounding on the transcript level of this gene, and the composition of leaf polar lipids.

Materials and Methods

Plant materials Seeds of hot pepper var. Dae Poong were soaked for 10 min in 1% sodium hypochlorite and then in deionized water for 24 h. The seeds were germinated in the dark at room temperature for up to 2 weeks. The seedlings were transferred to a growth chamber set at a 16/8 h day/night cycle with white light (100 μEinstein/mm^2/s), and a 28/25°C temperature cycle. The leaves of 4-week-old pepper were cut into about 5 mm long sections with a sterile razor blade and soaked in 50 mM sodium phosphate buffer, pH 7.0.

Isolation of nucleic acids Plant genomic DNA was isolated by the method of Doyle and Doyle (1990) from the young leaves of pepper. Total RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987).

Polymerase chain reaction Ten μl of a cDNA library was left in boiled water for 10 min and then centrifuged. Only the supernatant was taken and used as a template for the PCR reaction. Two degenerate oligonucleotides, CHU [5′-gTNT(A/T/g)AggCC(A/T)CT(C/T)CC(A/g/C)AgAAT] and CHL [5′-CCACAATCATg (A/T/g)CCAAgAACA] corresponding to two conserved regions of plastid *FAD* genes, were used as primers for amplifying the N-terminal of plastid *FAD*. Two other primers, CHF5U (5′-gCTTTTCTCTTTTTTTCTCC) and CHF5L (5′-AgATAgCTTgAATgggggTgg), were used to generate a *CachFAD* specific probe. The PCR reaction program was as follows; denaturation at 94°C for 5 min followed by 30 cycles of PCR consisted of annealing at 53°C for 1 min, polymerization at 72°C for 30 s, denaturation at 94°C for 45 s. The PCR product was ligated into a pGEM7 vector and nucleotide sequencing was carried out as described by Sanger *et al*. (1977).

Screening of cDNA library The method of Benton and Davis (1977) was used for the screening with some modifications. Approximately 5,000 pfu of a hot pepper cDNA library (Kim *et al*., 1997) were plated with XL-1 blue cells on a 80 × 100 mm Petri dish. A total of five plates were used. Following a 6–8 h incubation at 37°C, the plates were cooled at 4°C for 2 h. Phage DNA from each plate was transferred onto a Hybond N nylon membrane (Amersham, USA). DNA on the filters was denatured in 1.5 M NaCl, 0.5 N NaOH for 3 min, neutralized in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 5 min, rinsed in 2× SSC for 30 s and blotted briefly on Whatman 3 MM paper. The PCR product from CHU/CHL primers was labeled with ^{32}P using a random primer labeling kit, and was used as a probe for hybridization carried out by the method of Sambrook *et al*. (1989).

Nucleotide sequencing Nucleotide sequencing of positive clones was carried out as described by Sanger *et al*. (1977), using a Sequenase Version 2.0 DNA sequencing Kit (USB TM Ohio, USA), T3 (20-mer, 5′-AATTAACCCCCTCACTAA-Aggg), and T7 (22-mer, 5′-TAATACgACTCACTATAggg-CgA). Analysis was carried out by a Blast search (Altsch *et al*., 1990), DNAsis and PCgene program.

Southern and Northern hybridization DNAs and RNAs were separated on an 0.8% agarose gel and a 1% formaldehyde agarose gel, respectively. The gel was transferred onto a Hybond N nylon membrane (Amersham, USA) by the method of Sambrook *et al*. (1989). Hybridization and washing conditions were same both for Southern and Northern hybridization. The membrane was hybridized with the ^{32}P-labeled probe in a BEPS solution (Shah *et al*., 1997) (1% BSA, 1 mM EDTA, 0.5 mM sodium phosphate, pH 7.2 and 7% SDS) at 62°C for 4 h for prehybridization followed by 24 h for hybridization. Blots were then washed with 2× SSC/0.1% (w/v) SDS for 1 h, 1× SSC for 30 min and 0.5× SSC for 30 min at 62°C.

Fatty acid analysis The overall fatty acid composition was determined by following the method of Miquel and Browse (1992). Briefly, samples were heated at 80°C in 1 ml of 2.5% (v/v) H_2SO_4 in methanol for 90 min in screw-capped tubes. After adding 1.5 ml of 0.9% NaCl solution and 1 ml of hexane, fatty acids were extracted into the organic phase by centrifugation at low speed. Samples (1 μl) of the organic phase were separated by a Hewlett Packard gas chromatograph 6890.

Results and Discussion

cDNA library screening In the course of random sequencing of hot pepper leaf cDNA library (Kim *et al*., 1997), we selected a clone which showed 75% nucleotide sequence homology with tobacco microsomal *FAD*. To isolate a plastid *FAD* clone, we screened the cDNA library with a 435-bp PCR product as a probe. The probe was obtained by PCR of genomic DNA using conserved N-terminal sequences from plastid *FAD* genes as primers (CHF5U/CHF5L). Among six positive clones, one which showed high homology with other plastid *FAD* genes was selected and named *CachFAD*.

cDNA sequences of *CachFAD* The cDNA clone contained a 1,562-bp insert DNA. It consisted of a 39-bp 5′ untranslated region (UTR), a 203-bp 3′ UTR and a

1,317-bp putative open reading frame corresponding to 438 amino acid residues with a molecular mass of 50.5 kDa and a pI of 8.14 (Fig. 1). Consensus sequences surrounding the start codon, CCCAATG, were found in the 5′ UTR, while putative polyadenylation signal was found in the 3′ UTR. The N-terminal sequence of 61 residues has several characteristics of transit peptides of plastid-destined proteins (Keegstra *et al.*, 1989). These include a high content (21%) of hydroxylated residues, a low content (3%) of basic residues and highly conserved N-terminal dipeptide, Met-Ala (Yadav *et al.*, 1993). The amino acid sequence of CachFAD shows 80.9% identity with that of tobacco plastid FAD. Multiple alignment of amino acid sequences of plastid FADs exhibited the conserved nature of plant plastid FADs through entire proteins (data not shown), especially around three histidine motifs (Fig. 1). They are known to participate in the formation of active sites with iron molecules and to be conserved in all ω-3 type FADs (Los and Murata, 1998).

Genomic hybridization Genomic DNA was digested with *Eco*RI, *Hin*dIII and *Bam*HI, respectively, and separated on a 0.8% agarose gel. The DNA was transferred onto a nylon membrane and hybridized with the insert DNA of *CachFAD* labeled with [^{32}P]-dCTP as a probe. More than two fragments in each digest showed hybridization signals; 12.0-, 7.0-, 3.8-, 2.7-kb *Eco*RI-fragments, 4.3-, 3.0-, 2.8-kb *Hin*dIII-fragments, and 20.0-, 2.7-kb *Bam*HI-fragments (Fig. 2). Since there are no restriction sites for the three enzymes in the clone, these results indicate that more than two copies of the plastid *FAD* gene are present in the genome of hot pepper. Two plastid *FAD* genes, *FAD7* and *FAD8*, have been reported in *Arabidopsis* and maize (Berberich *et al.*, 1998; Gibson *et al.*, 1994).

Effect of wounding on the expression of *CachFAD* The mRNA corresponding to *CachFAD* was not detected in stem and root tissues, but was detected in leaf tissue (Fig. 3A). In *Arabidopsis* (Nishiuchi *et al.*, 1995), maize (Berberich *et al.*, 1998) and wheat (Horiguchi *et al.*, 1996) the transcripts of plastid ω-3 *FAD* were also detected in leaves but not at all in roots. Expression of the tobacco and *Arabidopsis* plastid *FAD* genes were upregulated in leaves by wounding (Hamada *et al.*, 1996; Nishiuchi *et al.*, 1997). Thus, the wound-responsive expression of hot pepper plastid *FAD* gene was examined. The pepper leaves were cut into sections with a razor blade and then soaked in sodium phosphate buffer for various times. RNA gel blot analysis with the 5′ UTR of *CachFAD* as a probe showed that full-size *CachFAD* mRNA levels started to increase at 20 min, reached a peak between 1–3 h, decreased rapidly thereafter, and slightly increased at 24 h after wounding (Fig. 3B). We cannot tell whether this pattern corresponds to monophasic or biphasic accumulation, since we do not have data on the mRNA levels after 24 h. In tobacco and *Arabidopsis* (Hamada *et al.*, 1996; Nishiuchi *et al.*, 1997), ω-3 *FAD* mRNA levels started to increase after 1 h and high levels were maintained more than 6 h. Biphasic accumulation, however, was observed for microsomal ω-6 *FAD* in cultured cells of parsley treated with elicitor (Kirsch *et al.*, 1997). The difference may be attributed to the differences in the sample, the wounding treatment and the specificity of the probe used.

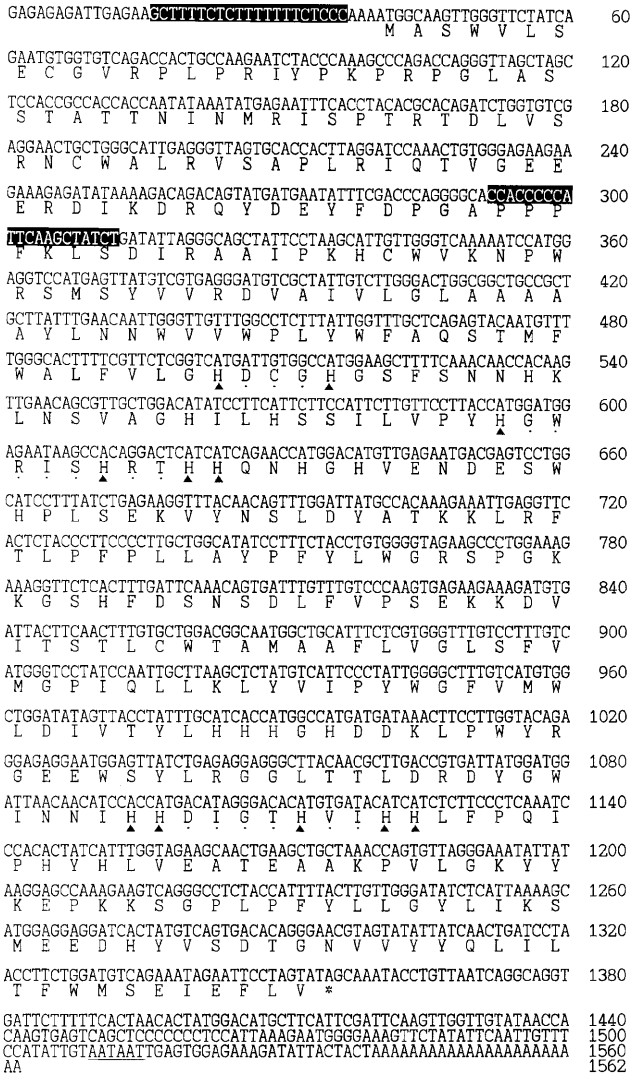

Fig. 1. Nucleotide and amino acid sequences of chloroplast ω-3 fatty acid desaturase from *C. annuum* (*CachFAD*). Amino acids are shown as standard one-letter abbreviations. A potential polyadenylation signal is underlined, and a stop codon by a star. Shaded regions indicate oligonucleotide sequences used for generating primers for 5′ UTR-containing probe. Three highly conserved histidine motifs are indicated by triangles and dots. The sequence has been deposited in GenBank as Accession No. AF222989.

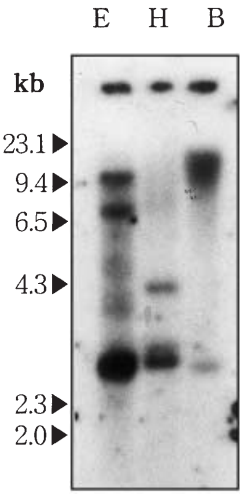

Fig. 2. Genomic Southern hybridization of *CachFAD*. Genomic DNA (10 μg) digested with restriction enzyme was separated on an 0.8% agarose gel and DNA fragments transferred on a nylon membrane was probed with ^{32}P-labeled full-length *CachFAD*. The size of DNA cut with *Hin*dIII is indicated in kb. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI.

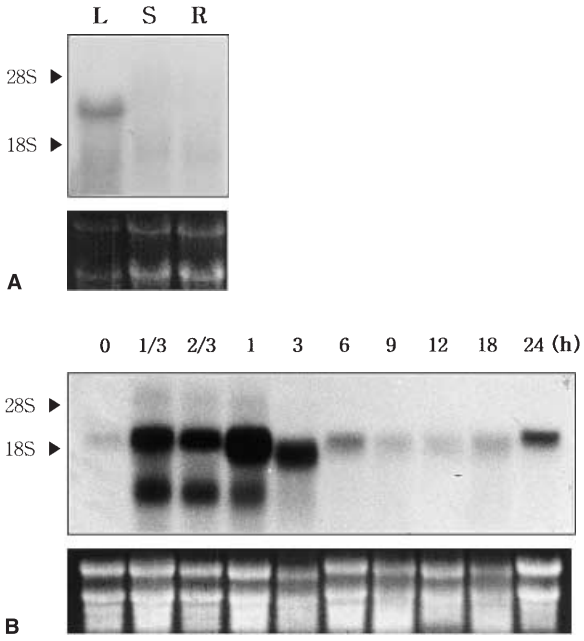

Fig. 3. Expression of *CachFAD* mRNA in different tissues (**A**) and in leaves left in phosphate buffer for various times after wounding (**B**). Total RNA (30 μg) from each tissue was analyzed by northern hybridization with ^{32}P-labeled 5′ UTR-containing probe of *CachFAD*. L, leaf; S, shoot; R, root.

An interesting feature of *CachFAD* expression was that smaller transcripts were detected only in a short period after wounding treatment (Fig. 3B). There are two ω-3 *FAD* genes in chloroplasts of *Arabidopsis*, tobacco and maize; *FAD7* and *FAD8*. Although we have not obtained a *FAD8* clone from red pepper, we believe that the full-size transcript originated from *FAD7* for following reasons. Firstly, *FAD7* has been shown to be rapidly induced by wounding treatment and exclusively expressed at normal temperature, while *FAD8* was expressed substantially at low temperatures both in *Arabidopsis* (Gibson *et al*., 1994) and maize (Berberich *et al*., 1998). Plant samples in our wounding experiments were never exposed to low temperature. Secondly, transcripts of *FAD8* in maize were present both at normal and low temperatures, but at normal temperature smaller transcripts prevailed suggesting posttranscriptional regulation of the *FAD8* gene (Berberich *et al*., 1998). The identity of the smaller transcripts should be addressed when the *FAD8 cDNA* clone from the hot pepper is available.

Effect of wounding on the fatty acid composition Since *CachFAD* mRNA levels increased in response to wounding, the effect of wounding on the fatty acid composition was investigated. The fatty acid compositions were determined in total lipids extracted from leaf tissues harvested at different times after wounding (Table 1). Unwounded hot pepper leaf had 39.4% of linolenic acid, characteristic of higher plant membranes (Miquel and Browse, 1992; Murata *et al*., 1982). The content of linolenic acid (18:3) increased to 49.2% at 6 h, decreased to control level at 9 h, then increased again to 45.9% at 18 h after wounding. The content of linoleic acid (18:2), however, decreased concomitantly. The ratio of 18:3/18:2 was high at 6 h and 12 h after wound treatments.

Although there is some discrepancy between mRNA accumulation and changes in trienoic fatty acid contents, these results indicated that wounding caused not only a rapid increase in plastid *FAD* mRNA but also an increase in the content of linolenic acid in hot pepper. But we do not know the exact physiological meaning of the increased trienoic fatty acid in wounded leaves. It may play a role in healing damaged tissue, the biogen-

Table 1. Change in the fatty acid composition of hot pepper leaves upon wounding.

Time (h)	Fatty acid composition (%)							18:3 /18:2
	14:0	16:0	18:0	18:1	18:2	18:3	Unidentified	
0.00	0.29	26.00	6.35	1.07	26.11	39.43	0.75	1.51
0.66	0.18	15.54	1.10	ND	24.84	42.61	15.73	1.72
1.00	0.38	13.40	1.06	ND	23.67	43.45	18.04	1.84
3.00	ND	13.50	1.03	ND	25.93	45.90	17.64	1.62
6.00	0.16	12.04	1.04	ND	22.62	49.15	14.77	2.17
9.00	2.68	15.64	0.83	ND	23.87	39.47	17.51	1.65
12.00	0.15	12.11	1.92	0.24	19.88	44.25	21.45	2.23
18.00	0.11	11.25	0.94	ND	21.98	45.89	19.83	2.09
24.00	0.25	13.75	0.97	ND	23.61	38.05	23.37	1.61

ND, not detected.

esis of a new membrane (Hugly and Somerville, 1992), or the production of a signal molecule like jasmonic acid (Nishiuchi *et al*., 1997). Further studies on the isolation of *FAD8* and microsomal *FAD* clones, the effects of wounding on their expression, and the changes in fatty acid in different types of lipids (Hamada *et al*., 1996) are necessary to elucidate the role of *FAD* genes in the wounding response of hot pepper.

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