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Characterization of a rice (*Oryza sativa* L.) gene encoding a temperature-dependent chloroplast ω-3 fatty acid desaturase ☆

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Abstract

A cDNA, designated *Osfad8*, encoding a chloroplast ω-3 fatty acid desaturase responsible for trienoic fatty acid formation, was isolated from the leaves of *Oryza sativa* L. by RT-PCR. Southern blot hybridization indicated that a small gene family composed of two copies or closely linked genes exists. RNA in situ hybridization showed that the accumulation of *Osfad8* mRNA was abundant in leaves but hardly detectable in roots. The *Osfad8* transcript level in leaves was much higher at 15 °C than at normal temperature (25 °C). In situ hybridization also showed particularly prominent expression of *Osfad8* in the palisade layer and spongy parenchyma cells of leaves when exposed to 15 °C conditions for 5 days and 10 days. Two transgenic lines (8S-52 and 8S-101) harboring the *Osfad8* ORF in sense orientation under the control of the CaMV 35S promoter contained increased levels of hexadecatrienoic (16:3) and linolenic (18:3) fatty acids. When exposed to 2 °C for 7 days, the damage observed to the control plants was significantly alleviated in the 8S-52 and 8S-101 lines. The amounts of trienoic fatty acids in an *Osfad8* antisense line (8A-35) declined 40.2% compared to the control plants. The 8A-35 plants survived after growth at 44 °C for 3 days while the control plants died. These data suggest that *Osfad8* encodes a temperature-dependent chloroplast ω-3 fatty acid desaturase.

Keywords: Fatty acid; In situ hybridization; *Osfad8*; *Oryza sativa* L.; ω-3 fatty acid desaturase; Transgenic tobacco

One of the major environmental stresses that plants can suffer is the exposure to a broad range of temperatures. Many species of a temperate origin can develop tolerance when exposed to changing environmental temperature for a period of time. This process is known as temperature adaption and is associated with complex biochemical and physiological changes. Among these changes, fluidity modification of the lipid membranes is a major factor for plants to acquire this tolerance. This modification is brought about by unsaturation level of the membrane lipids [1–4].

Fatty acid unsaturation is catalyzed by a group of fatty acid desaturases. In *Arabidopsis thaliana*, seven loci (*fad2*, *fad3*, *fad4*, *fad5*, *fad6*, *fad7*, and *fad8*) were identified as fatty acid desaturase-coding [5–9], among which *fad3*, *fad7*, and *fad8* are responsible for the production of trienoic fatty acids (TA) by unsaturation at the ω-3 position. The cDNAs corresponding to these three loci have been isolated [10]. The *fad3* gene encodes the microsomal ω-3 desaturase and the *fad7* and *fad8* genes encode the chloroplast isozymes. FAD8 was originally identified as a

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☆ *Abbreviations:* CTAB, cetyltrimethylammonium bromide; DTT, dithiothreitol; DA, dienoic fatty acid; MS, Murashige–Skoog; PBS, sodium phosphate buffer; RT-PCR, reverse transcriptase polymerase chain reaction; TA, trienoic fatty acids; 16:3, hexadecatrienoic; 16:0, palmitate; 18:3, linoleic acid; 18:2, linoleic acid; 18:0, stearate; ω-3 refers to the position of the double bond from the methyl end of a fatty acid.

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temperature-sensitive chloroplast isozyme by phenotypic analysis of the *fad7* mutant in which *fad8* is the only functional ω-3 desaturase in plastids [11]. Reduction in leaf TA level caused by the *fad7* mutation is less pronounced at lower temperatures, implying that FAD8 activity is induced by low temperature. It has been reported that RNA isolated from *Arabidopsis* grown at 20 °C contained levels of *fad8* mRNA that were detectable by Northern blot analysis, whereas RNA isolated from plants grown at 30 °C had no detectable *fad8* mRNA.

The physiological relevance of the responses to temperature has been demonstrated in transgenic plants with modified leaf TA contents. For example, the introduction of the *Arabidopsis thaliana fad7* gene into tobacco under the control of the constitutive promoter resulted in a significant elevation of TA levels and the ability to confer cold tolerance [12]. FAD7-deficient tobacco plants showed reduced leaf TA levels, but performed better with regard to growth and photosynthesis at high temperature [4].

Rice (*Oryza sativa* L.), one of the most highly grown crops, is cultivated mainly in tropical and subtropical regions, and its distribution is primarily determined by temperature. Therefore, there is considerable interest in improving broad temperature resistance of rice. Here we report the isolation and functional analysis of *Osfad8*, a putative rice chloroplast ω-3 desaturase gene, and discuss the possible role of *Osfad8* and its physiological significance.

Materials and methods

Plant growth conditions and low/high-temperature treatment. Rice plants (*Oryza sativa* L. cv Ey1 105. Japonica) were grown on wet gauze in a growth chamber (SPX-250-GB, Shanghai, China) at 25 °C with 12 h light/dark cycles. When the very small second leaf emerged, the plants were exposed to 15 °C for 5, 10 days, respectively. The leaves and roots were subjected to DNA and RNA analyses.

The wild-type and transgenic tobacco were grown on MS agar medium without or with 100 mg/L kanamycin and then transferred to wet gauze. During cold treatment, plants on wet gauze were exposed to 2 °C for 7 days in a low-temperature incubator. During high-temperature treatment, plants were exposed to 44 °C for 3 days. During different treatment plants were arranged randomly in the chamber to avoid local variations in the environment.

Southern hybridization. Genomic DNA was extracted by the CTAB method [13]. Aliquots (10 μg) of DNA were digested with *Dra*I, *Eco*RV, and *Hin*dIII. The genomic region of *Osfad8* has no restriction sites recognized by these enzymes. The 0.38 kb fragment of 3′ untranslated region (3′UTR) was used as a probe and the primers, 5′-ACC AGA CCG ACC CTG CTA ACT AA-3′ (forward), 5′-GAT ATA TCA ATA TGG CCT TGT CC-3′ (reverse), were designed according to the *Osfad8* genomic sequence (the Accession No. of the GenBank, EMBL, and DDBJ Data Libraries is AP004333). Southern blot analysis was according to the protocol of Gene Images Random Prime Labeling Module and Detection System (Amersham China, Shanghai, China).

Cloning of rice fad8 cDNA. To amplify a full-length cDNA of rice, RT-PCR was carried out using the primer 5′-ATG GCC CGG CTG CTA CTC TCC GGC G-3′ (forward) and 5′-TTA GTT AGC AGG GTC GGT CTG GTA G-3′ (reverse), which was designed from the ORF of predicted ω-3 fatty acid desaturase genes (the Accession No. of the GenBank, EMBL, and DDBJ Data Libraries is XM_506593). Five micrograms each of total RNA isolated from rice leaves was transcribed with oligo(dT) primer using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The first-strand cDNA served as a template. The PCRs were carried out for 30 cycles of 94 °C/1 min, 55 °C/1 min, and 72 °C/1 min in a volume of 25 μl. The PCR products were subcloned into the pGEM-T vector (Promega) and sequenced by the dideoxy chain termination method using an automatic sequencer (ABI100 DNA sequencer).

In situ hybridization. Leaves dissected from rice grown at 15 °C for 5 days and 10 days, and 25 °C, respectively, were fixed in 4% paraformaldehyde in PBS overnight, followed by dehydration in graded ethanol series, and cleaned in xylene. After soaking in xylene and paraffin, samples were embedded in paraffin. The paraffin blocks were sliced into 8 μm sections, which were mounted onto poly L-lysine coated slides. Digoxigenin-UTP labeled antisense and sense RNA probes were transcribed from the 0.45 kb fragment of the *fad8* N-terminal cDNA to prevent cross-hybridization to the RNA products of other fatty acid desaturase genes, using T7 and SP6 polymerase, respectively (Roche). The coding (sense) strand provides a negative control for the specificity of the positive, i.e., antisense probe. After deparaffination treatment with xylene, the sections were treated for 45 min with proteinase K (10 μg/ml). Hybridization of the sections was performed at 50 °C for 16 h with the riboprobe in hybridization solution (40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, and 1 mg/ml denatured and sheared salmon-sperm DNA). The washes, blocking, antibody incubation and detection were performed according to the procedure for In Situ Hybridization to Chromosomes, Cells, and Tissue Sections (Roche).

Plasmid construction. The 1.2-kb ORF of *Osfad8* was cloned into the *Xba*I–*Sac*I site of the binary plasmid pBI121 to replace the β-glucuronidase (GUS) gene in antisense and sense orientations relative to the CaMV 35S promoter, respectively. The resulting plasmids, pBI1218A, contained the *Osfad8* ORF in antisense orientation, and pBI1218S contained *Osfad8* in the sense orientation. The pBI121 was used as an empty vector.

Plant transformation. Tobacco (*Nicotiana tabacum*) was transformed by the leaf-disk method by using *Agrobacterium tumefaciens* EHA105 containing the plasmid pBI1218A, pBI1218S or pBI121 [14]. Individual kanamycin-resistant regenerated shoots were selected, and the plants were rooted in Murashige and Skoog (MS) agar plates and transferred to soil.

Characterization of the transgenic tobacco. Southern hybridization was carried out with 0.76 kb *nptII* gene from pBI121 as a probe and the primers, 5′ ATG ATT GAA CAA GAT GGA TTG CAC GCA GGT 3′ (forward), 5′ TCA GAA GAA CTC GTC AAG AAG GCG ATA GAA 3′ (reverse).

The expression of *Osfad8* in tobacco was confirmed by RT-PCR using the primer sets: *Osfad8*, 5′-CAT TCA CCA TCT TTT CCC CCA-3′ (forward) and 5′-TTA GTT AGC AGG GTC GGT CTG GTA G-3′ (reverse); *Ntactin*, 5′-CCA TTG GCT CAG AGA GGT TC-3′ (forward) and 5′-GTT GGA AGG TGC TGA GAG-3′ (reverse); *Ntfad*, 5′-TCT TTG TTC TTG GA/TC ATG ATT GT-3′ (forward) and 5′-ACA TAA/G TGA TCC TGC TTC AT-3′ (reverse). The primer set to identify *Osfad8* transcript in tobacco was designed to the *Osfad8*-specific region by aligning *Osfad8* with the ω-3 fatty acid desaturase genes (designated *Ntfad*) of tobacco. The primers to characterize the accumulation of endogenous *Ntfad* mRNA of tobacco were designed according to the homologous sequence of them but not homologous to *Osfad8* gene.

Fatty acid analysis. Fatty acid methyl esters of leaf samples were prepared as described by [15]. Samples (1 μl) were separated by gas chromatography on a 30 m × 0.32 mm capillary column (HP6890 Plus Hewlett–Packard, Wilmington, Del.) and quantified using a flame ionization detector. The gas chromatograph was programmed for an initial temperature of 150 °C for 3 min followed by an increase of 15 °C/min to 210 °C; this final temperature was maintained for a further 12 min.

Results

Isolation and sequence analysis of a rice cDNA clone of the putative chloroplast ω-3 fatty acid desaturase

In order to amplify the predicted ORF of the rice putative ω-3 desaturase by RT-PCR, primers were designed