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Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean

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Summary

In plants, the endoplasmic reticulum (ER)-associated oleate desaturase (FAD2) is the key enzyme responsible for the production of linoleic acid in non-photosynthetic tissues. In soybean three *FAD2*-like genes have been reported including two seed-specific genes, *FAD2-1A* and *FAD2-1B*, and a house-keeping gene *FAD2-2*. In this study, we isolated a novel gene encoding FAD2 isoform, designated as *FAD2-3*. The deduced amino acid sequences of the *FAD2-3* displayed the typical three histidine boxes characteristic of all membrane-bound desaturases, and possessed a C-terminal signal for ER retention. Phylogenetic analysis showed that *FAD2-3* is grouped within plant house-keeping FAD2 sequences. Yeast cells transformed with a plasmid construct containing the *FAD2-3* coding region accumulated a considerable amount of linoleic acid (18:2), normally not present in wild-type yeast cells, suggesting that the isolated gene encodes a functional FAD2 enzyme. Semi-quantitative RT-PCR and *in silico* analysis showed that *FAD2-3* gene is constitutively expressed in both vegetative tissues and developing seeds. In soybean leaves, the level of linolenic acid (18:3) increases with the decrease of linoleic aicd (18:2) under cold treatment. However, no significant change of transcript levels of *FAD2-2* and *FAD2-3* genes was detected. These results indicated that the altered polyunsaturated fatty acid levels in leaves treated with cold stress have no direct correlation with the expression of these two microsomal oleate desaturase genes.

Introduction

As in all other organisms, fatty acids in plants are the major structure components of membrane phospholipids and triacylglycerol storage oils. The relative quantities of the various saturated and

Abbreviations: DAF, days after flowering; ER, endoplasmic reticulum; FAD, fatty acid desaturase; PUFA(s), polyunsaturated fatty acid(s); RACE, rapid amplification of cDNA ends; *X*:*Y*, a fatty acyl group containing *X* carbon atoms and *Y cis* double bonds

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unsaturated fatty acids (PUFAs) are the major factors influencing the quality of plant oils. For example, oils high in oleic acid (18:1) and low in PUFAs appear to have improved nutritional benefits to human and animal consumption and increased stability (Liu and White, 1992). Fatty acid desaturases (FADs) are enzymes responsible for the insertion of double bonds into fatty acyl chains, following the removal of two hydrogen atoms. These desaturation processes take place in both the plastidial membrane and the endoplasmic reticulum (ER) membrane via two different pathways (Ohlrogge and Browse, 1995). The genes for ER- and plastid-derived Δ-12 FADs have been characterized from some plant species. Several different microsomal oleate desaturase (*FAD2*) genes may exist, depending on the particular plant. For instance, there is only one *FAD2* gene existing in Arabidopsis (Okuley et al., 1994), and two different *FAD2* genes in olive have been identified encoding seed-specifically and constitutively expressed microsomal oleate desaturases (Hernandez et al., 2005). Whereas, three different *FAD2* genes have been identified from both cotton and sunflower, with one expressed specifically in seed and the other two expressed in all tissues tested (Liu et al., 1999; Martinez-Rivas et al., 2001; Pirtle et al., 2001).

In soybean, one of the most important resources of vegetable oil, two different microsomal oleate desaturase genes have previously been reported: a constitutively expressed gene *FAD2-2*, and a seed-specific gene *FAD2-1*; the latter one plays a predominant role in determining the PUFA content of the seed-storage oil (Heppard et al., 1996). A seed-specific isoform of FAD2, designated as FAD2-1B, has been reported recently in soybean (Tang et al., 2005). However, it is not clear if there exist any other *FAD2*-like genes in soybean. Thus, in order to explore the regulatory mechanism of oleate desaturation, we isolated a novel microsomal oleate desaturase (*FAD2*) gene in soybean and demonstrated its function by expression in yeast (*Saccharomyces cerevisiae*). Meanwhile, the expression pattern of this gene was investigated in different tissues and during soybean seed development.

Materials and methods

Plant material and growth conditions

Soybeans (*Glycine max* L. cv Meng8206) were grown in growth chamber with day/night cycle of 32/28, 28/22, 18/12 and 12/8 °C. The light/dark cycle was 12/12 h. Developing seeds were harvested at 11, 14, 19, 20, 22, 24, 27 and 32 days after flowering (DAF) from field, chilled in liquid nitrogen and stored at −70 °C. Seeds at 22 DAF were dissected out to collect seed coat, embryo and cotyledon, frozen in liquid nitrogen and stored at −70 °C. Leaf, stem, and root tissues were collected from soybean seedlings grown at 28/22 °C with light/dark cycle 12/12 h. Soybean seedlings were grown at 28 °C for 12 days and then shifted to 8 °C, and leaves were collected at 0, 1, 2, 4, 8, 16, 24, 48 h and 1 week.

RNA extraction and RT reactions

Total RNA was isolated from different soybean tissues using Plant RNA extraction Kit (TianGen, Beijing) as described by manufacturer. RNA concentration was determined spectro-photometrically and verified by ethidium bromide staining of agarose gel. Total RNA was then treated with RNase-free DNase I (TaKaRa), and about 2 μg was used as template for the first cDNA synthesis using Superscript First Strand Synthesis system and oligo(dT) primers (TaKaRa) according to manufacturer's protocol.

Isolation of microsomal oleate desaturase partial cDNA clone

Two degenerate primers, P1 (5′-AAG AA [AG]G CGA T[ACT] CCG CCG CA[CT]TG-3′) and P2 (5′-GC [CT]T CCA TGG C[AG] T[GT] [AG]T A[AG] TG-3′), were designed from the comparison of known plant FAD2 amino acid sequences, corresponding to highly conserved regions (Fig. 1). This pair of primers, together with an aliquot of cDNA from 19 DAF seeds, young leaves, stems and roots, were used in a standard PCR amplification protocol with Hotstart *Ex* DNA polymerase (TaKaRa). One fragment was generated in each reaction, then subcloned into the vector pGEM-T (Promega) and sequenced.

Rapid amplification of cDNA ends (RACE)

The full-length cDNA clone was obtained by 5′ and 3′ RACE using the SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) as described by the manufacturer. The gene-specific primers used for RACE were designed from the above partial cDNA sequence. The primers 5R1 (5′-AGC CCA ATA GAT TGC CAT GCC ACG-3′) and 5R2 (5′-GGG TGG CAA CAT AAT AGA GGC AGA-3′) were used for 5′ RACE, and the primers 3F1 (5′-CGT ATG ATA GGT TTG CTT CCC ACC T-3′) and 3F2 (5′-AGA TGC AGG AGT ACT TGC AGT ATG-3′) were used for 3′ RACE. The PCR fragments were cloned into the pGEM-T vector (Promega) and sequenced by Invitrogen (Shanghai).