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Functional analysis and tissue-differential expression of four *FAD2* genes in amphidiploid *Brassica napus* derived from *Brassica rapa* and *Brassica oleracea*

Kyeong-Ryeol Lee [a], Soo In Sohn [a], Jin Hee Jung [b], Sun Hee Kim [a], Kyung Hee Roh [a], Jong-Bum Kim [a], Mi Chung Suh [b], Hyun Uk Kim [a,*]

[a] Department of Agricultural Biotechnology, National Academy of Agricultural Science, RDA, Suwon 441-707, Republic of Korea
[b] Department of Bioenergy Science and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea

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ABSTRACT

Fatty acid desaturase 2 (FAD2), which resides in the endoplasmic reticulum (ER), plays a crucial role in producing linoleic acid (18:2) through catalyzing the desaturation of oleic acid (18:1) by double bond formation at the delta 12 position. FAD2 catalyzes the first step needed for the production of polyunsaturated fatty acids found in the glycerolipids of cell membranes and the triacylglycerols in seeds. In this study, four *FAD2* genes from amphidiploid *Brassica napus* genome were isolated by PCR amplification, with their enzymatic functions predicted by sequence analysis of the cDNAs. Fatty acid analysis of budding yeast transformed with each of the *FAD2* genes showed that whereas BnFAD2-1, BnFAD2-2, and BnFAD2-4 are functional enzymes, and BnFAD2-3 is nonfunctional. The four *FAD2* genes of *B. napus* originated from synthetic hybridization of its diploid progenitors *Brassica rapa* and *Brassica oleracea*, each of which has two *FAD2* genes identical to those of *B. napus*. The *BnFAD2-3* gene of *B. napus*, a nonfunctional pseudogene mutated by multiple nucleotide deletions and insertions, was inherited from *B. rapa*. All BnFAD2 isozymes except BnFAD2-3 localized to the ER. Nonfunctional BnFAD2-3 localized to the nucleus and chloroplasts. Four *BnFAD2* genes can be classified on the basis of their expression patterns.

1. Introduction

Plant fatty acids are always synthesized from acetyl-CoA in plastids, and then acylated to glycerols to form glycerolipids, such as polar lipids and neutral lipids (Ohlrogge and Browse, 1995). In general, polyunsaturated fatty acids (PUFAs), such as linoleic acid (LA; $18{:}2\Delta^{9,12}$) and α-linolenic acid (ALA; $18{:}3\Delta^{9,12,15}$), are abundant in plant lipids. Because LA is a precursor of ALA, the synthesis of LA is the most important step for PUFA synthesis. Microsomal oleate 12-desaturase, commonly called fatty acid desaturase 2 (FAD2; EC 1.3.1.35), is responsible for the synthesis of LA (Okuley et al., 1994). This enzyme is localized in the endoplasmic reticulum (ER), accepts electrons from cytochrome *b5*, and then converts *sn*-2-oleoyl phosphatidylcholine (PC) into *sn*-2-linoleoyl-PC (Shanklin and Cahoon, 1998). Therefore, the *Arabidopsis fad2-1* mutant has low levels of PUFAs in phospholipids (Miquel and Browse, 1992), and the limited fluidity of its cell membranes prevent this mutant from surviving at a low temperature (6 °C) (Miquel et al., 1993). The *FAD2* gene was first identified from *Arabidopsis thaliana* (Okuley et al., 1994), with *FAD2* genes subsequently identified from oil crops such as soybean (*Glycine max*; Heppard et al., 1996; Li et al., 2007; Tang et al., 2005), sunflower (*Helianthus annuus*; Hongtrakul et al., 1998), cotton (*Gossypium hirsutum*; Liu et al., 1999; Pirtle et al., 2001; Zhang et al., 2009), sesame (*Sesamum indicum*; Jin et al., 2001), peanut (*Arachis hypogaea*; Lopez et al., 2000; Jung et al., 2000), olive (*Olea europaea*; Hernandez et al., 2005), flax (*Linum usitatissimum*; Krasowska et al., 2007; Khadake et al., 2009), camelina (*Camelina sativa*; Kang et al., 2011), Chinese cabbage (*B. rapa* ssp. pekinensis; Jung et al., 2011), and table grape (*Vitis labrusca*; Lee et al., 2012). Two or more *FAD2* genes were cloned and characterized in all of these plants, except sesame. Nonetheless, Southern blot analysis suggested that additional *FAD2* genes might exist in sesame (Jin et al., 2001). Plant *FAD2* genes comprise two exons and one intron that are located in the 5′-untranslated region (UTR; Okuley et al., 1994). Three histidine boxes (H boxes) are crucial for FAD2 desaturase activity. Displacement of even one of the histidines in these three H boxes can disrupt desaturase activity (Shanklin et al., 1994; Kurdrid et al., 2005).

The biotechnological relevance of *FAD2* relates to its importance in controlling the level of unsaturation of seed oil. High oleic vegetable oils, which are stable to oxidation and have a low potential to turn

Abbreviations: PUFA, polyunsaturated fatty acid; LA, linoleic acid; ALA, α-linolenic acid; ER, endoplasmic reticulum; PC, phosphatidylcholine; UTR, untranslated region; MYA, million years ago; eYFP, enhanced yellow fluorescent protein; cDNA, DNA complementary to RNA; FAME, fatty acid methyl ester; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; SSC, sodium chloride-sodium citrate; SDS, sodium dodecylsulfate; kb, kilo base; nt, nucleotide; bp, base pair; aa, amino acid; QTL, quantitative trait loci; ORF, open reading frame; TM, transmembrane; DAP, days after pollination.

* Corresponding author. Tel.: +82 31 299 1703; fax: +82 31 299 1672.
E-mail address: hukim64@korea.kr (H.U. Kim).

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rancid, are favored for food and industrial purposes, such as for frying food at high temperatures and producing biodiesel (Warner and Knowlton, 1997; Graef et al., 2009).

Several *Brassica* species, including oilseed rape, mustard, cabbage, Chinese cabbage, broccoli, cauliflower, and turnip, are commercially important vegetable crops throughout the world. In particular, rapeseed oil obtained from *Brassica napus* is one of the most important vegetable oils. According to the Food and Agriculture Organization (FAO), in 2012 the global cultivation area of oilseed rape was 34,257,051 ha, and the production quantity of oilseed rape was 64,813,233 M/T (http://faostat3.fao.org/home/index.html). Almost all seeds from members of the Brassicaceae contain large proportions of very-long-chain monounsaturated fatty acids, including erucic acid ($22{:}1\Delta^{13}$). In conventional rapeseed oil, erucic acid accounts for about 50% of the fatty acids. Although the high-erucic rapeseed oil is useful for certain industrial applications, consumption of erucic acid causes heart disease in humans and animals. In addition, conventional rapeseed contains glucosinolates, another antinutritional component. Varieties of *B. napus* with a low content of erucic acid and glucosinolates were bred by mutation of *FAE1*, with the process culminating in the generation of canola, which has limited amounts of erucic acid and glucosinolates (Stefansson et al., 1961). Oleic acid levels in canola seed are about 60–70%, with the increase occurring at the expense of reduced levels of the antinutritional component erucic acid (Katavic et al., 2002).

B. napus (AACC genome, 2n = 38) is an amphidiploid (or allotetraploid) species that originated from spontaneous hybridization of *B. rapa* (AA genome, 2n = 20) and *B. oleracea* (CC genome, 2n = 18) (U, 1935). It is estimated that the *Arabidopsis* genus and *Brassica* genus diverged from a common ancestral plant at 17–18 million years ago (MYA; Yang et al., 2006). After this event, whole genome triplication was estimated to have first occurred at approximately 13–17 MYA in Brassicaceae (Yang et al., 2006) and then *B. oleracea* and *B. rapa* diverged at approximately 3.75 MYA (Inaba and Nishio, 2002). Finally, *B. napus* emerged as a consequence of synthetic hybridization of these two species at approximately 5000–10,000 years ago (U.N., 1935; Rana et al., 2004; Xiong et al., 2011). This suggests that *B. napus* has multiple *FAD2* genes. Scheffler et al. (1997) reported that the *B. napus* genome encodes four *FAD2* genes and six *FAD3* genes. Schierholt et al. (2000) attributed that the high oleic acid content in mutant oilseed rape is attributed to a *fad2* mutation, and estimated that *B. napus* may have either four or six *FAD2* genes. The recent release of a draft genome sequence of *B. rapa* (The *Brassica rapa* Genome Sequencing Project Consortium, 2011) has facilitated the ease with which genetic information, such as the nucleotide sequence on *Brassica* crops, can be obtained. Based on the genome sequence database for *B. rapa* and *B. oleracea* (Cheng et al., 2011; http://brassicadb.org/brad), Yang et al. (2012) reported that *B. napus* may have four *FAD2* genes originated from two genes of *B. rapa* and *B. oleracea*, respectively. Resolution of the copy number and other features of *BnFAD2* genes should be of value in efforts to control the expression of *FAD2* in *B. napus* seed for industrial purposes.

In this study, we cloned four *FAD2* genes from *B. napus*, and also analyzed the *FAD2* genes of *B. rapa* and *B. oleracea*. The production of LA in yeast transformed with three of the *BnFAD2* genes confirmed their predicted functions, although one of the genes was nonfunctional. Our findings confirm that *B. napus* has four *FAD2* genes, and that these originated from two *FAD2* genes from *B. rapa* and two *FAD2* genes from *B. oleracea*.

2. Materials and methods

2.1. Plant materials

Oilseed rape (*B. napus* cv. Youngsan), Chinese cabbage (*B. rapa* ssp. pekinensis var. Chiifu), and cabbage (*B. oleracea* var. capitata IT100498) were grown under greenhouse conditions at approximately 25 °C. Rapeseed and cabbage seed were obtained from the Bioenergy Crop Research Center, National Institute of Crop Science (NICS), Rural Development Administration (RDA) and the National Agrobiodiversity Center, National Academy of Agricultural Science (NAAS), RDA, respectively. Chinese cabbage seed was kindly provided by Dr. Jin A. Kim, Department of Agricultural Biotechnology, NAAS, RDA. *Nicotiana tabacum* cv. Xanthi plants that were approximately four week old were used for transient expression of *FAD2* genes to determine the subcellular localizations of the enzymes they encode.

2.2. Preparation of genomic DNA and total RNA

Tissues of *B. napus*, *B. rapa*, and *B. oleracea* were ground under liquid nitrogen. The Plant RNA Purification Reagent (Invitrogen, CA, USA) was used for RNA preparation following the manufacturer's protocol. Extraction of DNA preparation was performed as described by Dellaporta et al. (1983).

2.3. Gene cloning

Given that all putative *BnFAD2* genes have identical 20-bp sequences at the 5′ and 3′ ends of their coding sequences, all of the *BnFAD2* genes could be amplified with the same pair of primers, KOD+ polymerase (Toyobo, Japan), and genomic DNA as a template. The primers used in this research are listed in Table 1. The PCR products were extracted using a QIAEX II gel extraction kit (Qiagen, Germany), and the eluted DNA was cloned into pCR-Blunt II-TOPO vector (Invitrogen, CA, USA), following the manufacturer's instructions.

2.4. Sequence analysis and phylogenetic analysis

DNASTAR MegAlign (Ver. 7.2.1) was used to assess the similarities of *FAD2* genes, and the ClustalW method was used to establish the phylogenetic relationships between FAD2 isozymes. Amino acid sequences were analyzed using a program in the Aramemnon Plant Membrane Protein Database (http://aramemnon.botanik.uni-koeln.de/seq_viewBlast.ep) and TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/), as well as the TMpred server (http://www.ch.embnet.org/software/TMPRED_form.html) to identify transmembrane domains. Subcellular sites of localization were predicted using the PSORT prediction (http://psort.hgc.jp/form.html) and WoLF PSORT (http://wolfpsort.org/).

2.5. Gene-specific PCR and semi-quantitative RT-PCR to characterize FAD2 isozymes

Primer sequences and amplicon sizes are listed in Table 1. For genomic PCR, FAD2 isozyme gene-specific PCR condition was as follows: 94 °C for 5 min, 25 cycles of 94 °C for 20 s, 54 °C for 30 s, and 72 °C for 30 s, and an additional extension at 72 °C for 5 min. For RT-PCR, the number of cycles was increased to 30 cycles. Semi-quantitative RT-PCR reactions were carried out as follows. First strand cDNA synthesis included 2 μg of total RNA and components of the PrimeScript II 1st strand cDNA synthesis kit (Takara, Japan) provided at the concentrations recommended by the manufacturer. After the first strand cDNA synthesis, PCR was performed with 2 μl of cDNA and 1 U of Ex Taq polymerase (Takara, Japan) in 20 μl of reaction volume. PCR conditions and primers were identical to those described above. Bactin primers (Yao et al., 2005) can amplify the highly conserved region of all *Brassica* actin genes.

2.6. Vector construction

The BnFAD2 in. F and R primers were designed for the subcloning of *BnFAD2* genes into a yeast expression vector using the In-fusion enzyme (Clontech, CA, USA) (Table 1). The PCR products were cloned into pENTR/D-TOPO (Invitrogen, CA, USA) digested with *Not*I and *Asc*I through the In-fusion reaction. The pENTR/BnFAD2s and pYES-DEST52