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CHARACTERIZATION AND TISSUE-DIFFERENTIAL EXPRESSION OF *FAD2* GENES IN *BRASSICA NAPUS*

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Abstract

In this study, genome DNA and RNAof *fad2* genes from three types of oleic acid content from *B. napus* were isolated by PCR amplification, respectively, the results showed that not only had nucleotides sequences little differences from three types of oleic acid content *B. napus*, but also that of genome DNA and cDNA had still little differences from *B. napus* as far as specific one type of rape. Different genotypes *fad2*-I and *fad2*-II could be easily distinguished by sequence analysis of the cDNAs in G type and CK type except in D type. By analysis on cDNAs, specific differences could be found in three types of rape when compared with the sequence from Genebank. Conserved domains prediction and phylogenetic analysis showed that both six transmembrane domains and three H boxes could be found in FAD2 protein from three types of oleic acid content *B. napus*, respectively. BnFAD2-I and BnFAD2-II belonged to different classes and class I could be divided into two kinds. By QPCR, expression pattern of *fad2* gene in different tissues showed that simple division of *fad2*-Iand *fad2*-II was not apply to all oleic acid content *B. napus*. By southern blot, there were differences in copy numbers of *fad2* genes on different oleic acid content *B. napus*.

Key words: _____

Introduction

Rapeseed (*B. napus* L.), soybean, peanut and sunflower are the most important four greatest oil crops in the world. In China, rapeseed production, cultivated area and total yield had occupied the first place in the world. According to their function, there were three kinds of fatty acids in rapeseed. Palmitic acid and stearic acid belonged to saturated fatty acid. Oleic acid, linoleic acid and linolenic acid belonged to unsaturated fatty acid. Eicosenoic acid and erucic acid belonged to super-long chain fatty acid (Peng, 2011). Oleic acid is the major fatty acid of rapeseed oil. High oleic vegetable oils, which is stable to oxidation and has a low potential to turn rancid, suitable for food and industrial purposes, such as for frying food at high temperatures and also producing biodiesel (Warner & Knowlton, 1997; Graef *et al*., 2009). Due to its nutritional and health value, breeders payed special attention for screening high oleic acid traits in the rapeseed for breeding process (Guan, 2006).

Microsomal oleate 12-desaturase, commonly called fatty acid desaturase 2 (FAD2), is the primary enzyme that controls the relative content of oleic acid, linoeic acid and linolenic acid (Hu *et al*., 2006; Stoutjesdijk *et al*., 2002). This enzyme is localized in the andoplasmic reticulum (ER), accepted electrons from cytochrome *b5*, and then converts *sn*-2- oleoyl phosphatidylcholine (PC) into *sn*-2-linoleoyl-PC (Shanklin & Cahoon, 1998). In higher plants, the copy number and expression of *fad2* genes are different. The *fad2* gene was first identified from *Arabidopsis thaliana* (Okuley *et al*., 1994), which was the only known exception as it harbored only one *fad2* gene copy (At3g12120) and constitutively and abundantly expressed (Beisson *et al*., 2003). Subsequently, *fad2* genes were indentified from crops such as soybean (*Glycine max*), sunflower (*Helianthus annuus*), cotton (*Gossypium hirsutum*), peanut (*Arachis hypogaea*), olive (*Olea europaea*), flax (*Linum usitatissimum*), camelina (*Camelina sativa*), Chinese cabbage (*B. rapa* ssp. Pekinensis), table grape (*Vitis labrusca*), cron (*Zea mays*), sesame (*Sesamum indicum*) and rapeseed (Heppard *et al*., 1996; Li *et al*., 2007; Tang *et al*., 2005; Hongtrakul *et al*., 1998; Liu *et al*., 1999; Pirtle *et al*., 2001; Zhang *et al*., 2009; Lopez *et al*., 2000; Jung *et al*., 2000; Hernandez *et al*., 2005; Krasowska *et al*., 2007; Khadake *et al*., 2009; Kang *et al*., 2011; Jung *et al*., 2011; Lee *et al*., 2012; Mikkilineni & Rocheford, 2006; Jin *et al*., 2001; Yang *et al*., 2012). Two or more *fad2* genes were cloned and characterized in all of these plants, except sesame. Different *fad2* gene copy numbers indicated different expression patterns. In soybean, expression patterns of two *fad2* genes were different (Heppard *et al*., 1996). In sunflower, *fad2-2* and *fad2-3* were the same in expression patterns, but *fad2-1* showed the different expression pattern (Martinez-Rivas *et al*., 2001). Different expression patterns of *fad2* genes had been reported in other crops such as maize (*Zea mays*), olive (*Olea europaea*), *Camelina sativa* and *B.napus* (Hernandez *et al*., 2005; Kang *et al*., 2011; Mikkilineni & Rocheford, 2006; Xiao *et al*., 2008). Interestingly, most *fad2* genes comprised two exons and one intron that were located in the 5'-untranslated region (Okuley *et al*., 1994; Kim *et al*., 2006; Schuppert *et al*., 2006; Jung *et al*., 2011; Kang *et al*., 2011; Suresha & Santha, 2013). The intron sequence regulated the *fad2* gene expression. Sesame *fad2* intron was related to seed-specific expression and enhanced the transcription of the *fad2* gene. Compared with the intron-less controls, the intron in the 5'-UTR increased the expression of the reporter gene by 2.5- to 1000-fold (Kim *et al*., 2006). In developing soybean seeds, a sense suppression construct of the *fad2* intron sequence was used to achieve more efficient and specific reduction of *fad2* transcripts (Mroczka *et al*., 2010). Three histidine boxes were crucial for FAD2 desaturase activity. Displacement of even one of the histidines in these three H boxes could disrupt desaturase activity (Shanklin *et al*, 1994; Kurdrid *et al*., 2005).

B. napus (AACC genome, 2n=38) is an amphidiploids species, that originated from spontaneous hybridization of *B. rapa* (AA genome, 2n=20) and *B. oleracea* (CC genome, 2n=18) (Nagaharu, 1935). This suggested that *B. napus* had multiple *fad2* genes. Scheffler *et al*. (1997) reported that the *B.napus* genome encoded four *fad2* genes. Schierholt *et al*. (2000) estimated that *B. napus* may have either four or six *fad2* genes. By cloning and sequencing, Xiao *et al.* (2008) reported there were eleven *fad2* genes in B. napus Xiang You 15 genome, and six *fad2* genes had no biological function because of premature termination among them. Yang *et al*. (2012) reported that *B. napus* may had 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 helpful to improve oleic acid content and cultivated high oleic acid content *B. napus*.

In this study, we cloned *fad2* genes from three types of oleic acid content *B. napus*, and also analyzed the conserved domains of BnFAD2 Protein. Specific primers were designed to study expression patterns of different types of *fad2* genes in different tissues, and the results confirmed that not all expression patterns of *fad2* genes in three types of rape were accorded with the expression patterns of HO1 and HO2. By southern blot, the copy numbers of *fad2* genes were analyzed and the findings confirmed that both high oleic acid content and control *B. napus* had three *fad2* gene copies, but low oleic acid content *B. napus* had four *fad2* gene copies.

Materials and Methods

Materials and reagents: The three types of rape seeds, including induced, high oleic acid content of rape (*Brassica napus*, maintainer line 100B), the original control of rape (Xie, 2008) and low oleic acid content of rape (Zhong You 821)were grown in natural condition for 120 days to study the tissue-specific gene expression, which oleic acid content were 62.60%, 56.57%, 25.17%, respectively. The three types of rape in this study were represented by G type, CK type and D type, respectively. Tissues of seedlings roots, stems, leaves, 27 d silique after blossom and 35 d silique after blossom were quickly put into centrifuge tubes, frozen in liquid nitrogen and kept at -80℃ in refrigerator after sampling. Genomic DNA was extracted from leaf tissue using extraction method of Murray & Thompson (1980). Total RNA were extracted from the tissues using TRIZOL reagent (Invitrogen, USA) and reverse-transcribed with a SuperScript III RT reagent kit (Invitrogen, USA) according to the manufacturer's instructions.

***Bnfad2* clone:** Primers were designed based on the predicted *Bnfad2* gene (gene bank number AY57731, Peng, 2011), using DNAMAN 6.0. The primers sequences amplified fragments of *BnFad2* were 5'-ATGGGTGCA GGTGGAAGAATGCAAG-3'/5'-TCATAACTTATTGT TGTACCAGAACACACC-3'. PCR amplification was done using ES Taq DNA Polymerase (CWBIO, Beijing) with proofreading activity. The temperature cycles were: 4 min at 94℃, 40 s at 94℃, 40 s at 60℃, 100 s at 72℃ for 35 cycles; and 7 min at 72℃. PCR products were purified by agarose gel recovery kit (TIANGEN, Beijing). The three types of cDNA fragments were ligated to p-EASY-simple-T (Transgen, Beijing), respectively. After positive identification, 65 genomic *fad2* clones and 45 cDNA *fad2* clones in three types of rape were randomly selected, respectively. The fragments were sequenced by Invitrogen Co. Ltd (Guangzhou, China), and sequencing results were analyzed using DNAMAN 6.0 software.

Conserved domain prediction and phylogenetic analysis: Using NCBI CDS bank (Conserved Domain Search, http:// www. ncbi. nlm. nih. gov/Structure/cdd), the conserved domain of *Bnfad2* was predicted. Using DNAMAN 6.0, amino acid sequence comparison with that of other species and phylogenetic analysis were conducted.

Real-time RT-PCR: According to Xiao's description (Xiao *et al*., 2008), all the three type *Bnfad2* genes were divided into two kinds, which named *Bnfad2*-I and *Bnfad2*-II, respectively. For real-time RT-PCR, two pairs of primers (5'-TGAACAAGGTCTTCCACAATATCAC-3'/5'-TGCGGCATGGTCGAGAA-3';5'-CTACTGTGGA TAGAGACTATGGA-3'/5'- TGGTCGCTTCCATCGCG TTATAATG-3') and two probes (5'- GGACACGCACGT GGCGCATCACCT-3'/5'-CATAACATCACGGACACG CACGTGGCGC-3') were designed to amplify and detect fragment of the three types of *Bnfad2*-I and *Bnfad2*-II, respectively. One pair of primers (5'- CCTGGAATTGC TGACCGTATG-3'/5'- TGCGACCACCTTGATCTTCA-3')and one probe (5'-CAAAGAGATCACGGCGCT CGCAC-3') were designed to amplify and detect fragment of *β-actin*, which was used as endogenous control for template standardization. After optimization of the parameters used for exponential amplification, the temperature cycle was designed as 45 cycles for *Bnfad2*-I, *Bnfad2*-II and *β-actin* of three types of rape. The temperature protocol of gene and endogenous controlwere one cycle of 3 min at 94℃, 45 cycles of 5 s at 95℃, 15 s at 57℃, 30s at 72℃.

Gene copy-number analysis: Southern blotting and hybridization were used to estimate the number of gene copies for three types of rape (*B. napus*) varieties. Genomic DNA was extracted from 1.5g of leaf tissue using the urea extraction method of Murray & Thompson (1980). DNA was digested using EcoRI or HindIII, electrophoresed on a 0.7% gel for overnight at 25V, respectively. The gel was sequentially soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, twice in neutralization solution [0.5 M Tris-HCl (PH 7.5), 1.5 M NaCl] for 15 min, and then in neutral-transformation buffer (2×SSC) for 5 min before blotted onto Hybond N^+ (GE Healthcare, USA). The filters were then probed with a selected segment from single cDNA of *Bnfad2* which was labeled with [Dig-dUTP] using random-primed oligonucleotide labeling. Hybridizations were carried out overnight at 37℃ followed by two low-stringency washes (2×SSC, 0.1% SDS) and two 15-min high-stringency washes (1×SSC, 0.1% SDS) at 65℃. Because of difficulty in resolving some of the higher-weight-molecular bands and the chance of partial digestion producing extraneous bands, all of the filters were prepared and examined independently at least twice.

The length of single cDNA was 746 bp and used as the labeled probe. The probe was generated by PCR amplification of the cDNA clone using oligonucleotide primers to specifically amplify the desired segment. The PCR products were purified using the agarose gel recovery kit (TIANGEN, Beijing). The probe was amplified using forward primer 5'-CCTTTCTTCTC ACCTTGCCTGTC-3' and reverse primer 5'-TCCTTCC TCCTCGTCCCT-3'. In order to ensure the confidence of the experiment result, plasmid of cDNA clone digested by single restriction enzyme was used as positive control.

Results

Analysis on sequences in *Bnfad2* genes: The nucleotides sequences of *Bnfad2* geneswere obtained from genomic DNA and cDNA showed that *Bnfad2* genes had no intron, which conform to the previous study (Xiong, 2002; Xiao, *et al*., 2008). And there were little differences by using different templates. *Bnfad2* genes in three types of rape were cloned by RT-PCR, which could be found obvious two kinds of difference in the gene sequence of three types of rape. Above all, ten nucleotides sequences differenced from 41 to 50 in G type and in CK type and 8 nucleotides sequences differenced from 41 to 48 in D type. In G type and in CK type, some clones were AAAAGTCTGA and other clones were GCTCCCCCGG. In D type, some clones were AAAAGTCT and other clones were GCTCCCCC. The other was fifteen nucleotides sequences differences. In G type and in CK type, the clones that owned GCTCCCCCGG from 41 to 50 defected TCCCTCACCCTCTCT from 231 to 245 and other clones that owned AAAAGTCTGA from 41 to 50 retained these nucleotides sequences. However, in D type, there was just only one clone that owned GCTCCCCC from 41 to 48 defected TCCCTCACCCTCTCT. But other two clones that owned GCTCCCCC from 41 to 48 retained these nucleotides sequences. Furthermore, a complete ORF of 1155 nucleotides were obtained from the clones that retained fifteen nucleotides and these nucleotides encoded 384 amino acid residues, respectively. The nucleotides were obtained from the clones that defected fifteen nucleotides appeared premature termination on different degree in protein level (Figs. 1-3).

Protein structure characteristic of BnFAD2: By DNAMAN 6.0, there are six transmembrane domains and three H boxes in three types of rape. In the C-termini, these proteins had the aromatic-amino-acid-enriched signal peptide YNNKL. But these domains and motifs were distributed differently in three types of rape. In G type, all clones belonged to *Bnfad2*-I have these domains and motifs. In D type, both all clones belonged to *Bnfad2*-I and two clones belonged to *Bnfad2*-II have these domains and motifs. In CK type, two clones belonged to *Bnfad2*-I have not these obvious domains and motifs (Figs. 4-6). By using NCBI's Conserved Domain Database tools, the conserved domains of BnFAD2-I and BnFAD2-II were analyzed for in-depth exploration of its function and distinguish difference between them. The result of our present study showed that both *Bnfad2*-I genes of three types of rape and *Bnfad2*-IIgenes of three types of rapes had the one coding conserved domain, namely membrane-FADS-like super-family. Several motifs were found in BnFAD2-I protein including three putative di-iron ligands. However, these motifs were not found in BnFAD2-II protein (Fig. 7). The phylogenetic analysis showed that the FAD2 proteins in these species were divided into two categories. *Brassica napus* FAD2-II and *Brassica rapa* FAD2-2 were attributed to one class, and other species were attributed to the other class, which named II and I, respectively. In I class, the FAD2 protein in these species were four sub-varieties. *Brassica rapa* FAD2-1, *Brassica rapa subsp. Pekinensis* FAD2 and *Brassica campestris* FAD2 had a completely consistency, which had a 99% consistency among *Brassica napus* FAD2-I, *Brassica oleracea* FAD2-1and *Brassica juncea* FAD2.The consistency between *Brassica carinata* FAD2 and them was 96%. The seven FAD2 protein in different species mentioned above were attributed to the first sub-variety. *Arabidopsis lyrata* FAD2 and *Arabidopsis lyrata subsp. lyrata* FAD2 had a completely consistency. The consistency between *Arabidopsis thaliana* FAD2 was 99%. The consistency between *Camelina sativa* FAD2 and *Capsella rubella* FAD2 was 98%, which had a 97% consistency when compared to the three *Arabidopsis thaliana* species FAD2 mentioned above. The five species and *Lepidium campestris* constituted the second sub-variety and the consistency among them was 94%. The first and the second sub-variety, which had a 90% consistency, constituted the third sub-variety together. The fourth sub-variety was consisted of single one species, which named *Brassica oleracea* FAD2-2. And the consistency among the three sub-varieties and the fourth sub-variety was 88%. In II, *Brassica napus* FAD2-II and *Brassica rapa* FAD2-2 had a completely consistency. The consistency between I and II was only 65% and was the lowest (Fig. 8).

Expression of *Bnfad2* in three types of rape in different tissues: The expression of *Bnfad2*-Iand *Bnfad2*-II in different tissues of three types of rape grown 120 days in natural outside room condition were examined independently in order to study the different type of gene's role in plants' development. The result indicated that in G type, the highest level of *Bnfad2*-I was in 35d silique after blossom. Expression in stem was respectively 70% of the highest level in 35d silique after blossom tissue. Expressions in leaves and in 27d silique after blossom were 40% and 50% when compared to that of the expression in 35 d silique after blossom. Expression of *Bnfad2*-I was the lowest in root. In D type, the highest level of *Bnfad2*-I was also in 35d silique after blossom. Expression in 27d silique after blossom was respectively 60% of the highest level in 35d silique after blossom tissue. Expressions in leaves and in stem were 50% and 45% when compared to that of the expression in 35 d silique after blossom. In D type, expression of *Bnfad2*-I was also the lowestin root. In CK type, the highest level of *Bnfad2*-I was in stem. Expression in 27d slilique after blossom was respectively 95% of the highest level in stem. Expressions in 35d silique after blossom and in root were 90% and 75%, respectively. In CK type, expression of *Bnfad2*-I was the lowest in leaves (Fig. 9a).

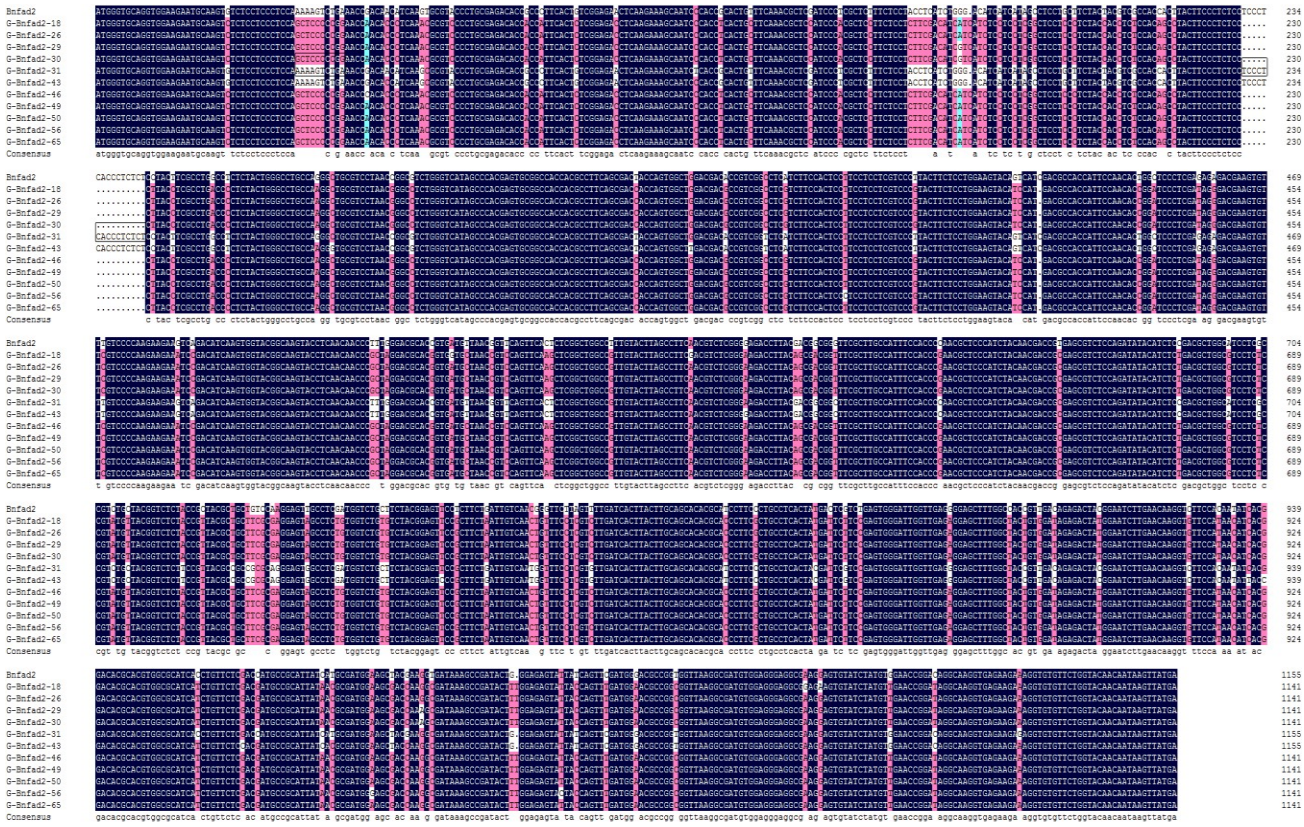

Fig. 1. Alignment of *Bnfad2* in G type of rape with *Bnfad2* gene in Gene bank.
The two major differences between *Bnfad2-I* and *Bnfad2-II* are framed. *Bnfad2*: CDS sequence in Gene bank; G-*Bnfad2* represents gene CDS sequence comes from induced, high oleic acid content of rape and latter numbers represent different clones.

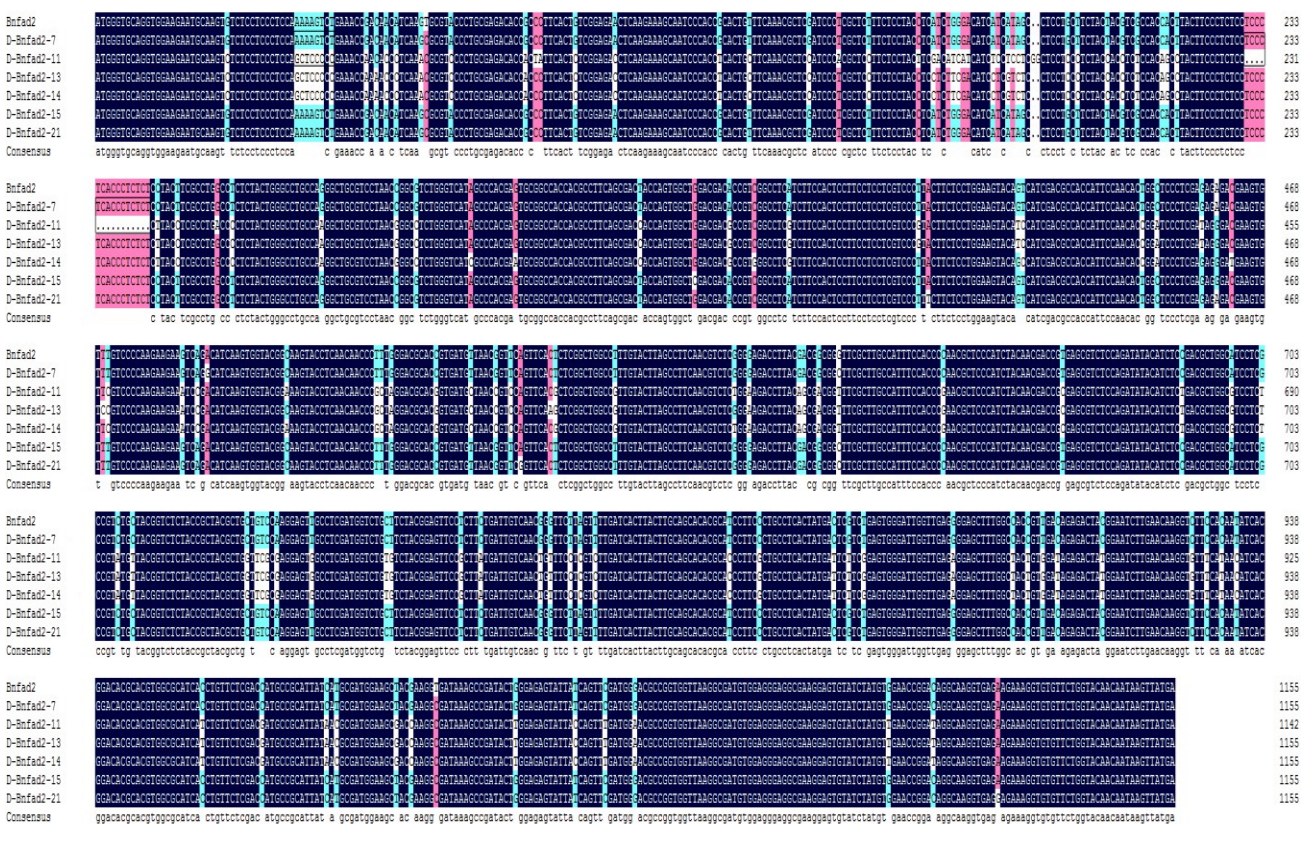

Fig. 2. Alignment of *Bnfad2* in D type of rape with *Bnfad2* gene in Gene bank.
The two major differences between *Bnfad2-I* and *Bnfad2-II* are framed. *Bnfad2*: CDS sequence in Gene bank; D-*Bnfad2* represents gene CDS sequence comes from low oleic acid content of rape and latter numbers represent different clones.

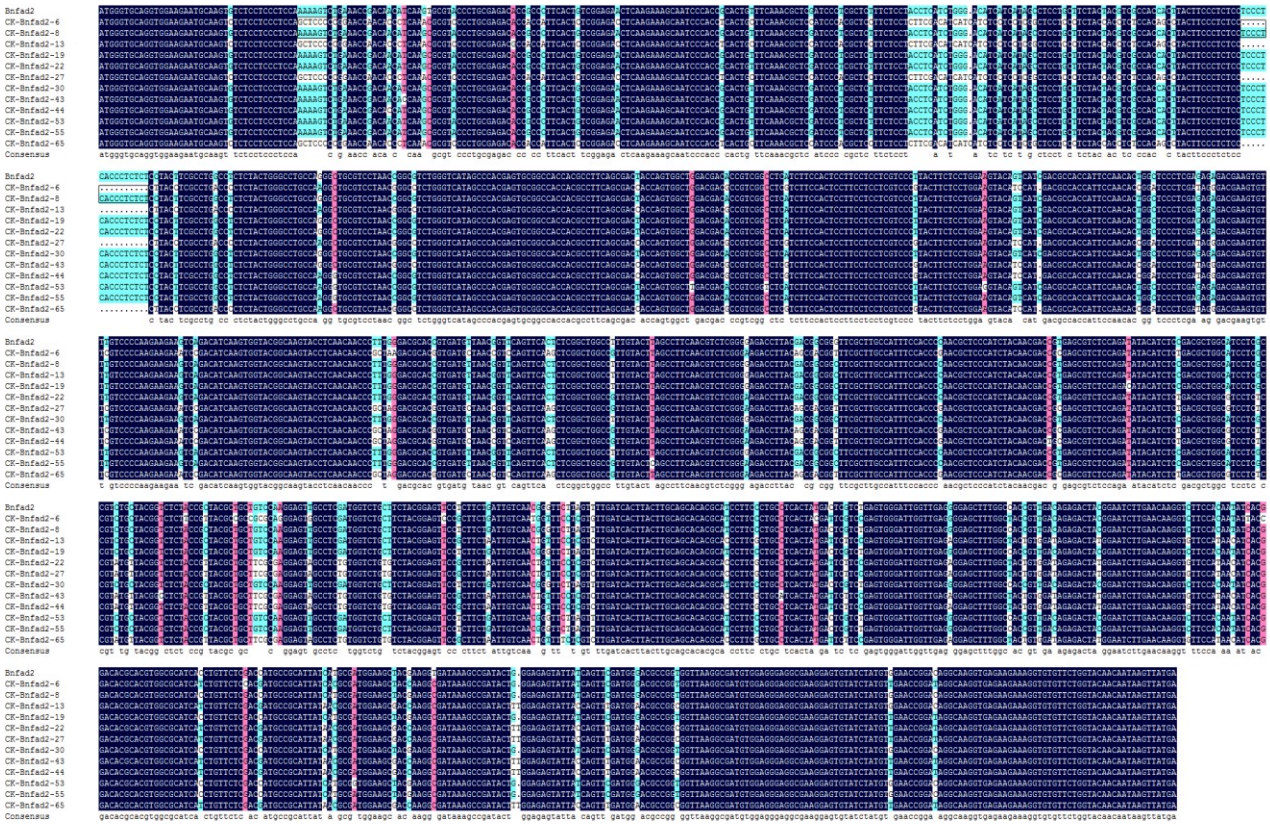

Fig. 3. Alignment of *Bnfad2* in CK type of rape with *Bnfad2* gene in Gene bank.
The two major differences between *Bnfad2-I* and *Bnfad2-II* are framed. *Bnfad2*: CDS sequence in Gene bank; CK-*Bnfad2* represents gene CDS sequence comes from the original control of rape and latter numbers represent different clones.

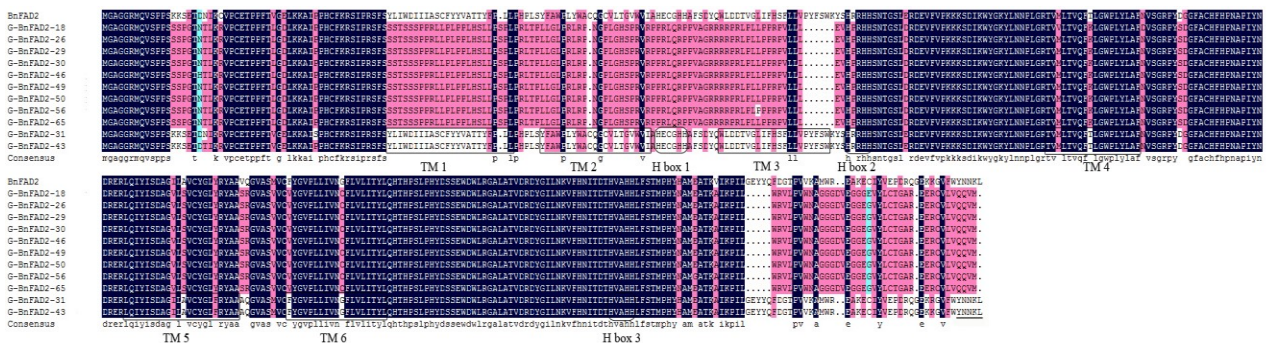

Fig. 4. Alignment of deduced amino acids of BnFAD2 in G type of rape and BnFAD2 in Gene bank.
Black background represents the identical amino acid residues. Rectangular and rhombus boxes indicate transmembrane domains (TM) and H boxes, respectively. The single line at the C-terminal end of the alignment denotes the ER retrieval motif.

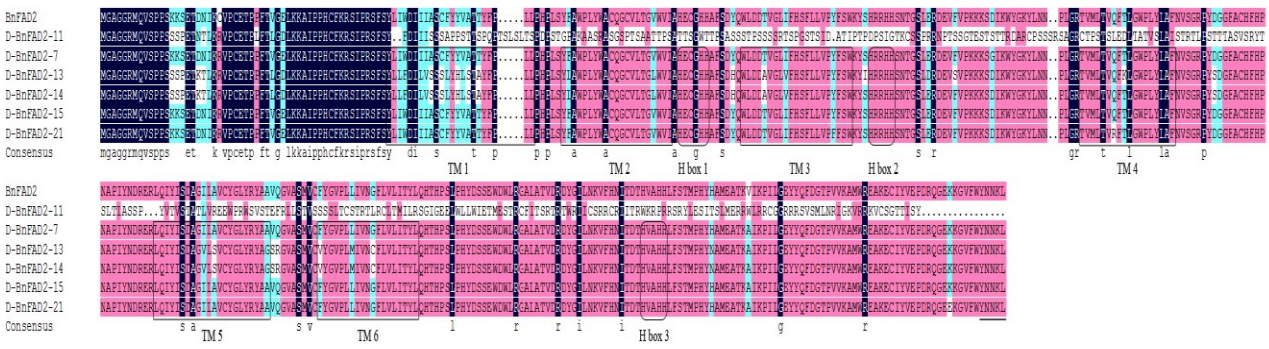

Fig. 5. Alignment of deduced amino acids of BnFAD2 in D type of rape and BnFAD2 in Gene bank.
Black background represents the identical amino acid residues. Rectangular and rhombus boxes indicate transmembrane domains (TM) and H boxes, respectively. The single line at the C-terminal end of the alignment denotes the ER retrieval motif.

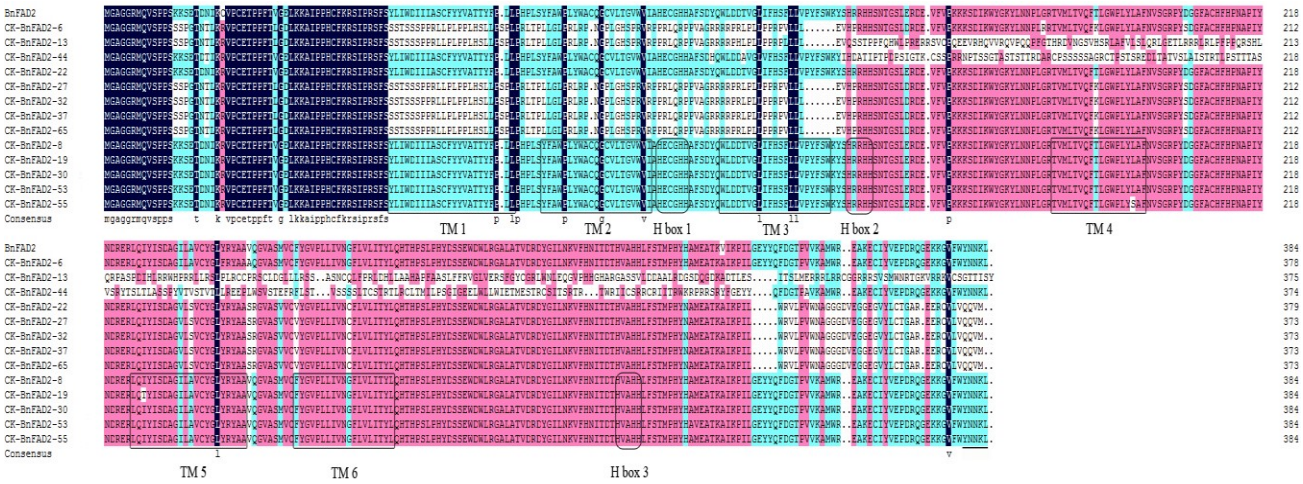

Fig. 6. Alignment of deduced amino acids of BnFAD2 in CK type of rape and BnFAD2 in Gene bank.
Black background represents the identical amino acid residues. Rectangular and rhombus boxes indicate transmembrane domains (TM) and H boxes, respectively. The single line at the C-terminal end of the alignment denotes the ER retrieval motif.

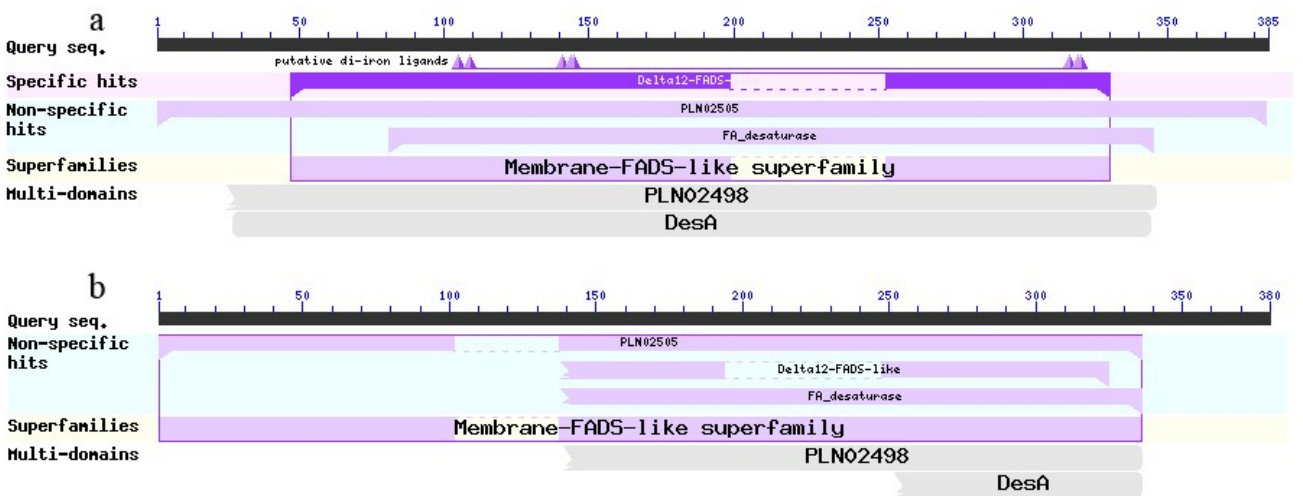

Fig. 7. The prediction conserved domain of BnFAD2-I and BnFAD2-II.
a. The prediction conserved domain of BnFAD2-I b. The prediction conserved domain of BnFAD2-II

In G type, the highest level of *Bnfad2*-II was in stem. Expressions in 27d silique after blossom and in 35d silique after blossom were closely equal, which respectively 95% of the highest level in stem. Expression in leaves was 90% when compared to that of the expression in stem. Expression was the lowest in root. In D type, the highest level of *Bnfad2*-II was in 27d silique after blossom. Expression in leaves was respectively 90% of the highest level in 27d silique after blossom. Expressions in 35d silique after blossom and in stem were 80% and 45% when compared to that of the expression in 27d silique after blossom. In D type, expression was also the lowest in root. In CK type, the highest level of *Bnfad2*-II was also in 27d silique after blossom. Expression in 35d silique after blossom was respectively 65% of the highest level in 27d silique after blossom. Expressions in leaves and in stem were 55% and 50% when compared to that of the expression in 27d silique after blossom. In CK type, expression of *Bnfad2*-II was also the lowest in root (Fig. 9b).

Expression of *BnalCR78* in three types of rape in the same tissues: The expressions of *Bnfad2*-I and *Bnfad2*-IIin different tissues of three types of rape were independently conducted. And results showed that expression levels in three types of rape in 35 d silique after blossom were closely equal. In order to distinguish the real difference among three types of rape, the expression of *Bnfad2*-I and *Bnfad2*-II in three types of rape in the same tissues were examined, respectively. The results showed that expression of *Bnfad2*-I in all detected tissues was not conformed to the results of and expressions of *Bnfad2*-I except that in root. In G type, the highest level of *Bnfad2*-I was in stem, and in CK type, expression level was closely equal to that in G type. Expression level in D type was 75% of that in G type. Expression of *Bnfad2*-IIin all detected tissues was not conformed to the results of and expressions of *Bnfad2*-II except that in root and stem. In G type, the highest level of *Bnfad2*-IIwas in root, and expression level in D type was closely equal to that in G type. Expression level in CK type was 65% of that in G type. The highest level of *Bnfad2*-IIin G type was in stem, and expression levels in CK type and D type 75% and 65% of that in G type (Fig. 10).

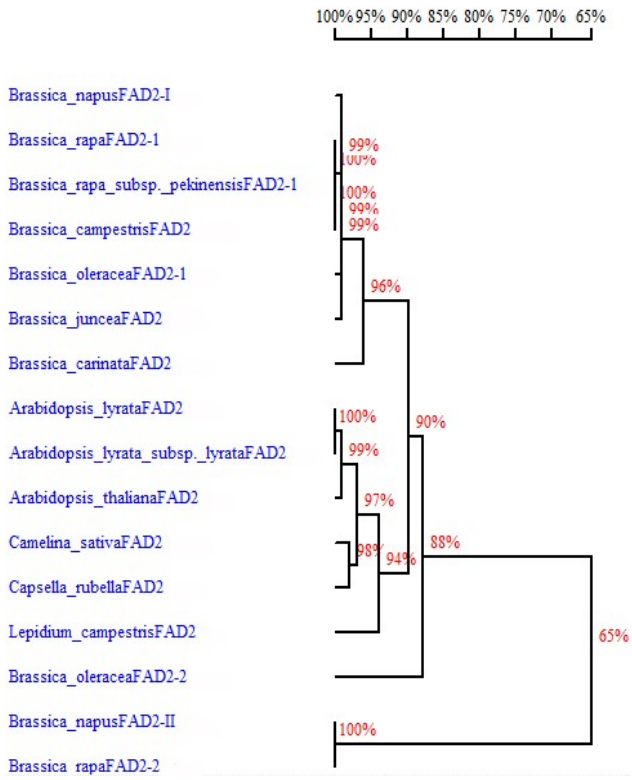

Fig. 8. Phylogenetic tree showing comparisions between predicted amino acid sequences from FAD2 protein in some species.

Note: *Brassica rapa* FAD2-1 (AFC41105); *Brassica rapasubsp. pekinensis* FAD2 (AEA76336); *Brassica campestris* FAD2(CAD30827); *Brassica oleracea* FAD2-1 (AFC41106); *Brassica juncea* FAD2 (ABR27357); *Brassica carinata* FAD2(AAD19742); *Arabidopsis lyrata* FAD2 (ADN10828); *Arabidopsis lyrata subsp. lyrata* FAD2 (XP_002884883); *Arabidopsis thaliana* FAD2 (AAA32782); *Camelina sativa* FAD2 (NP_001291954); *Capsella rubella* FAD2(ADN10827); *Lepidium campestre* FAD2 (ACR15954); *Brassica oleracea* FAD2-2 (AFC41107).

Copy-number estimates for desaturase genes in *B. napus*: To understand the copy number of Bnfad2 impacted on oleic acid content, southern blot was done. As *Bnfad2*-I could translate from ATG to TGA and *Bnfad2*-II appeared premature termination in different degree, the research object of southern blotfocused on *Bnfad2*-I. EcoRI and HindIII that do not cut within coding region of genes of three types of rape were used to digest genomic DNA. Using a single *Bnfad2*-I plasmid sequence as a probe, genomic Southern blot of *B.napus* revealed at least three hybridizing bands, three hybridizing bands and four hybridizing bands in G type, in CK type and in D type, respectively (Fig. 11).

Discussion

In this study, cDNA sequences of *Bnfad2* in three types of rape were analyzed by PCR, and the results showed that there were some obvious differences among them. One was ten nucleotides sequences differences from 41 to 50. Some clones were aaaagtctga and other clones were gctcccccgg. The former nucleotides sequences belonged to *Bnfad2*-I and the latter belonged to *Bnfad2*-II (Xiao *et al*., 2008; Schierholt *et al*., 2001). Furthermore, the clones belonged to *Bnfad2*-I owned tccctcaccctctct from 231 to 245 and could be translated from ATG to TGA. Those clones belonged to *Bnfad2*-II defected these nucleotides sequences could not be completely translated. Defection these nucleotides sequences resulted in premature termination. In this study, the result of analysis on nucleotides sequences of *Bnfad2* in three types of rape showed that in detected clones, the numbers of clones retained nucleotides sequences tccctcaccctctct in G, D and CK types of rape were two, five and eight, respectively. In D type of rape, rather than defected tccctcaccctctct from 231 to 245, the clones that owned gctccccc from 41 to 48 retained these nucleotides sequences, which resulted in increasing the number of *Bnfad2* completely translated and may reduce oleic acid content in D type of rape. The oleic acid content of double low rapeseed "Xiang you 15" was above 90% whose seeds were radiated by ray ^{60}Coγ. By analysis on nucleotides sequenceof *fad2* gene in the rape, termination codon appeared because the base in 270 was mutated (Guan *et al*., 2006). In three types of rape, *Bnfad2* was found diversity in numbers of clones that retained specific nucleotides sequences suggested that defection specific nucleotides sequences in *Bnfad2* might be one of the reasons that resulted in increasing in oleic acid content.

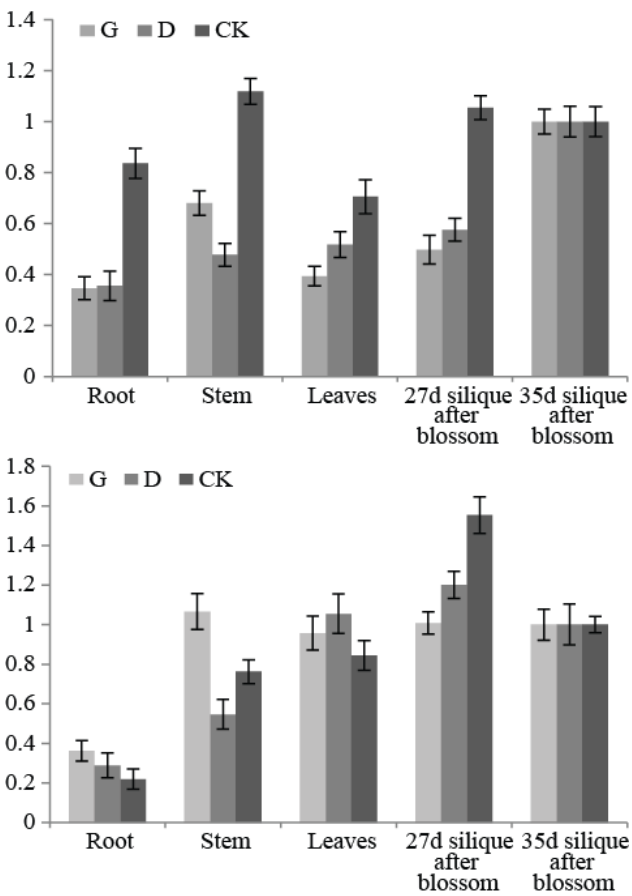

Fig. 9. Real-time RT-PCR profile of *Bnfad2-I* and *Bnfad2-II* in various tissues of three types of rapes (*β-actin* as a quantity control)
a. Real-time RT-PCR profile of *Bnfad2-I* in various tissues of three types of rapes
b. Real-time RT-PCR profile of *Bnfad2-II* in various tissues of three types of rapes
G、D、CK represent induced, high oleic acid content of rape, low oleic acid content of rape and the original control, respectively.

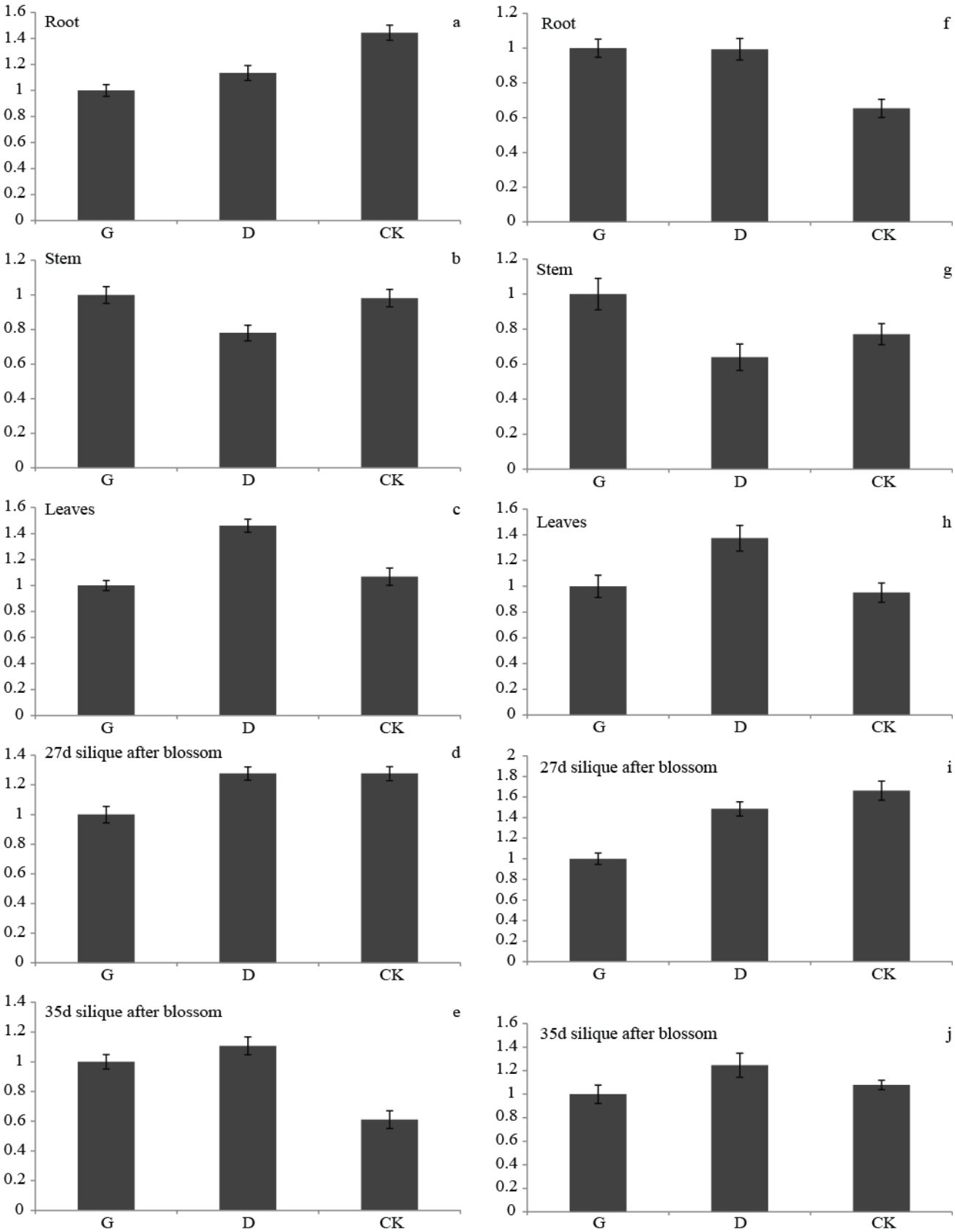

Fig. 10. Real-time RT-PCR profile of *Bnfad2-I* and *Bnfad2-II* in the same tissues of three types of rapes (*β-actin* as a quantity control)
a-e. Real-time RT-PCR profile of *Bnfad2-I* in root, stem, leaves, 27d silique after blossom and 35d silique after blossom of three types of rapes, respectively.
f-j. Real-time RT-PCR profile of *Bnfad2-II* in root, stem, leaves, 27d silique after blossom and 35d silique after blossom of three types of rapes, respectively
G、D、CK represent induced, high oleic acid content of rape, low oleic acid content of rape and the original control, respectively

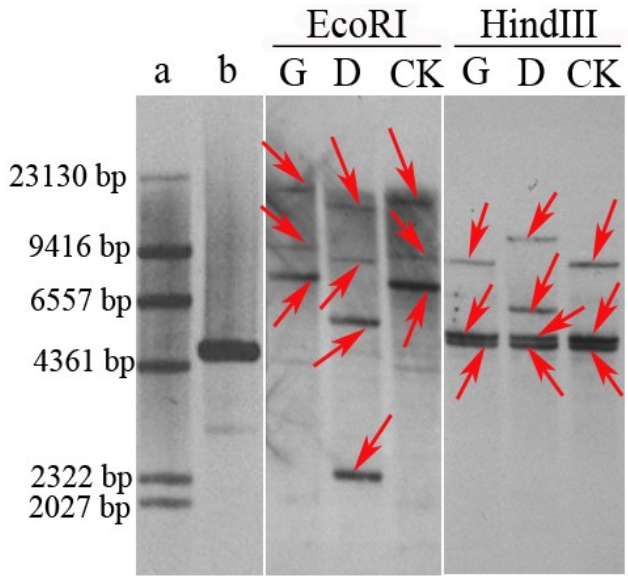

Fig. 11. Genomic Southern blot analysis of three types of rape
a. DNA molecular-weight marker b. Plasmid of cDNA clone digested by single restriction enzyme as control
G、D、CK represent induced, high oleic acid content of rape, low oleic acid content of rape and the original control, respectively;
Red arrows represent hybridizing bands in each type of rape with the same single restriction enzyme digestion

In AtFAD2, there could be **found** six transmembrane domains and three H boxes (Lee *et al*., 2013). By analysis with DNAMAN 6.0, some BnFAD2 in three types of rape have these same conserved domains. The H box motif is found in all acyl-lipid desaturases, with the conserved motifs $HX_{(3-4)}$ HH, $HX_{(2-3)}$HH, and $HX_{(2-3)}$ HH, found sequentially from the N-terminus to the C-terminus (Okuley *et al*., 1994; Los & Murata, 1998). In our present study, the sequences of the H boxes of BnFAD2 isozymes are HECGHH (closest to the C-terminus), HRRHH, and HVAHH (closest to the C-terminus), which conformed to previous study result. *Arabidopsis* FAD2 localizes to the ER membranes by virtue of the aromatic-amino-acid-enriched signal peptide YNNKL at its C-terminus (Okuley *et al*., 1994). The ER retrieval motif of ER membrane-bound fatty acid desaturase in plant is ΦXX(K/R/D/E)Φ at their C-termini, where Φ is the hydrophobic amino acid such as F, Y, W, I, L and V (Mccartney *et al*., 2004). The ER retrieval motifs of those clones coding protein in three types of rape are the same as that of *Arabidopsis*, respectively (Figs. 4-6). By using bioinformatics tool, conserved domains of BnFAD2-I and BnFAD2-IIwere analyzed, respectively, and results showed that BnFAD2-I had three conserved domains. The first was completely DesA domain, which was found on fatty acid desaturase and responsible for lipid transportation and metabolism. The second one was incompletely PLN02498domain, which was found on omega-3 fatty acid desaturase. The last one was incompletely delta 12-FADS-like domain. Furthermore, BnFAD2-I had three putative di-iron ligands. BnFAD2-II also had the two incompletely DesA domain and PLN02498domain. Both BnFAD2-I and BnFAD2-II belonged to membrane- FADS-like super family. There was not found delta 12-FADS-like domain in BnFAD2-II because of deficiency amino acids from 1 to 140, which suggested that these amino acids were important to form delta 12-FAD2S-like domain.

Koch studied the ITS sequences of thirty groups, which results showed that the relationship between *Capsella* in *Lepidieae* and *Arabidopsis* more close than other species (Koch *et al*., 2001). Yang analyzed nine genera of four families in *Cruciferae* by ITS, which results showed that the relationship among *Arabidopsis*, *Capsella* and *Lepidieae* more close than other species (Yang *et al*., 1998). Furthermore, *Arabidopsis*, *Lepidium* of *Lepidiese* and *Capsella* belonged to the same group (Yang *et al*., 1999). Our study also supported these viewpoints mentioned above, which showed that *Arabidopsis*, *near edge genus of Arabidopsis*, *Camelina*, *Capsella*, *Lepidium* belonged to one group, then *Brassica napus* FAD2-I, *Brassica campestris*, *Brassica juncea* and *Brassica carineta* belonged to another group, which belonged to the same category. Interestingly, *Brassica napus* FAD2-II and *Brassica rapa* FAD2-2 belong to the other category. As *Brassica napus* is produced by natural inter-specific hybridization from *rapa* and *oleracea*, high homologies between *Brassica napus* FAD2-II and *Brassica rapa* FAD2-2 suggests that relationship between the two species more closer than other species.

To study the expression patterns of *Bnfad2*-I and *Bnfad2*-II, *fad2*-specific RT-PCR was performed on RNA samples prepared from different tissues. In *Brassica napus*, the oleic acid content was affected by two genetic loci. One genetic loci named HO1, which mainly expressed in seed. The other genetic loci named HO2, which expressed not only in seed, but also expressed in leaves and root (Schierholt *et al*., 2001). Our study results showed that *Bnfad2*-I of G type and CK type were not only expressed in seed, especially in mature seed, but also expressed in other tissues such as in root, in stem and in leaves, which not conformed to expression pattern of HO1. However, *Bnfad2*-I of D type mainly expressed in seed, especially in mature seed, which conformed to expression pattern of HO1. *Bnfad2*-II of G type, D type and CK type were expressed not only in seed, but also expressed in other tissues such as in stem and in leaves, which incompletely conformed to expression pattern of HO2. The results of *Bnfad2*-I and *Bnfad2*-II expression patterns suggested that expression patterns of HO1 and HO2 might not be applied to study all oleic acid content of rape and impaction factors of oleic acid content of *B. napus* seed may need to be further studied.

By quantitative RT-PCR method, expression of *Bnfad2*-I and *Bnfad2*-II genes in three types of rape were analyzed, and results indicated expression of gene almost the lowest in root and nearly equal in 35 d silique after blossom. For better distinguish expression of the genes, further analysis of the genes in specific same tissue by quantitative RT-PCR method were conducted, and the results showed that the expression patterns of *Bnfad2*-I gene and *Bnfad2*-II gene in specific same tissues were different in three types of rape. The trend on independent expressions of *Bnfad2*-I gene in root only conformed to the expression of gene in three types of rape. In D type, the expression level of gene was obviously lower in stem tissue than that in G type and in CK type, which had no obvious difference in the latter two. In D type, the expression level of gene was obviously higher in leaves tissue than that in G type and in CK type. In G type, the expression level of gene was obviously lower in 27d

silique after blossom than that in D type and in CK type, which had no obviously difference in the latter two. In D type, the expression level of gene was obviously higher in 35d silique after blossom than that in G type and in CK type. However, the trend on independent expressions of *Bnfad2*-II gene in other tissues conformed to the expression of gene in three types of rape, expect in 35d silique after blossom. In D type, the expression level of gene was also obviously higher in 35d silique after blossom than that in G type and in CK type. Previously research showed that *fad2* gene was the key gene controlled the relative content of oleic acid in plant seed (Okuley *et al*., 1994; Ohlrogge & Browse, 1995; Ohlrogge & Jaworski, 1997; Hu *et al*., 2006). In this study, both results of quantitative RT-PCR of *Bnfad2*-Iand *Bnfad2*-II in same specific tissue showed that the expression of genes in G type was obviously lower than that in D type both in 27d silique after blossom and in 35d silique after blossom. The results mentioned above suggested that the expressions of *Bnfad2*-I and *Bnfad2*-II in silique were one of the mainly reasons resulted in differences in oleic acid content.

Because coded protein of *Bnfad2*-II had unfunctioned because of premature termination, southern blot was used to study the copy number of *Bnfad2*-I in three types of rape. Previous study showed that there were eight to twelve *fad2* gene copies of genomic DNA in *Brassica napus* (Scheffler *et al*., 1997). By using genomic DNA and cDNA as templates respectively, Xiao *et al*., 2008, studied copy number of *Bnfad2* in xiangyou 15, and result showed that there was 11 gene copies in xiangyou 15. In this study, by southern blot method, specific probe was used to hybrid with genomic DNA of three types of rape, respectively. The result showed that copy number of *Bnfad2*-I was four in D type. Both the copy numbers of same gene were three in G type and CK type, respectively. The copy number of gene in this study was less than the results of previous study may because of undistinguished independently copy numbers of *Bnfad2*-I and *Bnfad2*-II. The difference in copy number of gene may be another reason that resulted in difference in oleic acid content. Oleic acid trait of *B. napus* is controlled by polygene (Xiao *et al*., 2008), such as *fad2* and *fae1*. To cultivated high oleic acid content *B. napus*, the regulation mechanism to understand *fad2* gene need to be further studied.

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