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ARTICLE

Landscape of Sequence Variations in Homologous Copies of *FAD2* and *FAD3* in Rapeseed (*Brassica napus* L.) Germplasm with High/Low Linolenic Acid Trait

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ABSTRACT

Genetic manipulation (either restraint or enhancement) of the biosynthesis pathway of α-linolenic acid (ALA) in seed oil is an important goal in *Brassica napus* breeding. *B. napus* is a tetraploid plant whose genome often harbors four and six homologous copies, respectively, of the two fatty acid desaturases *FAD2* and *FAD3*, which control the last two steps of ALA biosynthesis during seed oil accumulation. In this study, we compared their promoters, coding sequences, and expression levels in three high-ALA inbred lines 2006L, R8Q10, and YH25005, a low-ALA line A28, a low-ALA/high-oleic-acid accession SW, and the wildtype ZS11. The expression levels of most *FAD2* and *FAD3* homologs in the three high-ALA accessions were higher than those in ZS11 and much higher than those in A28 and SW. The three high-ALA accessions shared similar sequences with the promoters and CDSs of *BnFAD3.C4* and *BnFAD3.A3*. In A28 and SW, substitution of three amino acid residues in BnFAD2.A5 and BnFAD2.C5, an absence of *BnFAD2.C1* locus, and a 549 bp long deletion on the *BnFAD3.A3* promoter were detected. The profile of *BnFAD2* mutation in the two low-ALA accessions A28 and SW is different from that reported in previous studies. The mutations in *BnFAD3* in the high-ALA accessions are reported for the first time. In identifying the sites of these mutations, we provide detailed information to aid the design of molecular markers for accelerated breeding schemes.

KEYWORDS

Brassica napus; linolenic acid; FAD2; FAD3; promoter; coding sequences; mutation

1 Introduction

Oil rapeseed (*Brassica napus*. L. AACC, 2n = 38) is the second leading edible oil crop in the world. The nutritional value of rapeseed to human beings has been greatly improved since the creation of canola oil, which contains a low level of erucic acid and has the great advantage of very high content of three major unsaturated fatty acids with reasonable ratio, i.e., approximately 65% oleic acid (OA, C18:1, ω-9), 20% linoleic acid (LA, C18:2, ω-6), and 9% α-linolenic acid (ALA, C18:3, ω-3) [1]. The production of rapeseed which is either high or low in ALA is now an important priority for breeders, based on the two

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major requirements of oxidative stability and high ω-3 nutrient content [1,2]. Because of the oxidative instability of ALA during long-term storage and high-temperature cooking, breeders have developed dozens of high-OA/low-ALA (HOLL) varieties [3–8]. However, defects such as poor resistance, late ripening, and low seed vigor have been reported in some low-ALA germplasms [9]. To better manipulate OA and ALA biosynthesis, therefore, it is necessary to find new genetic loci with fewer adverse effects on the biological and agronomic traits [10]. All previous studies on the genetic control of rapeseed ALA content have been based on plant accessions with low and medium levels of ALA content, but not high-ALA genotypes [4,5]. Nowadays, high-ALA trat is also desired by consumers because ALA is an essential nutrient that cannot be synthesized by the human body and must be obtained from foods [11]. ALA has been recommended by the Food and Agriculture Organization as a vegetable ω-3 fatty acid for dietary supplementation, to increase the ratio of ALA: LA (ω-3:ω-6) in food [11]. However, the availability of the main vegetable ω-3 sources, i.e., oilseed flax and Perilla, is restrained by stringent cultivation conditions. In light of this, an economical and effective alternative method may be to supplement ALA directly into people's diets through high-ALA canola oil [1–2]. It is possible to obtain high-ALA *B. napus* germplasm because high-ALA germplasm exists in some other *Brassica* species [12]. In recent years, several new germplasms of *B. napus* with ALA content levels of 15% to 21%, for example, two yellow-seeded *B. napus* inbred lines YH25005 and R8Q10, have been created through various breeding methods [1,2,13,14].

For better-quality canola breeding, therefore, the genetic mechanisms underpinning the formation of either high- or low-ALA resources in *B. napus* must be comprehensively understood, and more knowledge is required about the genetic network that regulates ALA synthesis. ALA biosynthesis in Arabidopsis seed is carried out by two types of fatty acid desaturase (FAD). The ω-6 type FAD2 and FAD6 catalyze the first desaturation step of OA to LA; the ω-3 type FAD3, FAD7, and FAD8 catalyze the further desaturation of LA to ALA. The FAD2 and FAD3 proteins work in the endoplasmic reticulum and determine the content of the three major unsaturated fatty acids in seed oil; their isoenzymes FAD6, FAD7, and FAD8 are located on the chloroplast membrane and exert less influence on seed oil deposition. Dysfunction of FAD2 and FAD3 is the main method used to increase OA content or reduce ALA content [3–5]; hence, previous studies have mainly focused on the relationship between the traits of low ALA or high OA and coding sequence (CDS) variations in *FAD2* or *FAD3* [3–8]. However, the promoter information of *BnFAD2*/*BnFAD3* and the regulation of their transcription levels have been less studied. Higher ALA content and lower OA content can be achieved both by overexpression and by increasing the copy numbers of Arabidopsis *FAD3* [15]. The upregulation of *FAD3* expression in flax seeds has been associated with high ALA levels [16]. In one recent study, researchers found that more ALA was accumulated in a transgenic *B. napus* plant with overexpressed *BnFAD*3; however, the seeds also shriveled, with decreased oil content and lower levels of seed germination [17].

Although *FAD2* and *FAD3* are the key factors in controlling OA reduction and ALA biosynthesis, the inheritance of OA and ALA content in the allotetraploid species rapeseed is more complex than the model plant Arabidopsis because most *B. napus* germplasm maintain four or six homologous copies of *FAD2* and *FAD3* [5] from its progenitors. The duplicated genes, such as *BnFAD2.A5* and *BnFAD2.C5*, often have redundant functions and show additive effects on the accumulation of OA and ALA [18]. Therefore, OA or ALA content in rapeseed is controlled by multiple copies of *BnFAD2* and *BnFAD3* and function variation in different copies may affect the phenotype of OA or ALA content. For instance, in the progenies of the two crosses 'YH25005 × ZH9' and 'R8Q10 × D636' (lines ZH9 and D636 are wildtypes containing about 9% ALA), the inheritances of OA and ALA content are controlled by a lot of minor-effect genes, following a genetic model of 'additive-dominant-epistatic polygene' [14]. However, the results of our subsequent study [1] showed that, if ZH9 and D636 were replaced by a HOLL cultivar SW, the inheritances of OA and ALA changed to another genetic model of 'two pairs of additive major genes

+ additive-dominant polygenes'. It indicates that the HOLL parent SW might contain two loss-of-function mutations in *BnFAD2* and/or *BnFAD3* and the mutations had a strong effect of 'two pairs of additive major genes' to reduce ALA and increase OA content [1].

In the present study, we focused on the mutation and expression regulation of *BnFAD2* and *BnFAD3*, which are the most critical factors in controlling ALA content. The expression levels of multiple copies of these genes were detected by real-time quantitative PCR (qPCR). We cloned the coding sequences (CDSs) and promoters of all copies of *BnFAD2* and *BnFAD3* in six *B. napus* accessions with high-, medium- or low-ALA traits to find important mutations including SNPs (single-nucleotide polymorphisms) and indels (insertions/deletions) in the CDS region, affecting protein functions, and also in promoter cis-regulatory elements (CREs), affecting changes in expression levels.

2 Materials and Methods

2.1 Materials

Six plant accessions (Table 1) were included in the study: three high-ALA inbred lines (R8Q10, 2006L, and YH25005); an elite cultivar ZS11 whose genome had been sequenced (representing a wildtype genotype); a low-ALA line A28; and a special-line SW with high-OA and low-ALA traits. The plants were grown in the autumn of the year 2021 in the experimental field of Northwest A&F University. In the next spring, several batches of flowers opening on the same day were selected in the six accessions and bagged to obtain their self-pollinated seeds. The content of fatty acid components in mature seeds of the six plant accessions was determined using a GC-2010 Plus gas chromatographer (Shimadzu, Kyoto, Japan) as it had been described [1].

Table 1: Fatty acid content in the seeds of the six rapeseed genotypes

Accession	OA(C18:1)	LA(C18:2)	ALA(C18:3)	C16:0	C18:0	C20:0	C22:1
ZS11	57.97	24.06	9.88	3.88	1.57	0.39	0.14
A28	67.10	21.15	3.34	3.92	2.55	0.25	0.23
SW	86.32	2.03	3.46	3.05	1.30	0.51	0.16
YH25005	49.86	26.12	15.20	4.75	1.09	0.43	0.25
2006L	46.49	25.84	18.16	5.21	1.09	0.45	0.30
R8Q10	47.48	24.57	20.11	3.93	1.09	0.44	0.30

2.2 Methods

2.2.1 Gene Cloning and Sequencing

Different reference genes for *BnFAD2* and *BnFAD3* were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov) and the *B. napus* pan-genome database (BnPIR) (http://cbi.hzau.edu.cn/bnapus). For example, the ZS11 genome possesses four copies of *BnFAD2* (*BNaA01g00369500zs, BNaA05g0427800zs, BNaC01g00461200zs* and *BNaC05g0480500zs*) and six copies of *BnFAD3* (*BNaA03g0143900zs, BNaA04g0019190zs, BNaA05g0136600zs, BNaC03g0167300zs, BNaC0480200zs*, and *BNaC0496200zs*). These were henceforth referred by chromosome, i.e., *FAD2.A1, FAD2.C1, FAD2.A5, FAD2.C5, FAD3.A3, FAD3.A4, FAD3.A5, FAD3.C3, FAD3.C4*, and *FAD3.C4-* (antisense strand of C4). The PCR primers (Table 2) were designed for full-length or overlapping partial fragments of gDNA, CDSs, and promoter sequences of different gene copies using the online Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 2: The primers designed for the CDS and promoter of *BnFAD2* and *BnFAD3* genes

Gene	Primer	Direction	Sequence (5′-3′)
BnFAD2	HY267*	Forward	CAGGATCCATGGGTGCAGGTGGAAGAAT
	HY267-2*	Forward	CAGGATCCATGGGCGCAGGTGGAAGAAT
	HY268*	Reverse	CAGAGCTCTCATAACTTATTGTTGTACCAG
BnFAD3	A3C3-F	Forward	GTTGTTGCTATGGACCAACGCAC
	A3C3-R	Reverse	GAAAGTTTAATTGATTTTCGATTTGA
	A4C4-F	Forward	CAGGATCCATGGTTGTTGCTATGGACCAGCGC
	A4C4-R	Reverse	GTTAATTGATTTTGGATTTGTCAGAAGC
	A5C4+F	Forward	ACACATCTAGAGAGAGAAACTTCG
	A5C4+R	Reverse	GAGAAGGAGTGTTTATTCCTAAT
P-*BnFAD2*	P2C1-F1	Forward	GTCTGTTGTGCGTGAGTCAGACACATCAT
	P2C1-R1	Reverse	TCATAACTTATTGTTGTACCAGAACACACC
	P2C1-F2	Forward	GGCATCAACTACCATCTTCTTCTT
	P2C1-R2	Reverse	CGAGGATGTCGAAGAGGAGGT
	P2C5-F	Forward	GAGATGAGTTGGAGATTAGAAATAG
	P2C5-R	Reverse	TTGATGGTGTCGGTTTCAGACTTC
	P2A5-F	Forward	AAATGAAATGAAATCATGGTAGGTGATG
	P2A5-R	Reverse	CGCGCTTGATGTTGTCGGTTTCAGACTT
P-*BnFAD3*	PA4-F	Forward	CTAGAAGAAATAGTGGTTAC
	PA4-R	Reverse	TCGTCTCCGTTCGCATTGCTA
	PA5-F	Forward	GTGTTCGCGTCAACTTAATTAT
	PA5-R	Reverse	TGCTTAGGAATCGCCGCCCTG
	PC4-F	Forward	TGTTTAACACGCTTGCAGATTCTTCC
	PC4-R	Reverse	TGGTCCATAGCGACAACCATCGCC
	PA3-F	Forward	TATTTGAGTTAACAGACTATAGAACC
	PA3-R	Reverse	TTGGTCCATAGCAACAACCATCCTG
	PC4+F	Forward	TAATCTCGCAAGACCCATTTCGGC
	PC4+R	Reverse	CCGTTAACATTGCTGCGCTGGTA
	PC3-DF	Forward	TCTGCTTGATGCATAAATGTCTCC
	PC3-ZF	Forward	CGAAATGATTCTTTCGACGTACTG
	PC3-R	Reverse	GCCCTTATGTCCCCGATCTTAAA

Note: ‘P-’ before a gene indicates the promoter for the corresponding gene. Symbol * indicates the primers from Yang et al. [5].

DNA was extracted from seedlings of the six genotypes using a standard protocol using hexadecyl trimethylammonium bromide lysis buffer. RNAs in the seed samples at 20, 27, and 34 DAP (days after pollination) were extracted by using E.Z.N.A.® Plant RNA Kit (Omega Bio-tek Inc., GA, USA) and were transcribed into cDNA for cloning of CDS region. PCR amplification was performed on a thermal cycler using Phanta® Max Super Fidelity DNA polymerase (Vazyme Biotech, Nanjing, China). The PCR products were separated via agarose gel electrophoresis, and the target fragments were extracted using the

D2500 Gel Extraction Kit (Omega Bio-tek Inc, GA, USA). The fragments were inserted into pEASY ® Blunt Zero Cloning Vector (TransGen Biotech., Beijing, China). The positive clones were identified by PCR using specific primers, and the molecular weights of PCR products were confirmed again using agarose gel electrophoresis. The DNA sequencing was conducted by SANGON Biotech (Shanghai, China) and conformed with the results of two or three clones per gene copy. The CDS region of each gene was identified after sequence alignment using MEGA 7.0 (https://www.megasoftware.net/) and Vector NTI 11.0 (Invitrogen, CA, USA). The 1.5–2 kb sequence before the first open reading frame (ORF) of a gene was defined as the candidate promoter region. Each promoter was named by adding 'P-' before the corresponding gene. The prediction of promoter structures and cis-regulatory elements (CREs) was conducted on the PLANTCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

2.2.2 Detection of Gene Expression Levels by qPCR

Immature seeds from the six plant accessions were quickly collected at 20, 27, and 34 DAP, immersed in liquid nitrogen, and then stored at −80°C. Each plant accession had three biological replications. Total RNAs were extracted using RNA Isolation Kit (Omega Bio-tek Inc., GA, USA) and then transcribed into cDNA. The primers designed for the various homologous copies of *FAD2* and *FAD3* (Table 3) were used to detect their expression levels by qPCR. *BnFAD2.A1*, encoding a truncated protein without function [5,18], was not detected here in light of that very low abundance in the transcriptome of developing seeds [18]. The PCR reaction system consisted of NovoStart SYBR qPCR Supermix plus (Novoprotein, Shanghai, China) with SYBR Green I, Rox II, primers, cDNA template, and RNase-free water. The Rapeseed *beta-actin7* gene was used as the internal reference. The PCR reactions were completed on a QuantStudio 7 Flex PCR thermal cycler (ABI, CA, USA).

Table 3: The primers used in the qPCR assay

Gene	Forward prime	Reverse prime
Actin7	CGCGCCTAGCAGCATGAA	GTTGGAAAGTGCTGAGAGATGCA
FAD3.C3	CCAATGTGAACGGAGATG	GCGGCAATGGCCAAAGC
FAD3.C4-	TGAACGGAGATTCCAAGGAC	ACGACGGAGAAAATGTCTCTC
FAD3.C4+	TCATCATGGTCACGATGAG	CGTGATGGATGTTGTTGAAG
FAD3.A3	AGCTTATTGCAACTTCGACT	TCCAACCACATCACAAAGATA
FAD3.A4	CCTCTCTATCTGTGGGTA	ACACATGTTTAGCAGATTTC
FAD3.A5	GCTTATTGCAACTTCAACA	TACAAAGATAATGTAAGGAAC
FAD2.C1	GTACCTCAACAACCCGCTA	ATCCCACTCCGAAGAATCAT
FAD2.A5	ATTGTCAACGGGTTCTTAGTT	CAGTTTCTTCTTTGCTTCATAAC
FAD2.C5	ATTGTCAATGGTTTCCTCGTG	CTTTCACCATCATCATATCCTC

3 Results

3.1 Expression Levels of BnFAD2 and BnFAD3 Copies during Seed Development

qPCR was carried out to detect the expression levels of individual copies of *BnFAD2* and *BnFAD3* in immature seeds (20, 27 and 34 DAP) of 2006L, R8Q10, YH25005, ZS11, A28 and SW (Fig. 1). The expression levels of *FAD2* copies, especially *BnFAD2.C1*, in the two low-ALA accessions SW and A28 were significantly lower than in the others. The expression of individual *BnFAD3* copies in the three high-ALA accessions was higher than in the low-ALA seeds of A28 and SW. The expression of *BnFAD3.A4* and *BnFAD3.A5* in the three high-ALA accessions was significantly higher than in ZS11.

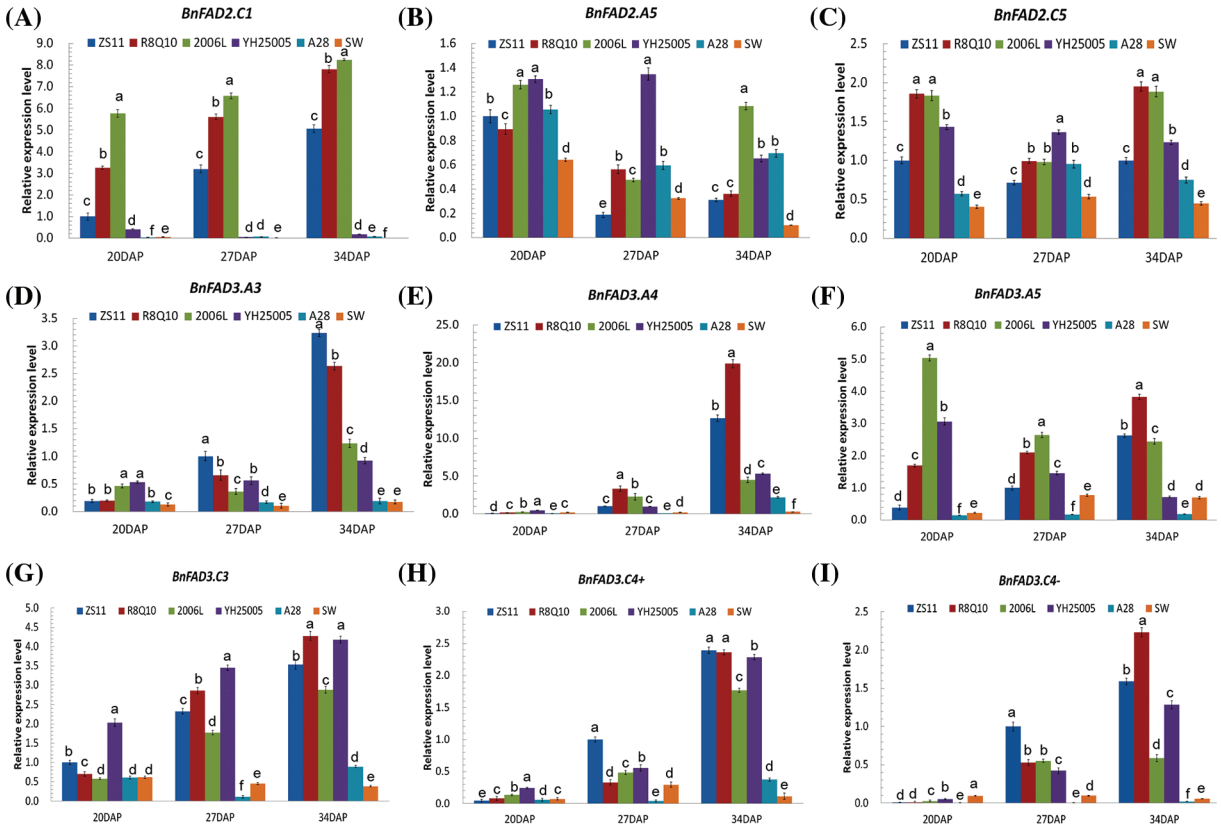

Figure 1: Expression level of *BnFAD2* and *BnFAD3* copies in the six genotypes with different ALA contents. The letters a, b, c, etc., indicate significant differences at a 95% confidence level

3.2 CDS Region Variations in BnFAD2 Copies

Due to the close similarity of the four *BnFAD2* copies, they were amplified by PCR using two forward primers (HY267 and HY267-2) and one reverse primer (HY268) [5], and then identified after monoclonal sequencing. The *BnFAD2.A1* (*BNaA01g00369500zs* in ZS11 genome deposited in BnPIR website http://cbi.hzau.edu.cn/bnapus/) is a short pseudogene encoding a truncated protein without enzyme function [5,6]. An identical 1155 bp long CDS region of *BnFAD2.C1* (*BNaC01g00461200zs*) was found in ZS11, 2006L, and R8Q10, but was not detected in SW, A28, and YH25005. This copy is also absent in the Darmor genome in the BnPIR database, in line with a previous report of *BnaC.FAD2.b* absence in cv. Cabriolet [7], indicating the possible deletion of this copy in many germplasm during the evolutionary process. Both obtained sequences of *BnFAD2.A5* and *BnFAD2.C5* were 1155 bp long and encoded 385 amino acids, in line with the results of previous studies [5–7]. *BnFAD2.A5* (NCBI accession No. OR676828) of SW had a G > A SNP at nucleotide 316 of the CDS region, resulting in E (Glu) > K (Lys) substitution in the 106th residue (abbreviated as E106K) of the amino acid (Table 4, Fig. S1) compared to ZS11. This was an amino acid mutation from acidic to alkane residue which may have caused the defect in this protein function. *BnFAD2.C5* (OR676829) in both SW and A28 had a G > A SNP at 908 bp of the CDS (Fig. S2), resulting in a mutation from G (Gly) to E (Glu) in the 303rd residue (Fig. S3). Finally, *BnFAD2.C5* (OR676830) in A28 had a C > T mutation at 1103 bp of the CDS, resulting in a mutation P (Pro) > L (Leu) in the 368th residue (Fig. S3).

Table 4: The CDS mutations of *BnFAD2* and *BnFAD3* in the five accessions in comparison to ZS11

Accession	*FAD2.C1*	*FAD2.A5*	*FAD2.C5*	*FAD3.A4*	*FAD3.C3*	*FAD3.C4*	*FAD3.C4-*
R8Q10	–	–	–	–	G13E, Q18R, N160I	Y6D	S9I
2006L	–	–	–	–	G13E, Q18R, N160I	Y6D	S51I, V53F
YH2005	Absent	–	–	–	–	Y6D	–
A28	Absent	–	G303E, P368L	S620L	G13E, Q18R, N160I	A67V	S9I
SW	Absent	E106K	G303E	–	–		D6Y

Note: 'E106K' and so on indicate substitution of the former residue by the latter. The symbol '–' indicates no mutation.

3.3 Sequence Comparison of BnFAD3 Copies

There are six copies of the *BnFAD3* gene in the ZS11 reference genome, and these exhibited 99%, 97.6%, and 85.7% nucleotide identity in a pairwise comparison of the homologous copies of chromosome A5 *vs*. C4, A4 *vs*. C4-, and A3 *vs*. C3, respectively. Thus, three pairs of specific primers with main differences at the 3-prime end were designed for the three groups of *BnFAD3* copies, and the PCR products using each pair of primers obtained two different nucleotide sequences. The six accessions had the same CDSs as *BnFAD3.A3* (*Bna03g0143900ZS*) and *BnFAD3.A5* (*Bna05g0136600ZS*) in the BnPIR database, but different CDSs of the other copies.

The CDSs of *BnFAD3.A4* in the six accessions were 1134 bp long and encoded 378 amino acids. The CDS sequences of *BnFAD3.A4 in* R8Q10, 2006L, and SW were identical to those of *BnFAD3.A4* in ZS11 (*BNaA04g0019190zs*). The *BnFAD3.A4* CDS in YH25005 had a T > C SNP at 309 bp, compared with that of ZS11; however, this was a synonymous mutation. *BnFAD3.A4* in A28 (OR676831) had three SNPs at 309, 620, and 870 bp of the CDS region, compared with that of ZS11 (Fig. S4). The shift of C > T at the 620th base led to the substitution of Ser (S) with Leu (L) in the 207th residue (Fig. S5), but the other two mutations were synonymous.

The CDS sequences of *BnFAD3.C3* in YH25005 and SW were as same as in ZS11 (*BnaC03g0167300zs*), with 1152 nucleotides coding for 384 amino acids. However, 2006L, R8Q10, and A28 (OR676832) had another CDS haplotype (Table 4, Fig. S6) of *BnFAD3.C3*, the same as that of cv. Tapidor (*BnaC03t0127700ta*), Westar (*BnaC03g0107200we*), and No. 2127 (*BnaC03t0094100no*) in the BnPIR database. There were six SNPs in the *BnFAD3.C3* CDS of 2006L, R8Q10, and A28 when compared with the CDS of ZS11; three of these SNPs were synonymous mutations, and the remainder caused amino acid substitution (Fig. S7). Notwithstanding this result, it may be that these SNPs do not affect ALA synthesis, because both cv. Tapidor and Westar have a common phenotype of ALA content.

The CDS of the *BnFAD3.C4* gene is also 1152 bp long and encodes 384 amino acids. Compared to ZS11, the *BnFAD3.C4* CDS of R8Q10 (OR676834), 2006 L and YH2500 had one T > G SNP at 16 bp, resulting in the mutation of the sixth residue from Y (Tyr) to D (Asp) (Table 4, Fig. S8). *BnFAD3.C4* in A28 (OR676833) had one C > T SNP at 200 bp of the CDS region, resulting in Ala (A) to Val (V) mutation at the 67th residue (Fig. S8).

The CDS of the *BnFAD3.C4-* gene of YH25005 is the same as that of ZS11, with 1143 bp encoding 381 amino acids (Fig. S9). In comparison, the *BnFAD3.C4-* CDS of R8Q10 (OR676837) has one G > T SNP at 26 bp, resulting in a mutation of the ninth amino acid from Ser (S) to Ile (I) (Fig. S10). There were two G > T SNPs at 152 and 157 bp in *BnFAD3.C4-* CDS of 2006L (OR676838), resulting in the mutation of the 51st residue from Ser (S) to Ile (I), and of the 53rd residue from Val (V) to Phe (F). The *BnFAD3.C4-* CDS of SW (OR676836) had an SNP of G > C at 16 bp, resulting in the mutation of the sixth amino acid from D (aspartic acid) to Y (tyrosine) (Table 4).

3.4 Variations in BnFAD2 Promoter Sequences

The promoter sequences of *BnFAD2.A5* (OR676843) and *BnFAD2.C5* (OR676846) obtained from the six accessions were 100% identical, except a depletion of three bp in P-*BnFAD2.A5* of A28 (OR676845) and insertion of seven bp in P-*BnFAD2.A5* of Yh25005 (OR676847). P-*BnFAD2.A1* of SW (OR676839) and A28 (OR676840) showed five SNPs and a deletion of four nucleotides (TAAT) compared to ZS11 and P-*BnFAD2.A1* of 2006L and R8Q10 (OR676841) possessed three SNPs. However, these SNPs are not located on any known CREs and all known CREs in the six accessions were arranged in the same pattern in the promoter. The promoter of *BnFAD2.C1* was successfully amplified by designing a specific nucleotide at the 3' end of the primer. After separation via agarose gel electrophoresis, a band with identical molecular weight of ca. 2 kb—as well as an identical sequence (OR676844)—was produced in R8Q10, 2006L, and ZS11; however, no band was produced in YH25005, A28, and SW (Fig. 2). The divergence of two alleles of *BnFAD2.C1* promoters was consistent with their CDS sequence being absent, as mentioned above, indicating that this copy of R8Q10, 2006L, and ZS11 possibly has an ancestry different from that of YH25005, A28, and SW.

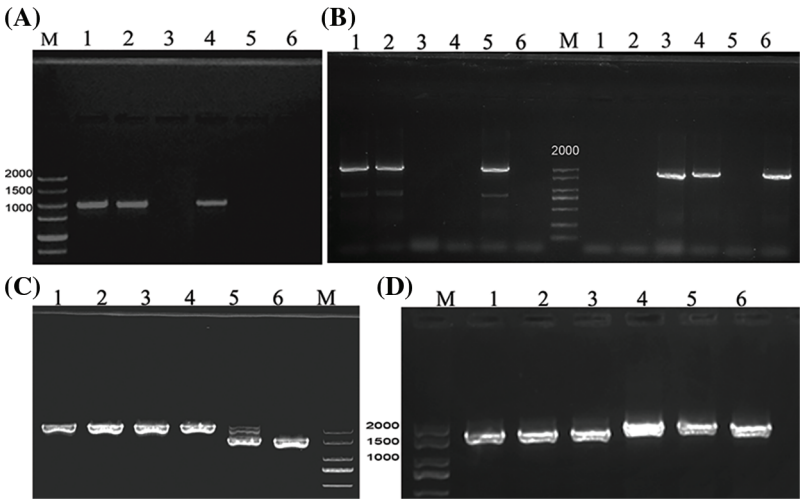

Figure 2: Electrophoretic profile of PCR products for the promoter P-*BnFAD2.C1* (A), P-*BnFAD3.C3* (B) using primer pairs PC3-DF/PC3-R (left panel) and PC3-ZF/PC3-R (right panel), P-*BnFAD3.A3* (C), and P-*BnFAD3.C4*- (D), respectively. M: DNA Marker; Lane 1: R8Q10; Lane 2: 2006L; Lane 3: YH25005; Lane 4: ZS11; Lane 5: A28; Lane 6: SW

3.5 Variations in BnFAD3 Promoter Sequences

There are great differences in the transcription start sites in the promoter sequences of the six *BnFAD3* copies of the ZS11 reference genome, so we designed six pairs of specific PCR primers to amplify them. The similarity in the promoter sequences of *BnFAD3.A4*, *BnFAD3.A5*, and *BnFAD3.C4*- (OR676854-OR676866) within the six accessions was very high, i.e., about 99%, with a few SNPs located not on any known CREs.

According to two different promoter sequences of the *BnFAD3.C3* gene found in the public database, two pairs of specific primers were designed, i.e., PC3-DF/PC3-R and PC3-ZF/PC3-R; agarose gel for electrophoresis resulted in amplification bands for R8Q10, 2006L, and A28 in the case of PC3-DF/PC3-R, while bands for YH25005, ZS11, and SW (OR676868) were obtained in the case of PC3-ZF/PC3-R (Fig. 2). The promoter sequences of the three accessions R8Q10 (OR676867), 2006L, and A28 (Fig. S11) were the same as those for the corresponding fragment of P-*BnFAD3.C3* of cv. Tapidor, Westar, and No. 2127 in the BnPIR database. Comparing the two types of promoter sequences, except the

322 bp before the transcription initiation site, the remaining promoter sequences were found to be very different. In the prediction of CREs, there was also a large gap between the two alleles (Fig. 3).

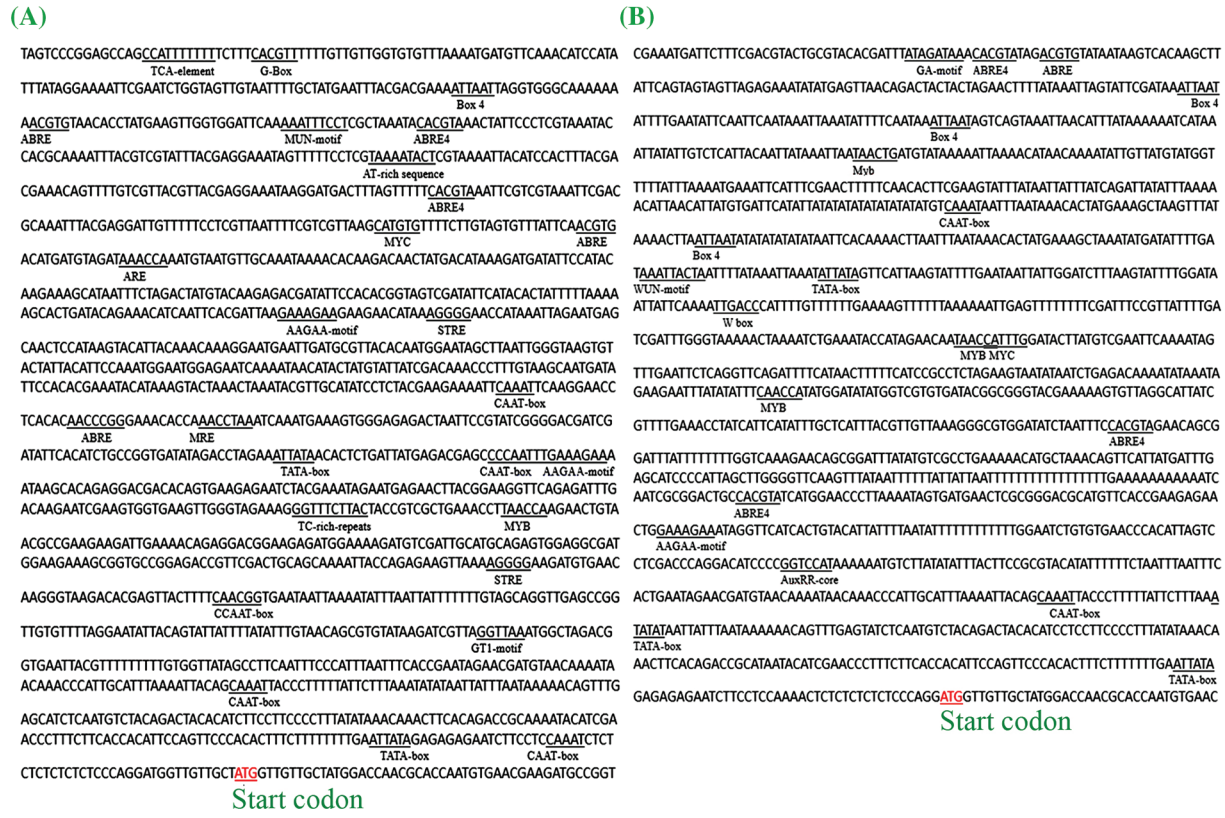

Figure 3: The predicted CREs on the promoter of *BnFAD3.C3* of R8Q10 (A) and ZS11 (B)

The PCR products for P-*BnFAD3.A3* of A28 and SW had a smaller size of 1470 bp on gel electrophoresis (Fig. 2) than the band of 2016 bp in ZS11, YH25005 (OR676851), 2006L, and R8Q10 (OR676848). P-*BnFAD3.A3* of A28 (OR676849) and SW (OR676850) had a 549 bp fragment deletion and an (AT) insertion (Fig. S12). The depletion included two key CREs, namely, TATA-box (TATAAAT) and A-box (CCGTCC) of polymerase II transcription, and several important CREs such as the MYB binding site MBSI (AAAAAACCGATATGTA), the abscisic acid response element ABRE4 (CACGTA), the auxin response element TGA element (AACGAC), the WRKY homologous action element W-box (TTGACC), and the stress response element STRE (AGGGG) (Fig. 4).

The PCR products for P-*FAD3.C4* of six accessions showed two bands of slightly different size, separated by agrarose gel electrophoresis (Fig. 2). The shorter band produced by R8Q10 (OR676852), YH25005 and 2006L was 1750 bp long, identical to cv. Darmor and Tapidor in the BnPIR database; however, A28 and SW (OR676853) shared with ZS11 a longer sequence of 2018 bp (Fig. S13). The divergent patterns of the two alleles, with a low similarity of 83.0%, are consistent with the CDS differences mentioned above, further indicating that the *BnFAD3.C4* gene of these six genotypes has two different ancestries. Compared with ZS11, the promoter sequences of the three high-ALA accessions lacked the module ACE and the MYB-binding site MRE involved in light response, as well as the MYB-binding site MBSI involved in flavonoid biosynthesis regulation (Fig. 5).

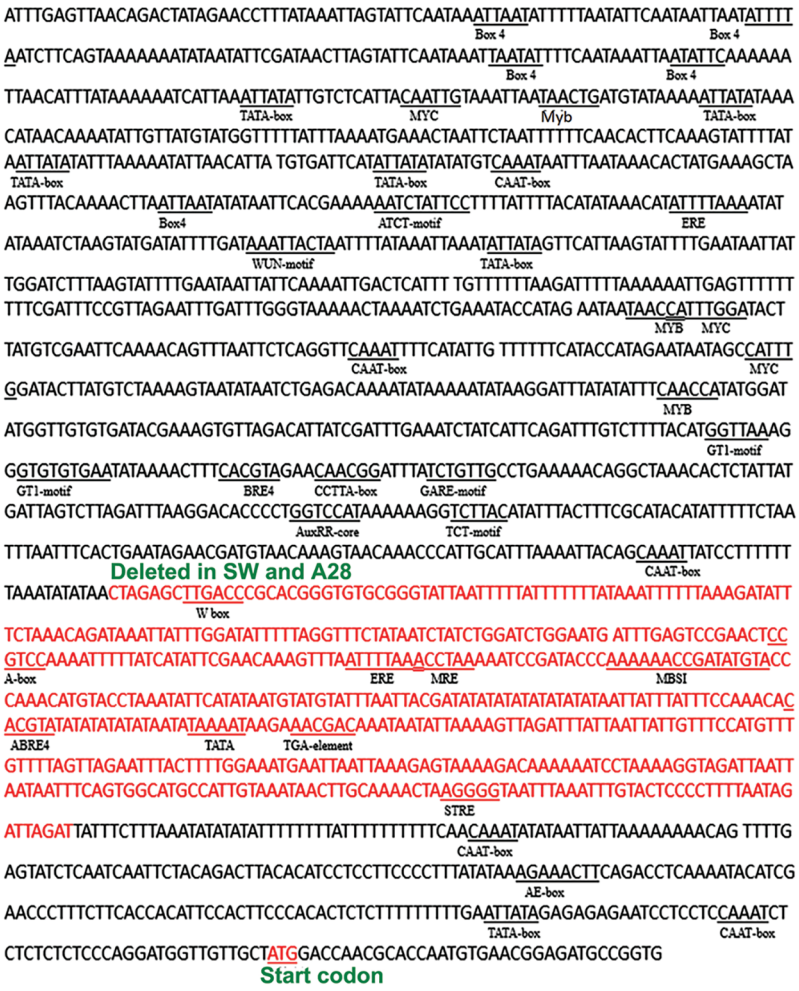

Figure 4: The predicted CREs on the promoter of *BnFAD3.A3* of ZS11. The red region was deleted in SW and A28

4 Discussion

4.1 Both BnFAD2 and BnFAD3 are Hotspots of Genetic Modification in the Production of High-OA or Low-ALA Phenotypes

The in-tandem steps of OA and ALA biosynthesis are contradictory when total levels of unsaturated fatty acid are fixed. Therefore, when producing a high-OA and/or a low-ALA phenotype, either *FAD2* or *FAD3* will be the hotspot of genetic modification [3,5,8,18–20]. The results of the present study show that, compared to ZS11, the expression of most *BnFAD3* copies was upregulated in the three high-ALA accessions, but downregulated dramatically in the low-ALA line A28 and the HOLL line SW, at either one or two of the three stages of seed development. Generally, the different expression levels of these genes under the same background may be caused by differences in their promoter sequences. For example, the obvious differences between high and low-ALA plant groups in terms of indels and SNPs in the *BnFAD3.C4* promoter may explain their differences in expression levels. Compared with ZS11, the promoter of *BnFAD3.A3* in the low-ALA SW and A28 has a 549 bp deletion and a 2 bp (AT) insertion. The causal relationship between the promoter and the level of gene expression level may be further analyzed by investigating the activity of a mutated promoter tagged by a reported gene such as *GUS* or

GFP. The activity of some upstream transcription factors such as FUS3 or bZIP67 should also be studied because these can regulate the expression of *FAD3* or even *FAD2* [21–24]. In addition to considerable changes in gene expression levels, some non-synonymic amino acid residue mutations may also affect the catalytic function of enzymes. Protein and enzyme functions can be validated by expressing the mutated CDS of *BnFAD2* and *BnFAD3* in yeast and then measuring the catalytic speed of extracted proteins on their substrate OA or LA. In addition, the association between these SNP or indel mutations and phenotype can be validated in various segregation populations and thus there need for many allele-specific populations to keep unanimous genetic background (at least the same background of non-target *BnFAD2* and *BnFAD3* copies) in each population.

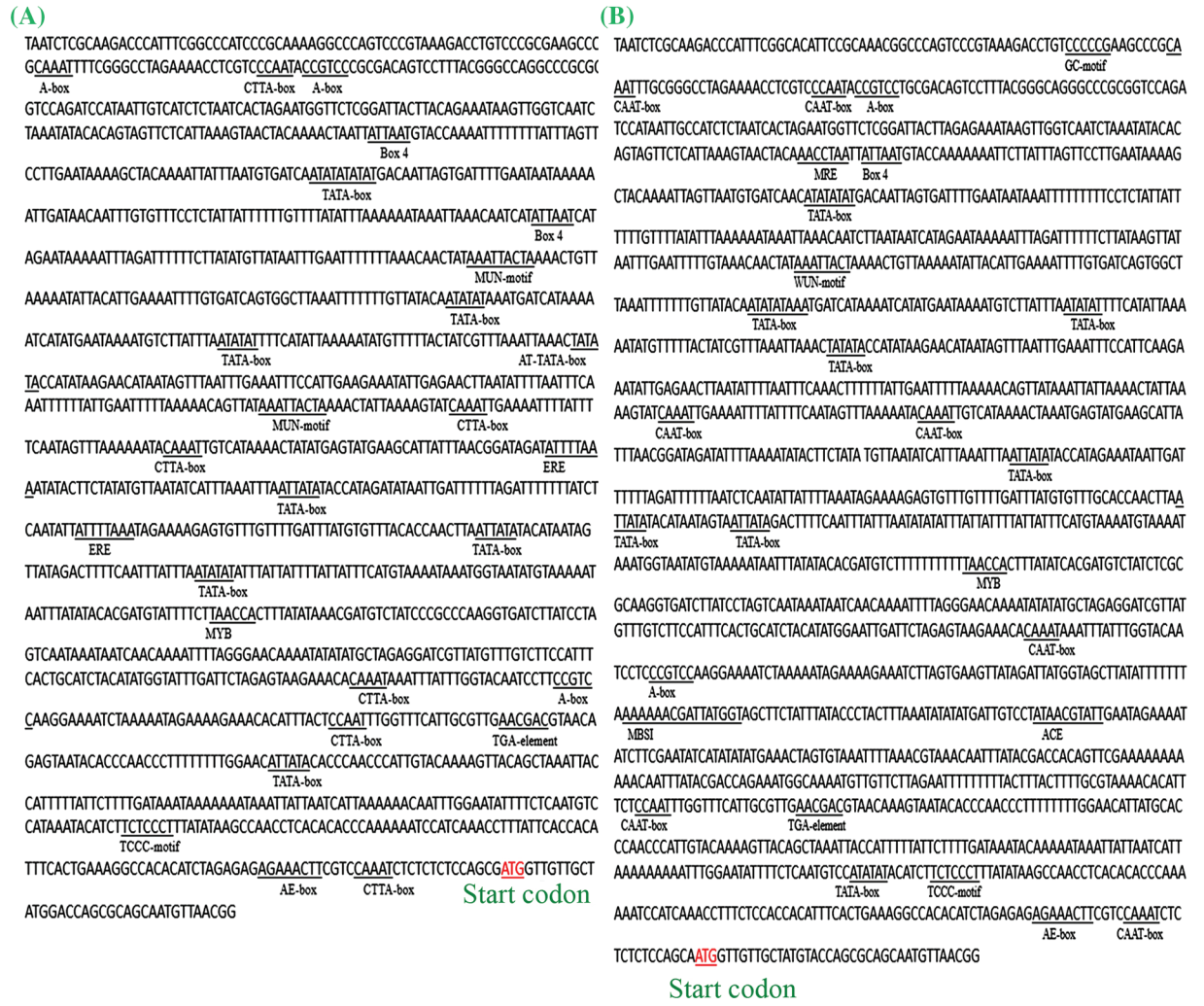

Figure 5: The predicted CREs on the promoter of *BnFAD3.C4* of R8Q10 (A) and ZS11 (B)

4.2 Relationship between Variations in BnFAD2/BnFAD3 and the Low-ALA Phenotype

Loss-of-function mutation in *FAD2* leads to an increase in OA but a reduction in ALA. In a previous study, it was found that the high-OA mutant $M_6$04-855 harbors SNPs at nucleotides 270, 1044, and 1062 in a *BnFAD2* copy, leading to the production of a stop codon [3]. In another high-ALA rapeseed accession FC81, it was found a C > T substitution at nucleotide 421 of *BnFAD2.A5*, leading to His141Tyr

mutation in the amino acid residue, as well as a G > A substitution at nucleotide 1073 of *BnFAD2a.C5*, leading to Arg358Lys mutation [25]. In the HOLL cv. SW-hickory, Yang, et al. [5] found that a 4 bp insertion at the 567 bp of *BnaA.FAD2* (chromosome A5) resulted in a frameshift that led to a misreading. They also found a C > T substitution in the seventh exon, resulting in a missense mutation of *BnaA.FAD3.b*, as well as a G > A transition in the 5' splice donor site of the sixth intron, resulting in abnormal splicing in *BnaC.FAD3.b*. Two identical C >T and G > A SNPs were also validated in another low-ALA germplasm [19].

The mutation sites of *BnFAD2* and *BnFAD3* found in the two low-ALA accessions A28 and SW (Table 4 and Figs. 3–5) are different from those described in previous studies [3–5]. In the present study, the HOLL accession SW had four obvious mutation sites including the absence of *BnFAD2.A1*, Glu106Lys mutation of FAD2.A5 protein, Gly303Glu mutation of BnFAD2.C5, and Asp6Tyr mutation of BnFAD3.C4, leading to stringent limitations in LA and ALA synthesis and very high OA accumulation. Compared to SW, the low-ALA accession A28 also had an absence of *BnFAD2.A1* and a mutation of Gly303Glu in BnFAD2.C5 but a normal copy of BnFAD2.A5. The normal BnFAD2.A5 can synthesize LA, resulting in a limited accumulation of OA (67.10%) (Table 1).

Compared with 2006L and R8Q10, SW, YH25005, and A28 all lacked a copy of *BnFAD2.C1*, which has a normal CDS encoding 384 amino acids like the A5 and C5 copies, and thus may have a certain enzymic function. The results of qPCR detection of *BnFAD2.C1* showed that it is expressed in 2006L, R8Q10, and ZS11 but not in SW, YH25005, and A28. In addition, ALA content in YH25005 is slightly lower—by two to four percentage points—compared with R8Q10 and 2006L. Meanwhile, the content of OA plus LA in YH25005 is slightly higher than R8Q10 and 2006L. We speculated, therefore, that *BnFAD2.C1* also plays a minor role in ALA biosynthesis.

4.3 Upstream Transcription Factors May Regulate the Expression of Various BnFAD3 Copies

The variation in *BnFAD2* and *BnFAD3* promoters and the corresponding transcriptional regulation may be an important reason for the elevated expression levels of *BnFAD2* and *BnFAD3* in the three high-ALA accessions. However, the SNP and indel variations in the promoters of the three *BnFAD3* copies (chromosomes A3, C3, and C4) in the three high-ALA accessions (Fig. 5) cannot explain the upregulation of the other copies on chromosomes A4, A5 and C4-. Therefore, there may be an activation of upstream transcription regulatory factors (for example transcription factor), which may play important roles both in the upregulation of the former three copies (chromosomes A3, C3, and C4) and the latter three copies chromosomes (A4, A5 and C4-). Previous studies have found that some *Transparent Testa* mutations in Arabidopsis have significant effects on key transcription factors related to embryonic development and that such transcription factors as FUS3, LEC1, ABI3, and bZIP67 can regulate the expression of *FAD3* [21–24]. Our high-ALA plants, R8Q10, 2006L, and YH25005, all have yellow-seed phenotypes with lowered expression of many *Transparent Testa* homologs and upregulation of the genes encoding transcription factors including FUS3, LEC1, ABI3, and bZIP67 in the three accessions [26]. We found that, in thousands of rapeseed samples, the yellow-seeded population often had an ALA content around two percentage points higher than that of their black- or brown-seeded siblings (unpublished). Thus, yellow-seededness may also be an important indirect factor in the elevation of ALA content. However, this does not mean that yellow seedness is necessary for the elevation of ALA content because some other high-ALA accessions have brown-colored seeds [2,13].

5 Conclusions

In the present study, detailed sequence mutation profiles for the CDSs and promoter regions of *BnFAD2* and *BnFAD3* in six genotypes with high- or low-ALA phenotypes were produced. Most of these have not previously been reported in the literature. The profile of *BnFAD2* mutation in the two low-ALA

accessions A28 and SW is different from that reported in previous studies. The CDSs of *BnFAD2.C5* and *BnFAD2.A5* in SW and A28 are quite different from those in the wild type and may lead to significant defects in their catalytic function. Some mutations in *BnFAD3* in the high-ALA accessions are reported for the first time here. The SNPs or indels in the *BnFAD3* promoters of high-, medium-, and low-ALA accessions may affect their binding situation concerning corresponding transcription factors and transcriptional levels. The influence on the enzymic activity by residue substitution of several *BnFAD3* CDSs of 2006L, R8Q10, and YH25005 needs a further study. The mutation sites may be considered useful information for the design of molecular markers for our next breeding program.

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Availability of Data and Materials: All data generated or analyzed during this study are contained in the paper and the additional information. The CDS and promoter sequences of *BnFAD2* and *BnFAD3* copies have been deposited in the NCBI database and the accession numbers are provided in the paper.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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