Detection and Molecular Characterization of Two FAD3 Genes Controlling Linolenic Acid Content and Development of Allele-Specific Markers in Yellow Mustard (Sinapis alba)

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Abstract
Development of yellow mustard (Sinapis alba L.) with superior quality traits (low erucic and linolenic acid contents, and low glucosinolate content) can make this species as a potential oilseed crop. We have recently isolated three inbred lines Y1127, Y514 and Y1035 with low (3.8%), medium (12.3%) and high (20.8%) linolenic acid (C18:3) content, respectively, in this species. Inheritance studies detected two fatty acid desaturase 3 (FAD3) gene loci responsible for variation of C18:3 content. QTL mapping revealed that the two FAD3 gene loci responsible for 73.0% and 23.4% of the total variation and were located on the linkage groups Sal02 and Sal10, respectively. The FAD3 gene on Sal02 was referred to as SalFAD3.LA1 and that on Sal10 as SalFAD3.LA2. The dominant and recessive alleles were designated as LA1 and la1 for SalFAD3.LA1, and LA2 and la2 for SalFAD3.LA2. Cloning and alignment of the coding and genomic DNA sequences revealed that the SalFAD3.LA1 and SalFAD3.LA2 genes each contained 8 exons and 7 introns. LA1 had a coding DNA sequence (CDS) of 1143 bp encoding a polypeptide of 380 amino acids, whereas la1 was a loss-of-function allele due to an insertion of 584 bp in exon 3. Both LA2 and la2 had a CDS of 1152 bp encoding a polypeptide of 383 amino acids. Allele-specific markers for LA1, la1, LA2 and la2 co-segregated with the C18:3 content in the F2 populations and will be useful for improving fatty acid composition through marker-assisted selection in yellow mustard breeding.

Introduction
Yellow mustard (Sinapis alba L., 2n = 24) is cultivated as an important condiment crop. It has many desirable agronomic traits such as resistance to cabbage aphids [1], flea beetles [2,3] and blackleg diseases [4]. In addition, it is drought tolerant and resistant to pod shattering. Yellow mustard germplasm with canola quality (low erucic and low glucosinolate contents) was developed at Agriculture and Agri-Food Canada-Saskatoon Research Centre (AAFC-SRC) [5], which makes yellow mustard accessions contain 6.9–12.4% linolenic acid of total fatty acids in the seed [7]. Current low C18:3 canola cultivars have been developed using this low linolenic gene source.

Linolenic acid content is determined mainly by the embryonic genotype with some influence from temperature, maternal genotype and cytoplasm in B. napus [8–10]. QTL mapping identified two major QTLs, accounting for 25.2–28.8% and 52.4–62.7% of the C18:3 variation, located on the linkage groups A4 and C4, respectively, in B. napus [11,12]. It was reported that the low C18:3 variant resulted from mutations of FAD3 genes in B. napus [11–13]. The FAD3 gene on A4 harboured a C to T substitution in exon 7, which when translated causes the wild type amino acid arginine to be replaced by cysteine. The FAD3 gene on C4 contained a G to A substitution in the 5’ splice site of intron 6 in the low C18:3 B. napus line. FAD3 allele-specific markers based on the sequence variation were developed and proved to be useful for identification of different C18:3 genotypes in canola B. napus [11,12]. Yellow mustard accessions contain 6.9–12.4% linolenic acid of total fatty acids in the seed [14,15]. Recently, inbred lines with high (18.5%), medium (13.8%) and low (3.8%) linolenic acid content, respectively, have been obtained through inbreeding of heterozygous open-pollinated plants in yellow mustard [16].
The low linolenic acid variant (3.8%) is a valuable gene source for breeding canola-quality yellow mustard with high stability oil (high oleic and low linolenic acids) as that of canola B. napus. The knowledge about genetic and molecular bases of the variation in C18:3 content and development of FAD3 allele-specific markers will greatly facilitate the development of low linolenic canola-quality yellow mustard. The objectives of this study were: 1) to determine the inheritance and perform QTL mapping of the C18:3 content; and 2) to clone the FAD3 genes and further develop allele-specific markers for marker assisted selection.

Materials and Methods

Plant Materials

Linolenic acid contents of the three parental lines Y1127, Y514 and Y1035 are shown in Table 1. Y1127 is an S4 inbred line produced by selfing of the low linolenic S2 line Y158 for two generations and has a low C18:3 content (average: 3.8%). Y514 is the doubled haploid line SaMD3 [17] and has a medium C18:3 content (average: 12.3%). Y1035 is an S4 inbred line and has a high C18:3 content (average: 20.8%).

The F1 seeds of the three crosses Y1127 (low)×Y1035 (high), Y1127 (low)×Y514 (medium) and Y514 (medium)×Y1035 (high) were produced. To produce the BC1 seeds, the F1 plants of the three crosses were crossed as the female with the parental line with a lower C18:3 content. All plants were raised under the same conditions in the greenhouse at AAFC-SRC.

Regional Linkage Mapping

Regional linkage mapping of the linolenic acid content was performed using intron length polymorphism (ILP) markers and bulked segregant analysis (BSA) [18]. A total of 1478 ILP primer pairs: 380 from Arabidopsis thaliana [19] and 1098 from B. napus [20] were used to screen the three parental lines for polymorphic markers. The high bulk was made by mixing equal amount of DNA from 10 F2 plants with the highest C18:3 content, while the low bulk was formed from 10 F2 plants with the lowest C18:3 content for each of the three crosses. The primers detecting polymorphic markers between the two bulks were subsequently used to genotype individual plants of the three F2 populations. Genomic DNA was extracted from young leaves of the parental lines Y1127, Y514 and Y1035, F1 and F2 plants using a modified sodium dodecyl sulfate method [21]. Each PCR (20 μl) contained 1x standard PCR buffer (NEB), 1 U of Taq polymerase (NEB), 0.25 μM forward primer, 0.25 μM reverse primer, 100 μM each dNTP and 50 ng of genomic DNA in a total volume 20 μl. The PCR amplification consisted of an initial denaturation at 94°C for 5 min, 35 cycles consisting of 94°C (45 sec), 55°C (45 sec), 72°C (1 min) terminating with 72°C for 7 min. All PCR products were analyzed by electrophoresis in 2% agarose gels in 1x Tris-acetate-ethylenediaminetetraacetic acid buffer. Gels were visualized by staining in ethidium bromide and photographed on a digital gel documentation system.

The regional linkage map of C18:3 content was constructed using JoinMap 4.0 [22] with a minimum LOD threshold of 4.0. QTL analysis of C18:3 content was performed using the interval mapping method of MapQTL 6.0 [23]. A Chi-square test was used for evaluating the genetic model of C18:3 content in the BC1 population, and the ILP markers in the F2 populations.

Cloning the Coding Region of the FAD3 Gene

Primer pair No 1 (Table S1) was designed based on the conserved coding regions of the FAD3 genes in B. napus and A. thaliana. It was used to clone the coding DNA sequence (CDS) of the FAD3 gene in yellow mustard. Immature seeds at 22 days after pollination were collected from two individual plants from each of the parental lines. Total RNA was extracted from the immature seeds using the RNeasy Plant Mini Kit (Qiagen) as per the manufacturer’s instructions. 750 ng of RNA from each of the parental lines was used to prepare the cDNA using Qiagen’s Omniscript RT Kit as per the manufacturer’s instructions. Each PCR (20 μl) contained 1x PCR standard buffer (NEB), 100 μM of each dNTP, 0.25 μM of each forward and reverse primer, 1 U of Taq polymerase (NEB) and 50 ng of cDNA. Polymerase chain reaction was performed with an initial denaturation at 94°C for 5 min followed by 35 cycles of 45 s at 94°C, 30 s at 55°C and 1 min at 72°C with a final extension cycle of 72°C for 10 min.

Cloning of the 5’ and 3’ Flanking Sequences and the Genomic DNA Sequences of the FAD3 Genes

Primer pairs No 2 and 3 (Table S1) were designed based on the 5’ coding sequences of the cloned SalFAD3:L1 and SalFAD3:L4 genes, respectively. They were used to clone the 5’ upstream sequences by PCR walking according to the protocol of Siebert et al. [24]. Primer No 4 (Table S1) was designed based on the 3’ coding sequences of the cloned SalFAD3:L1 and SalFAD3:L4 genes, and was used to clone the 3’ flanking sequence by PCR walking. Primer pairs No 5 and 6 (Table S1) were designed based

| Genotype | Generation | Linolenic Acid Content* (% of total fatty acids) |
|----------|------------|-----------------------------------------------|
| Y1127    | S4         | 3.8±0.7                                       |
| Y514     | DH         | 12.3±0.7                                      |
| Y1035    | S4         | 20.8±0.8                                      |
| Y1127×Y1035 | F1       | 13.7±1.3                                      |
| Mid parent value |           | 12.3                                           |
| Y1127×Y514 | F1       | 8.9±0.7                                       |
| Mid parent value |           | 8.0                                            |
| Y514×Y1035 | F1       | 15.3±0.7                                      |
| Mid parent value |           | 16.5                                           |

*: Linolenic acid content is expressed as mean value ± standard deviation.

Table 1. Linolenic acid contents of the parental lines Y1127, Y514, Y1035 and F1 seeds, and the mid-parental value in yellow mustard.

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on the 5' flanking sequence and the 3' flanking sequence of the cloned SolFAD3.LA1 and SolFAD3.LA2 genes, respectively, and were used to clone the genomic DNA sequences of SolFAD3.LA1 and SolFAD3.LA2 genes. The standard protocol from the Clontech kit (website: www.clontech.com, Protocol PT 3042, Version PR 03300) by Gwyneth Ingram and Karine Coenen was followed to facilitate the PCR walking.

DNA Sequencing

The expected PCR bands were cloned using the pGEM-T Vector System I (Promega) following the provided instructions. The plasmids were extracted using the QiaSpin Kit (Qiagen) following the manufacturer’s instructions and sequenced using the primer pairs No 7–11 (Table S1) at the Plant Biotechnology Institute, National Research Council, Canada.

Phylogenetic Tree

The multiple alignments were performed using ClustalW (http://www.ebi.ac.uk/clustalw/). MEGA software (version 4.0) (http://www.megasoftware.net/index.html)[25] was used to construct a phylogenetic tree with the aligned protein sequences. The neighbor-joining method was used with the pairwise deletion option, poisson correction model, and the 1000 bootstrap replicates test.
The BC1 seeds of (Y1127×Y1035)×Y1127 were classified into two groups: seeds with medium to high (5.4–19.1%) C18:3 content and seeds with low (3.0–4.9%) C18:3 content (Figure 1), fitting with a segregation ratio of 3:1 ($\chi^2 = 2.00, p = 0.16$). The F2 seeds of Y1127×Y1035 ranged from 2.9% to 20.4% in C18:3 content (Figure 1) with a segregation ratio of 15:1 (seeds with 4.5–20.4% versus seeds with 2.9–4.3% C18:3 content) ($\chi^2 = 5.07, p = 0.08$). Therefore, the segregation patterns of C18:3 content in the BC1 and F2 populations supported a digenic inheritance model in this cross.

The BC1 seeds of (Y1127×Y11035)×Y1127 showed a segregation ratio of 1:1 (seeds with 2.7–5.2% versus seeds with 6.4–9.7% C18:3 content) (Figure 2) ($\chi^2 = 3.38, p = 0.07$), suggesting that the C18:3 content was controlled by one gene locus in this cross. The F2 seeds of Y1127×Y11035 showed a continuous distribution ranging from 3.0% to 16.5% in the C18:3 content (Figure 2). The BC1 seeds of (Y11035×Y1035)×Y11035 and the F2 seeds of Y314×Y1035 exhibited a continuous frequency distribution in the C18:3 content (Figure 3). Therefore, it was not possible to classify the seeds into discrete groups.

Two QTLs Accounting for the Variation of C18:3 Content are Mapped to Linkage Groups Sal02 and Sal10, Respectively

In the F2 population of Y1127 (low)×Y1035 (high), eighteen ILP primer pairs were polymorphic between the high (16.6–20.4%) and low (2.9–4.0%) C18:3 bulks and generated 18 markers (Table 2). The 18 markers were mapped to two linkage groups, each of which carried one QTL for the C18:3 content (Figure 4). Based on the common ILP markers, the two linkage groups were referred to be Sal02 and Sal10 of the constructed S. alba map [28]. One QTL (LOD = 45.43) accounting for 73.0% of the total variation of C18:3 content was localized between BnapPIP685 and BnapPIP881 in Sal02 (Figure 4). The other QTL (LOD = 9.28) responsible for 23.4% of the total variation was located between BnapPIP1012 and BnapPIP363 in Sal10 (Figure 4). Together, the two QTLs explained 96.4% of the total variation for C18:3 content in the F2 population.

In the F2 population of Y1127 (low)×Y11035 (medium), 10 polymorphic ILP primer pairs between the low (3.0–4.0%) and medium (14.5–16.5%) C18:3 bulks produced 10 markers (Table 2). The 10 markers were all mapped to one linkage group corresponding to Sal02. The QTL (LOD = 46.53) was localized between BnapPIP685 and BnapPIP881 in the linkage group (Figure 4). In the F2 population of Y11035 (medium)×Y1035 (high), 11 markers were generated by 10 polymorphic primer pairs between the medium (10.4–11.6%) and high (16.7–19.2%) C18:3 bulks. The 11 markers were mapped to the linkage group Sal10. The QTL (LOD = 6.09) was located between BnapPIP1012 and At3g43520 in Sal10 (Figure 4). The two FAD3 gene loci controlling the QTLs in Sal02 and Sal10 were referred to as SalFAD3.LA1 and SalFAD3.LA2, respectively. The dominant and recessive alleles of the SalFAD3.LA1 gene were accordingly designated as $L^A$ and $l^A$, while that of the SalFAD3.LA2 gene as $L^B$ and $l^B$. Therefore, it could be inferred that the C18:3 genotypes of Y1127 (low), Y314 (medium) and Y1035 (high) were $l^A l^A l^A l^A$, $L^A l^B l^A l^B$ and $L^A L^A l^B l^B L^B$, respectively.

The SalFAD3.LA1 and SalFAD3.LA2 Genes are Cloned and Exhibit Differences in the Exon and Intron

The coding regions of the dominant alleles $L^A$ and $L^B$ were cloned from Y1033, while those of the recessive alleles, $l^A$ and $l^B$, from Y1127 using primer pair No 1 (Table S1). $L^A$ had a coding...
Table 2. Polymorphic ILP primers used for regional linkage mapping of C18:3 content in the three F₂ populations of Y1127×Y1035, Y1127×Y514 and Y514×Y1035.

| Primer Name | Locus Name | Y1127 × Y1035 | Marker type | \( \chi^2 \) Value* | Y1127 × Y514 | Marker type | \( \chi^2 \) Value | Y514 × Y1035 | Marker type | \( \chi^2 \) Value |
|-------------|------------|----------------|-------------|----------------------|--------------|-------------|-----------------|--------------|-------------|----------------|
| At2g22640   | At2g22640  | Codominant     | \( \chi^2 = 2.15 \) | Codominant           | \( \chi^2 = 0.03 \) | -            |
| At2g3490    | At2g3490   | Codominant     | \( \chi^2 = 0.00 \) | Codominant           | \( \chi^2 = 0.21 \) | -            |
| BnapPIP1236 | BnapPIP1236| Dominant       | \( \chi^2 = 0.21 \) | Dominant             | \( \chi^2 = 0.61 \) | -            |
| BnapPIP685  | BnapPIP685 | Dominant       | \( \chi^2 = 1.57 \) | Dominant             | \( \chi^2 = 0.94 \) | -            |
| BnapPIP690  | BnapPIP690 | Dominant       | \( \chi^2 = 1.28 \) | Dominant             | \( \chi^2 = 0.63 \) | -            |
| BnapPIP881  | BnapPIP881 | Dominant       | \( \chi^2 = 2.74 \) | Codominant           | \( \chi^2 = 0.41 \) | -            |
| At2g0765    | At2g0765   | Codominant     | \( \chi^2 = 1.30 \) | -                     | -            | Dominant     | \( \chi^2 = 1.90 \) | -            |
| At3g4320    | At3g4320   | Codominant     | \( \chi^2 = 0.46 \) | -                     | -            | Dominant     | \( \chi^2 = 0.83 \) | -            |
| At3g5765    | At3g5765   | Codominant     | \( \chi^2 = 0.20 \) | -                     | -            | Codominant   | \( \chi^2 = 3.27 \) | -            |
| BnapPIP100  | BnapPIP100 | Dominant       | \( \chi^2 = 0.10 \) | -                     | -            | Dominant     | \( \chi^2 = 0.27 \) | -            |
| BnapPIP1012 | BnapPIP1012| Dominant       | \( \chi^2 = 0.30 \) | -                     | -            | Dominant     | \( \chi^2 = 1.16 \) | -            |
| BnapPIP115 | BnapPIP115 | Codominant     | \( \chi^2 = 1.28 \) | -                     | -            | Dominant     | \( \chi^2 = 1.63 \) | -            |
| BnapPIP1488 | BnapPIP1488| Dominant       | \( \chi^2 = 0.05 \) | -                     | -            | Dominant     | \( \chi^2 = 0.94 \) | -            |

*: Codominant markers: Expected Mendelian segregation of 1:2:1, \( \chi^2 (0.05, 2) = 5.99 \); Dominant marker: Expected Mendelian segregation of 3:1, \( \chi^2 (0.05, 1) = 3.84 \).

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DNA sequence (CDS) of 1143 bp encoding a polypeptide of 380 amino acids. \( \text{la}^{1} \) had a CDS of 1171 bp. Sequence alignment with \( \text{LA}^{1} \) indicated that \( \text{la}^{1} \) harboured an indel involving a 64 bp insertion and a 36 bp deletion at position 412 (Figure 5 and Figure S1). A stop codon at the beginning of the 64 bp insertion might have resulted in the termination of protein translation after the 137th amino acid residue. Therefore, \( \text{la}^{1} \) is a loss-of-function allele.

The 5' flanking sequences from the translation start site were cloned for \( \text{LA}^{1} \) and \( \text{la}^{1} \) using the primer pair No 2 (Table S1). The 5' fragment of \( \text{LA}^{1} \) was 1250 bp, while that of \( \text{la}^{1} \) was 621 bp. A 435 bp 3' flanking sequence from the translation stop codon was cloned for \( \text{LA}^{1} \) and \( \text{la}^{1} \) using the primer pair No 4 (Table S1). The two alleles didn’t exhibit any differences in the cloned 3' flanking sequences. The genomic DNA sequences of the \( \text{LA}^{1} \) and \( \text{la}^{1} \) were amplified using the primer pair No 5 (Table S1) which was designed based on the 5' flanking sequence and the conserved 3'

Figure 4. Mapping QTLs controlling C18:3 content. A. The QTL in Sal02 was located between BnapPIP685 and BnapPIP881 in Y1127×Y1035 and Y1127×Y514. B. The QTL in Sal10 was located between BnapPIP1012 and BnapPIP363 in Y1127×Y1035, and between BnapPIP1012 and At3g43520 in Y514×Y1035. 1-LOD and 2-LOD supporting intervals of each C18:3 QTL were marked by thick and thin bars, respectively. The SalFAD3.LA1 and SalFAD3.LA2 genes co-localized with their C18:3 QTL peaks in the linkage groups Sal02 and Sal10.
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Figure 5. Structure of the SalFAD3.LA1 and SalFAD3.LA2 alleles \( \text{LA}^{1} \), \( \text{la}^{1} \), \( \text{LA}^{2} \), and \( \text{la}^{2} \) in yellow mustard. The black boxes represented the exons and the lines between the black boxes indicated the introns. The numbers on the top of black box of \( \text{LA}^{1} \) (\( \text{LA}^{2} \)) indicated the beginning and ending of each exon of \( \text{LA}^{1} \) (\( \text{LA}^{2} \)) and \( \text{la}^{1} \) (\( \text{la}^{2} \)). The number above each black line indicated the intron length. The nucleotide sequences of 9 bp deletion at position 45 of exon 1 of the alleles \( \text{LA}^{1} \) and \( \text{la}^{1} \), and the 64 bp insertion and the 36 bp deletion at position 412 of exon 3 of \( \text{la}^{1} \) were displayed on the top. Allele specific markers were developed based on the variation in intron 3 of the SalFAD3 alleles \( \text{LA}^{1} \), \( \text{la}^{1} \), \( \text{LA}^{2} \), and \( \text{la}^{2} \).
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flanking sequence specific to the candidate SalFAD3.LA1 gene. Comparison of the coding and genomic DNA sequences indicated that the candidate SalFAD3.LA1 gene contained 8 exons and 7 introns (Figure 5). Alignment of the genomic DNA sequences of LA1 and la2 revealed that la2 had an insertion of 584 bp in the third exon. This insertion contained a new intron splicing site GT (Figure S4), which resulted in a 64 bp insertion and a 36 bp deletion (nucleotide 412–447) at position 412 in the CDS (Figure S4), which resulted in a 64 bp insertion and a 36 bp deletion (nucleotide 412–447) at position 412 in the CDS (Figure 5). The inserted fragment contained a 5 bp direct repeat (5'-AGAAC-3') at each end, which is a typical LTR retroelement insertion site (Figure S4). In addition to differences in the CDS, LA1 and la2 exhibited variation in the length of the introns (Figure 5).

Both LA1 and la2 had a CDS of 1152 bp encoding a polypeptide of 383 amino acids (Figure S1 and S5). Six point mutations at positions 567, 579, 666, 699, 777 and 1059 were observed in the CDS of la2 when compared with that of LA1, but did not lead to any amino acid changes. The 5' flanking sequences from the translation start site were cloned for LA1 and la2 using the primer pair No 3 (Table S1). The two alleles didn't show any differences in the cloned 5' flanking sequences. The genomic DNA sequences of LA1 and la2 were cloned using primer pair No 6 (Table S1) which was designed based on the 5' flanking sequence and the conserved 3' flanking sequence specific to the candidate SalFAD3.LA2 gene (Figure S3). Comparison of the coding and genomic DNA sequences indicated that the candidate SalFAD3.LA2 gene also contained 8 exons and 7 introns (Figure 5). Variation in the length of the introns was observed between LA1 and la2 (Figure 5). For instance, the third intron of la2 was 530 bp, while that of LA1 was 1165 bp.

Sequence alignment of LA1 and LA2 indicated that LA1 harboured a 9 bp deletion at position 46 (Figure 5 and Figure S1), which resulted in the loss of the three amino acids glycine-arginine-lysine at position 16. In addition, 77 point mutations were observed between LA1 and LA2 (Figure S1), of which 19 mutations led to amino acid changes (Figure S5). The candidate SalFAD3.LA1 and SalFAD3.LA2 genes exhibited differences in the cloned 5' flanking sequences (Figure S2), but had the same 3' flanking sequences. Variation in the length of the introns was observed among the four alleles LA1, la1, LA2 and la2 (Figure 5).

Phylogenetic analysis based on the polypeptide sequences encoded by LA1 and LA2 implied that SalFAD3.LA1 and SalFAD3.LA2 genes in yellow mustard were clustered with FAD3 genes in *Brassica* species (Figure 6). The SalFAD3.LA1 gene was grouped together with the FAD3 genes of *B. oleracea* (Genbank accession No.AGH20189), the C genome in *B. napus* (Genbank accession No.AFJ19037.1) and *B. juncea* (Genbank accession No.ADJ58020.1), whereas the SalFAD3.LA2 gene was in the same cluster with the FAD3 genes of *B. rapa* (Genbank accession No.Bra018348) and the A genome in *B. napus* (Genbank accession No.AFJ19039.1).

Co-segregation of the SalFAD3.LA1 and SalFAD3.LA2 Allele-specific Markers with C18:3 Contents in the F2 Populations

Primer pair 12 (Table S1) produced co-dominant markers of 742 bp, 510 bp, 626 bp and 1273 bp specific for LA1, la1, LA2 and la2, respectively, which co-segregated with the C18:3 content in all of the F2 populations. In the cross of Y1127 (low)×Y1035 (high), all of the nine possible genotypes were identified using the markers specific for LA1, la1, LA2 and la2 (Figure 7A; Table 3). The homozygous F2 plants (LA1LA1LA2LA2) had a significantly higher C18:3 content (average: 17.1%) than the heterozygous F2 plants of LA1LA1LA2la2 (average: 13.1%) (t = 6.12, p < 0.01) and of LA1LA1- LA2la2 (average: 15.5%) (t = 2.23, p = 0.04) (Table 3). The homozygous F2 plants of LA1LA1la2la2 had a higher average C18:3 content (14.0%) than those of la1la1LA2LA2 (average: 9.1%) (t = 5.81, p < 0.01). In the cross of Y1127 (low)×Y514 (medium), the three genotypes for C18:3 content were differentiated with the markers specific for LA1 and la2 (Figure 7B; Table 3). The average C18:3 content of the homozygous F2 plants (LA1LA1la2la2) was 12.7%, which was significantly higher than the heterozygous F2 plants (LA1la1la2la2), average: 9.2% (t = 5.02, p < 0.01) (Table 3). In the cross of Y514 (medium)×Y1035 (high), the markers specific for LA2 and la2 distinguished the three C18:3 genotypes (Figure 7C; Table 3). The homozygous F2 plants (LA1LA1LA2LA2) had an average C18:3 content of 15.8%, which was higher than the heterozygous F2 plants (LA1LA1la2la2), average: 13.8% (t = 2.23, p = 0.04) (Table 3). The SalFAD3.LA1 and SalFAD3.LA2 genes colocalized with the QTL peaks on Sal02 and Sal10, respectively (Figure 4). A new band was observed in the F1 and F2 plants with the heterozygote genotype of LA2la2 (Figs. 7A and 7C).
### Table 3. Co-segregation of the SalFAD3.LA1 and SalFAD3.LA2 allele-specific markers with C18:3 contents in the F2 populations of Y1127×Y1035, Y1127×Y514 and Y514×Y035.

| F2 populations | Allele-specific Markers | Genotype | No. of Plants | C18:3 Content (% of total fatty acids) |
|----------------|-------------------------|----------|---------------|---------------------------------------|
|                | LA1 LA2 | LA1 LA2 | LA1 LA2 | LA1 LA2 | Mean | Range |
| Y1127×Y1035   | +         | -       | +       | -       | 9    | 17.1  | 15.4–20.4 |
|                | +         | +       | +       | +       | 22   | 15.5  | 11.5–19.5 |
|                | -         | +       | -       | +       | 5    | 14.0  | 10.0–18.7 |
|                | +         | -       | +       | +       | 14   | 13.1  | 10.0–15.5 |
|                | +         | -       | +       | +       | 44   | 11.1  | 8.5–13.4 |
|                | -         | -       | +       | +       | 20   | 11.1  | 8.3–12.5 |
|                | +         | -       | +       | +       | 12   | 9.1   | 7.7–10.6 |
|                | +         | -       | +       | +       | 19   | 9.1   | 7.1–10.6 |
|                | -         | +       | -       | +       | 19   | 5.7   | 3.1–8.5 |
|                | +         | -       | +       | +       | 12   | 3.9   | 2.9–4.5 |
|                | -         | +       | -       | +       | 36   | 12.7  | 8.6–16.5 |
|                | +         | -       | +       | +       | 82   | 9.2   | 5.6–14.8 |
|                | -         | -       | +       | +       | 37   | 4.6   | 3.0–5.9 |
|                | +         | -       | +       | +       | 90   | 15.8  | 13.7–19.2 |
|                | -         | -       | +       | +       | 30   | 13.8  | 10.1–16.0 |
|                | +         | -       | -       | +       | 30   | 12.9  | 10.4–15.0 |

FAD3 Genes and Allele-Specific Markers in Yellow Mustard

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Discussion

The present paper reported on the inheritance and QTL mapping of C18:3 content as well as molecular characterization of the FAD3 genes in yellow mustard. Linolenic acid content was controlled by the nuclear genotype of the embryo in yellow mustard as reported in B. napus [8]. Two nuclear gene loci were detected and functioned independently and additively to determine the total C18:3 content in the seeds. However, maternal effects on the C18:3 content couldn’t be ruled out since appropriate progeny tests were not performed in the present study. QTL analysis further revealed that the two gene loci SalFAD3.LA1 and SalFAD3.LA2 had a different magnitude of effect and together explained 96.4% of the total variation for C18:3 content. The residual 3.6% variation of C18:3 content beyond the QTLs could be resulted from maternal and environmental effects. It has been reported that temperature, maternal genotype and cytoplasm have effects on C18:3 content. The residual 3.6% variation of C18:3 content beyond the QTLs could be resulted from maternal and environmental effects. It has been reported that temperature, maternal genotype and cytoplasm have effects on C18:3 content.

Interestingly, LAI and LAc were clustered into different groups. LAI was grouped together with the FAD3 genes of B. oleracea and the C genome in B. napus, whereas LAc was in the same cluster with the FAD3 gene of B. rapa and the A genome in B. napus. In our study, the LAI gene controlled a higher C18:3 content than the LAc gene. It was reported that the FAD3 gene of the C genome in B. napus also contributed more to the total C18:3 content than that of the A genome [11,12]. This suggested that the molecular divergence of the LAI and LAc genes occurred before the speciation of yellow mustard and Brassica species.

In conclusion, our study revealed the existence of two FAD3 gene loci contributing to the genetic variation of linolenic acid content in yellow mustard. The SalFAD3.LA1 gene was located in the linkage group Sal02, while the SalFAD3.LA2 gene in Sal10. We have cloned the SalFAD3.LA1 and SalFAD3.LA2 genes and developed allele-specific markers for the detection of desirable genotypes, which will be valuable for marker assisted breeding in yellow mustard.

Supporting Information

Figure S1 Alignment of the coding DNA sequences of the SalFAD3.LA1 alleles LA1, la1 and the SalFAD3.LA2 alleles LA2 and la2 in yellow mustard. The nucleotide sequence alignment was carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (PDF)
**Figure S2** Alignment of the 5’ upstream sequences of the *SalFAD3.LA1* and *SalFAD3.LA2* genes in yellow mustard. **UP*LA1** and **UP*LA2** represented the 5’ upstream sequences of the alleles *LA1* and *LA2* of the *SalFAD3.LA1* gene. **UP*LA1** and **UP*LA2** indicated the 5’ upstream sequences of the alleles *LA1* and *LA2* of the *SalFAD3.LA2* gene. The nucleotide sequence alignment was carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). (PDF)

**Figure S3** PCR amplification of the *SalFAD3.LA1* and *SalFAD3.LA2* genes. A. PCR amplification of the genomic DNA sequences of *SalFAD3.LA1* gene using the primer pair No 5 (Table S1). Lanes 1–2: 4628 bp fragment of *la1* from Y1127; Lanes 3–6: 4534 bp fragment of *LA1* from Y1035. B. PCR amplification of the genomic DNA sequences of *SalFAD3.LA2* gene using the primer pair No 6 (Table S1). Lanes 1–4: 4608 bp fragment of *la2* from Y1127; Lanes 5–6: 4042 bp fragment of *LA1* from Y1035. (PDF)

**Figure S4** Nucleotide sequences of intron 3 and its flanking *SalFAD3.LA1* and *SalFAD3.LA2* genes in yellow mustard. The sequence of exon 3 was underlined in red while that of exon 4 was lined in blue. The nucleotide sequence of the inserted fragment in exon 3 of *la1* was underlined in pink. The new intron splicing site GT in the inserted fragment was indicated in green rectangle box. The nucleotides in blue rectangle box indicated the inserted fragment that remained in the CDS of exon 3 of *la1*. The nucleotides in red rectangle box indicated the 5 bp direct repeat (5’-AGAAC-3’). The first and the last nucleotides of intron 3 were indicated by arrowhead and arrow, respectively. The intron 3 of *LA1*, *la1* *LA2*, *la2* are 647 bp, 415 bp, 530 bp and 1165 bp in length, respectively. (PDF)

**Table S1** Primers used in this study. (PDF)

**Author Contributions**

Conceived and designed the experiments: BC. Performed the experiments: ET FZ KM VR. Analyzed the data: ET FZ VR. Wrote the paper: ET FZ KM BC.

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