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# Cleaved Amplified Polymorphic Sequences (CAPS) Markers for Characterization of the *LuFAD3A* Gene from Various Flax (*Linum usitatissimum* L.) Cultivars

**Magdalena Walkowiak *,†, Marcin Matuszczak †, Stanisław Spasibionek *, Alina Liersch and Katarzyna Mikołajczyk**

Plant Breeding and Acclimatization Institute—National Research Institute, Department of Oilseed Crops, Strzeszyńska 36, 60-479 Poznań, Poland; m.matuszczak@ihar.edu.pl (M.M.); a.liersch@ihar.edu.pl (A.L.); k.mikolajczyk@ihar.edu.pl (K.M.)

* Correspondence: m.walkowiak@ihar.edu.pl (M.W.); s.spasibionek@ihar.edu.pl (S.S.)

† These authors contributed equally to this work.

**Abstract:** Depending on the variety, flax (*Linum usitatissimum* L.) provides an oil rich in omega-3 acids (especially 50% α-linolenic acid) with proven health properties, and is used as a raw industrial material. Alpha-linolenic acid is a polyunsaturated fatty acid easily subject to oxidative transformation. The auto-oxidation of α-linolenic acid is the main process contributing to off-flavor, color loss, and change in the nutritional value of flax oil. We used six flax genotypes differing in fatty acid content in our research. For all the DNA samples extracted from the leaf tissue of the studied flax, the new, cleaved amplified polymorphic sequences (CAPS) markers specific to the *LuFAD3A* desaturase gene were applied. A specific PCR product from the *LuFAD3A* flax gene comprising fragments of exon 5, exon 6, and the intron between these exons was digested using six various restriction enzymes. These experiments could differentiate between some of the studied varieties of flax. We also proved that the *LuFAD3A* gene mutation previously detected by other authors in the low-linolenic forms of flax (solin line 593–708) was absent in the low-linolenic forms of flax that we studied (Linola KLA and Linola KLB).

**Keywords:** *Linum usitatissimum* L.; cultivars; molecular genetics; genes; *LuFAD3A*



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## 1. Introduction

Flax (*Linum usitatissimum* L.) is one of the oldest cultivated plants in the world. It is an important source of oil rich in omega-3 fatty acids (especially α-linolenic acid, accounting for >50% of all the fatty acids) that have proven health benefits; it is also utilized as a raw industrial material. Depending on the variety, the composition of flaxseeds varies, but they are always rich in fat (30–45%), proteins (20–31%), and dietary fiber (20–46%), being responsible for their high-energy value of about 5100 kcal $kg^{-1}$ [1,2].

Among the edible oils, flaxseed oil is the richest source of α-linolenic acid (C18:3, omega-3), accounting for 57% of all fatty acids, and hence has high health-promoting properties [3–6]. However, an important factor limiting the large-scale commercialization of flax oil is its low durability, resulting from the high content of polyunsaturated acids in the oil. Thus, many efforts were undertaken to obtain low-linolenic forms of flax.

The α-linolenic acid (C18:3, omega-3) is formed as a result of linoleic acid desaturation with the participation of Δ15 desaturase, referred to as FAD3 (fatty acid desaturase 3) [7]. This reaction occurs both in the plastids and endoplasmic reticulum (ER). Vrinten et al. [8] identified two genes (*LuFAD3A* and *LuFAD3B*) coding the fatty acid desaturase 3 protein in the flax genome. Also, the mutations of these genes resulting in the low linolenic acid content in seeds were found, and the cleaved amplified polymorphic sequences (CAPS) markers [9] were designed for the detection of these mutants [8].

Other flax plants carrying this type of mutations were also studied. Following ethyl methanesulfonate (EMS) mutagenesis, Rowland [10] identified M2 flax carrying mutations of both *LuFAD3* genes. The resulting line had a linolenic acid content of less than 2% compared with levels of approximately 49% in the wild-type parent McGregor. Similarly, Green and Marshall [11] isolated two EMS-derived mutants with linolenic acid contents of approximately 30%; by recombining these lines, they could obtain plants with linolenic acid contents <2% [12]. Low-linolenic acid oil is less subject to rapid oxidation than oil from traditional flax cultivars and is thus more competitive as a cooking or salad oil [4].

Here, we present the analysis results of six forms of flax (*Linum usitatissimum* L.) using the newly designed CAPS markers. These markers are based on the sequences of flax *LuFAD3* genes obtained by Vrinten et al. [8] and on the genomic assembly obtained by Wang et al. [13]. We followed all the steps from the previously performed protocol for rapeseed [14]. We also redesigned the primer pairs previously used by Vrinten et al. [8] for analyzing these genes. Our experiments focused on the *LuFAD3A* flax gene because we failed to amplify the *LuFAD3B* gene fragment.

## 2. Materials and Methods

### *2.1. Plant Material*

The six cultivars Szafir, Oliwin, Linola KLA, Linola KLB, Escalina, and Modran were used as parents. Plant material was harvested from the field at PBAI-NRI (Poznań; 52°24′30″ N 16°56′03″ E). Based on the classification and origin of the studied cultivars, their characteristics are presented in Table 1. The cultivars of flax used in our research were previously described by Walkowiak et al. [15] and selected for the marker analysis.

**Table 1.** The origin of flax genotypes used as genetic material and fatty acid composition in half-seeds.

| Name | Type | Seed Colour | Origin |
|---|---|---|---|
| Szafir | Oil | Brown | Poland |
| Oliwin | Oil | Yellow | Poland |
| Linola KLA | Oil | Yellow | Canada |
| Linola KLB | Oil | Yellow | Canada |
| Escalina | Fiber | Brown | Netherlands |
| Modran | Fiber | Brown | Poland |

The two cultivars of flax Szafir and Oliwin have a very high α-linolenic acid content (>58%). The two cultivars of fiber flax Escalina and Modran have a medium content of α-linolenic acid (up to 51%). The next two similar genotypes are flax Linola KLA and Linola KLB, with a high linoleic acid content (about 70%) and extremely low α-linolenic acid content (about 2%).

### *2.2. Half-Seed Method*

Donor seeds were placed in Petri dishes and placed in a growth room at 22 °C/17 °C (16 h day/8 h night) for 24 h. Afterward, the seeds were cut crosswise (using Jousse et al.'s [16] method), and part of the seed with the germinal root was placed into the pot filled with garden soil (5 × 5 cm dimension, 5 half-seeds in one pot). and the cotyledon into a hexane vial. The flax plants were grown in a growth chamber at 22 °C/17 °C (day/night) under a 16-h photoperiod until the material was collected for DNA isolation (Figure 1). In parallel, the vials with the second half of the seeds were sent to the Biochemical Laboratory, where the analysis of fatty acid content was performed.

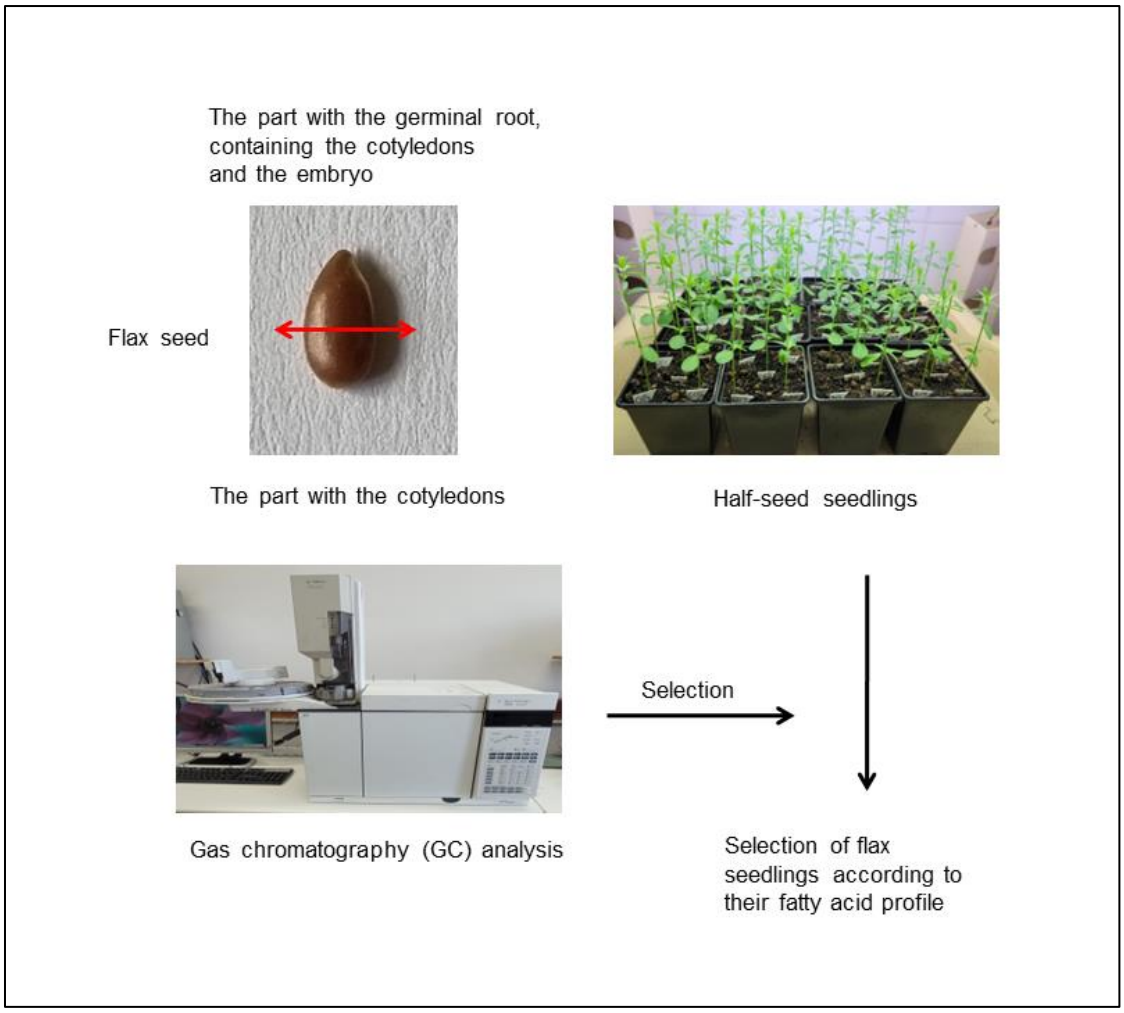


**Figure 1.** Diagram of the half-seed method—recreated based on the figure presented by Jousse et al. [16].

### *2.3. Biochemical Analyses (Fatty Acids Composition)*

The composition of fatty acids was determined using gas chromatography [17,18]. Fatty acids from seeds were extracted using hexane, and methyl esters of the extracted fatty acids were obtained. The Hewlett Packard Agilent Technologies 6890 N Network GC System was used to separate the extracted fatty acids. The results were calculated as the percentage of each fatty acid compared to the sum of all fatty acids.

Flax seedlings for the analyses of molecular markers were selected depending on their agronomic traits and obtained fatty acids profile.

### *2.4. Molecular Marker Analyses*

#### 2.4.1. Extraction of DNA

The DNA was extracted from leaves of flax using a standard procedure (with CTAB) as previously described by Doyle and Doyle [19], albeit with some modifications. The small fragments of flax leaves were homogenized in 1.5 mL Eppendorf tubes using 750 µL of ice-cold washing buffer [0.5 M sorbitol, 0.1 M Tris-base, 70 mM ethylenediaminetetraacetic acid (EDTA), final pH 7.5, with freshly added 20 mM sodium metabisulfite] and autoclavable plastic micropestles. After 2 min of centrifugation, the supernatant was discarded, and the pellet was resuspended in 750 µL of hot (65 °C) 2× CTAB extraction buffer [2% (*w*/*v*) cetyl trimethylammonium bromide (CTAB), 100 mM Tris-HCl (pH 8.0), 20 mM ethylene-diaminetetraacetic acid (EDTA) (pH 8.0), 1.4 M NaCl, 1% (*w*/*v*) polyvinylpyrrolidone (PVP), with freshly added 1% (*v*/*v*) β-mercaptoethanol]. The samples were incubated

at 65 °C for 30 min. After mixing with one volume of chloroform-octanol (24:1) (*v*/*v*), they were centrifuged at 11,500× *g* for 10 min. DNA from the collected aqueous phase was precipitated with a two-thirds volume of isopropanol. After 10 min of centrifugation, the supernatant was discarded, and the pellet was resuspended in TE buffer containing 40 µg/mL of RNAse A. After the digestion of RNA contaminants (at 37 °C for 1 h), the DNA was again precipitated with isopropanol supplemented with NaCl and finally rinsed with 70% cooled ethanol. The pellet was then resuspended in 100 µL of TE buffer.

The quality and concentration of all the DNA samples were tested by separation on a 0.8% agarose gel in Tris-borate-EDTA (TBE) buffer, followed by ethidium bromide staining. All the samples were diluted around 10 times with distilled water (the actual dilution rate depended on the results of concentration tests), and molecular marker analyses followed the dilution.

#### 2.4.2. CAPS Analyses

The cleaved amplified polymorphic sequences (CAPS) [9] marker analyses were performed for the prepared DNA samples. To obtain the marker, the *LuFAD3A* gene fragment comprising fragments of exon 5, exon 6, and the intron between these exons was amplified using two specific PCR primers in the reaction mixture. The forward primer, complementary to the sequence from exon 5 of the *LuFAD3A* gene, was the same as the one described by Vrinten et al. [8]; the reverse primer, complementary to the sequence from exon 6 of the *LuFAD3A* gene, was created de novo based on the sequences of the studied gene [8] and the genomic sequence of flax [13]. The sequences of the primers were as follows: FA3e5VF forward primer—5′-CAGTGACCTGTTCGCACCG-3′ [8] and FA3e6R reverse primer—5′-ACAGCCTGCAGCATACATCA-3′.

The reaction mixture (total reaction volume: 20–40 µL) contained PCR buffer (Fermentas/Thermo Scientific), 1.25 mM of $MgCl_2$ (Fermentas/Thermo Scientific), 0.2 mM of each dNTP (Sigma or Fermentas), 0.6 µM of each specific primer (FA3e5VF forward and FA3e6R reverse), 1.2–2.4 U of *Taq* polymerase (Fermentas/Thermo Scientific), and template DNA (4–8 µL of the diluted sample). Amplification was conducted using an Eppendorf Mastercycler ep Gradient thermal cycler with the following thermal profile: initial denaturation for 4 min at 94 °C (samples were directly transferred from ice to the hot block), 30 cycles for 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing), and 30 s at 72 °C (polymerization), and a final polymerization for 5 min at 72 °C.

Following PCR, a simplified procedure without purification of the amplified product was used [14]. The PCR product was digested using six restriction enzymes (Thermo Scientific and New England BioLabs) (Table 2). Approximately one-third of the initial PCR reaction mixture was used (7–15 µL, depending on the procedure) for the digestion reaction. Special care was taken to maintain an equal amount of DNA in both undigested (control) and digested samples loaded on the agarose gel. The digestion reaction mixture (total reaction volume: 14–30 µL) was prepared by the addition of a dedicated enzyme buffer (Thermo Scientific or New England BioLabs) and 10.5–22.4 U of the restriction enzyme (Thermo Scientific or New England BioLabs). The enzyme was added separately to each sample as the last component in order to avoid deactivation.

**Table 2.** The restriction enzymes used to study the *LuFAD3A* gene fragment from flax.

| Enzyme Name | Enzyme Source | Recognized Sequence | Buffer | Incubation Conditions |
|---|---|---|---|---|
| *Pvu*I | New England BioLabs | 5′-CGAT↓CG-3′ | NEBuffer r3.1 | 37 °C |
| *Hin*dIII | Thermo Scientific | 5′-A↓AGCTT-3′ | R | 37 °C |
| *Bsu*RI (=*Hae*III) | Thermo Scientific | 5′-GG↓CC-3′ | R | 37 °C |

**Table 2.** *Cont.*

| Enzyme Name | Enzyme Source | Recognized Sequence | Buffer | Incubation Conditions |
|---|---|---|---|---|
| *Hin*1II (=*Nla*III) | Thermo Scientific | 5′-CATG↓-3′ | G | 37 °C |
| *Vsp*I (=*Ase*I) | Thermo Scientific | 5′-AT↓TAAT-3′ | O | 37 °C |
| *Tru*1I (=*Mse*I) | Thermo Scientific | 5′-T↓TAA-3′ | R | 65 °C |

The procedure for electrophoresis was described previously by Matuszczak et al. [14]. Samples containing undigested or digested PCR products were analyzed by separation on a 1.8% agarose gel in the TBE buffer, followed by ethidium bromide staining. The undigested (control) and digested samples from the same plant were always placed side by side on the agarose gel for better comparison. Only about one-third of the initial PCR mixture (undigested or digested) was added to each well of the agarose gel (after mixing with an appropriate amount of the Loading Dye Solution—MBI Fermentas). Lambda DNA digested with *Hin*dIII and *Eco*RI restriction enzymes (Fermentas) (250 ng of digested DNA per well) was used as the size marker. The gels were photographed under UV light using a Vilber Lourmat Quantum ST4 1000 gel imaging system. The estimated sizes of the observed DNA fragments were calculated based on their relative position using a molecular weight analysis module of a Vilber Lourmat Quantum-Capt software. The bands from the lanes closest to the size marker lane were always considered for the calculations.

## 3. Results and Discussion

Before the CAPS analyses were performed, the sequences of two flax desaturase genes (*LuFAD3A* and *LuFAD3B*) were studied. The mRNAs of these genes were previously sequenced [8] and based on these sequences, we aligned them to the chromosomes Lu7 and Lu12 of the flax genome, respectively. The alignment was performed with a standard BLAST tool using the DQ116424.1 accession for the *LuFAD3A* gene [8], the CP027631.1 accession for the chromosome Lu7 of flax [13], the DQ116425.1 accession for the *LuFAD3B* gene [8], and the CP027622.1 accession for the chromosome Lu12 of flax [13]. The genome assembly used in our analysis (created for the flax cultivar CDC Bethune, Assembly Name: ASM22429v2, GenBank Assembly Accession: GCA_000224295.2, [13]), is an older one. We are aware that some better assemblies have recently become available (for example, the representative genome assembly made for the flax cultivar Longya-10, Assembly Name: ASM1066527v2, GenBank Assembly Accession: GCA_010665275.2, [20]). However, our purpose in using these data was to identify the chromosomal positions of the studied genes and to obtain genomic sequences that include introns. Therefore, we supposed the data obtained by Wang et al. [13] are of sufficient quality to achieve that. The sequences of the two desaturase genes were compared, and they appeared to be quite similar. Therefore, only some specific sequences can be used as templates for amplifying individual gene fragments. We performed the amplification step with the protocol and primers previously used by Vrinten et al. [8] for analyzing these genes. For the *LuFAD3A* gene, the initial results were quite optimistic (F3Ae5VF and F3A5i6VR primers, data not shown), whereas, for the *LuFAD3B* gene, we failed to amplify the product for most of the samples (data not shown). Therefore, we focused on the *LuFAD3A* gene for the other experiments presented here, however, we do still plan to analyze the *LuFAD3B* gene in the future. Based on the gene sequences, we identified the exon-intron structure of the *LuFAD3A* gene, with the help of the Splign tool. Using the Primer3 tool and forward primer sequence taken from Vrinten et al.'s [8] protocol, we designed additional primers for amplifying specific fragments of this gene. Various primer pairs were initially tested—all with the same forward primer. The amplification results obtained using these new primer pairs were generally in accordance with our expectations. Among the tested primer pairs, the most interesting one amplified the fragment comprising exon 5 and exon 6 of the *LuFAD3A* gene. This primer pair was used for all subsequent CAPS analyses (F3Ae5VF and F3Ae6R primers). The overview of

the presented CAPS markers concerning the structure and sequence of the flax *LuFAD3A* gene and genome, based on the model created using the sequence data from Vrinten et al. [8] and the genomic sequence from Wang et al. [13], is presented in Figure 2.

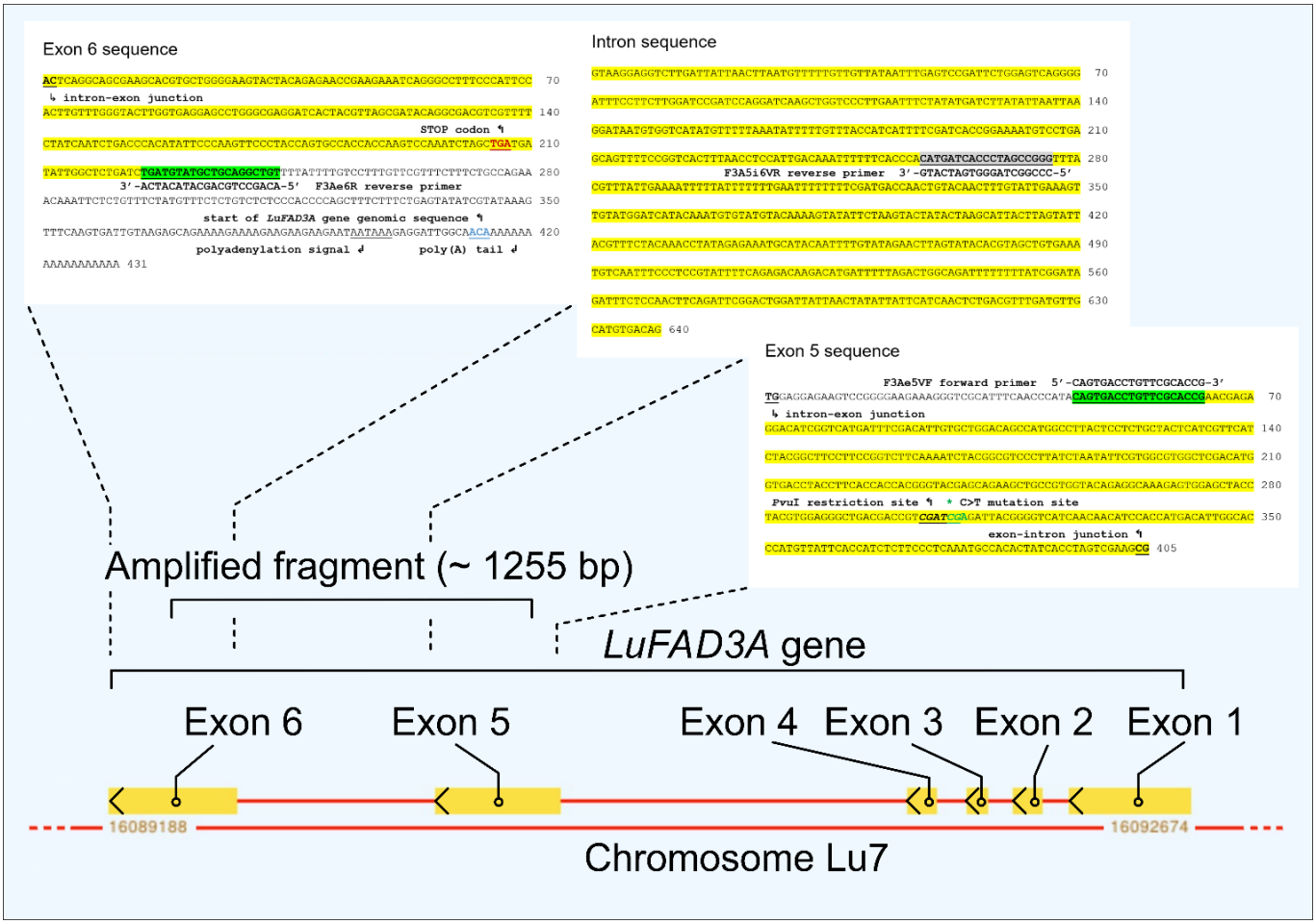


**Figure 2.** An overview of the presented CAPS markers concerning the structure and sequence of the flax *LuFAD3A* gene. The sequences shown for exon 5 and exon 6 of the *LuFAD3A* gene are based on the Vrinten et al.'s [8] mRNA sequence, and the sequence of intron was deduced from the genomic sequence presented by Wang et al. [13]. The sequence of the amplified gene fragment is marked yellow; the sequences complementary to primers are marked green for the pair of primers described in Materials and Methods (F3Ae5VF forward primer is the original Vrinten et al's [8] primer and the F3Ae6R reverse primer is a newly designed one), and marked grey for the Vrinten et al.'s [8] original F3A5i6VR reverse primer. Additionally, the *Pvu*I restriction site and the C>T mutation site identified by Vrinten et al. [8] in exon 5 are indicated (green asterisk). It must be noted that the *LuFAD3A* gene is encoded on the opposite strand when looking at the genomic sequence of the flax chromosome Lu7. Therefore, the start of the *LuFAD3A* gene genomic sequence from exon 6 [13] is also shown (underlined blue).

We performed analyses of the molecular markers for the basic set of six flax lines/varieties. Two individual plants represented each line/variety in the studied set, so for each restriction enzyme, 12 samples were analyzed. The standard DNA extraction procedure (with CTAB) was performed for all analyses; it was previously recommended for analyzing CAPS markers in *Brassica napus* L. [14]. During the extraction, some samples exhibited an excess of a gel-like substance from the flax tissue that interfered with obtaining the clean DNA product. These impurities were probably complex sugars extracted together with the flax DNA, increasing the difficulty of extraction. The analysis of the DNA samples from flax also

indicated problems with quality and concentration. When using the agarose gel (data not shown), some bands of genomic DNA were faint, or there were no bands at all. However, despite the observed variation in the quality and concentration of DNA crude samples, their functionality in the downstream application was generally good. It must be noted that the DNA concentration in the samples was equalized with distilled water according to the results of concentration tests prior to the molecular marker analyses (the final concentration was approximately 9 ng/µL), and that step was crucial to the obtained results.

For the initial amplification results obtained using the set of primer pairs from Vrinten et al. [8] and the set of newly designed primer pairs, the size of the amplified fragments matched the size deduced from the sequence; however, for some samples, no amplification products were obtained (data not shown). We supposed that this result was due to the inhibition of PCR. Nonetheless, there is a chance that it was due to some sequence alterations in the allelic forms of the *LuFAD3A* gene in the studied plant material. To avoid any mistakes during our interpretation of the results, we continued the molecular marker analyses using the primer pair that provided a distinct product for all the tested samples. The chosen pair amplified the fragment comprising both exon 5 and exon 6 of the *LuFAD3A* gene and the intron sequence between them. In this way, we hoped to detect some polymorphism among the studied lines, because the introns are known to be more polymorphic than exons. Some polymorphisms were detected during the first amplification step of the CAPS marker protocol; we hoped that the digestion step of the CAPS marker protocol would bring even more interesting results.

The 12 DNA samples were analyzed using cleaved amplified polymorphic sequences (CAPS) markers. A simplified procedure without purification of the amplified product was used for all the samples [14]. The results of these analyses are presented in Figure 3. After the amplification and digestion with the restriction enzymes, the bands were clearly visible for most samples in the resulting gel images.

Various band profiles were obtained and visualized after the digestion reactions (Table 3). These profiles depended on the alleles of the *LuFAD3A* gene present in the studied plants. We checked the theoretical size of the amplified DNA fragment based on the genome sequence [8,13], and found that the calculated size of the undigested product (control) generally matched the value deduced from the sequence (1261 bp experimental and 1255 bp theoretical size value) (compare Figures 2 and 3). However, the sum of calculated sizes for the digested DNA fragments differs from the calculated size of undigested DNA fragment (control) for almost all profiles. It is possible that the calculation method used for the estimation of sizes based on migration in agarose gel is not precise enough to obtain better results. Moreover, this may be the result of the presence of some undetected small fragments. In some cases (e.g., results for *Hin*1II and *Tru*1I enzymes, see Figure 3D,F, respectively), the larger fragments may mask themselves if they are of the same size and migrate together in the gel. Therefore, the actual number of restriction sites in the amplified DNA fragment is difficult to estimate, based only on these results.

In addition to the molecular marker analyses, the seeds of the studied plants were analyzed using gas chromatography to determine the actual fatty acid content. Our results confirmed that the Oliwin and Szafir cultivars are the standard oil flax cultivars, and the Linola KLA and Linola KLB lines are oil flax with low linolenic acid content in their seeds. The Escalina and Modran cultivars are standard flax for fiber cultivars (Table 4).

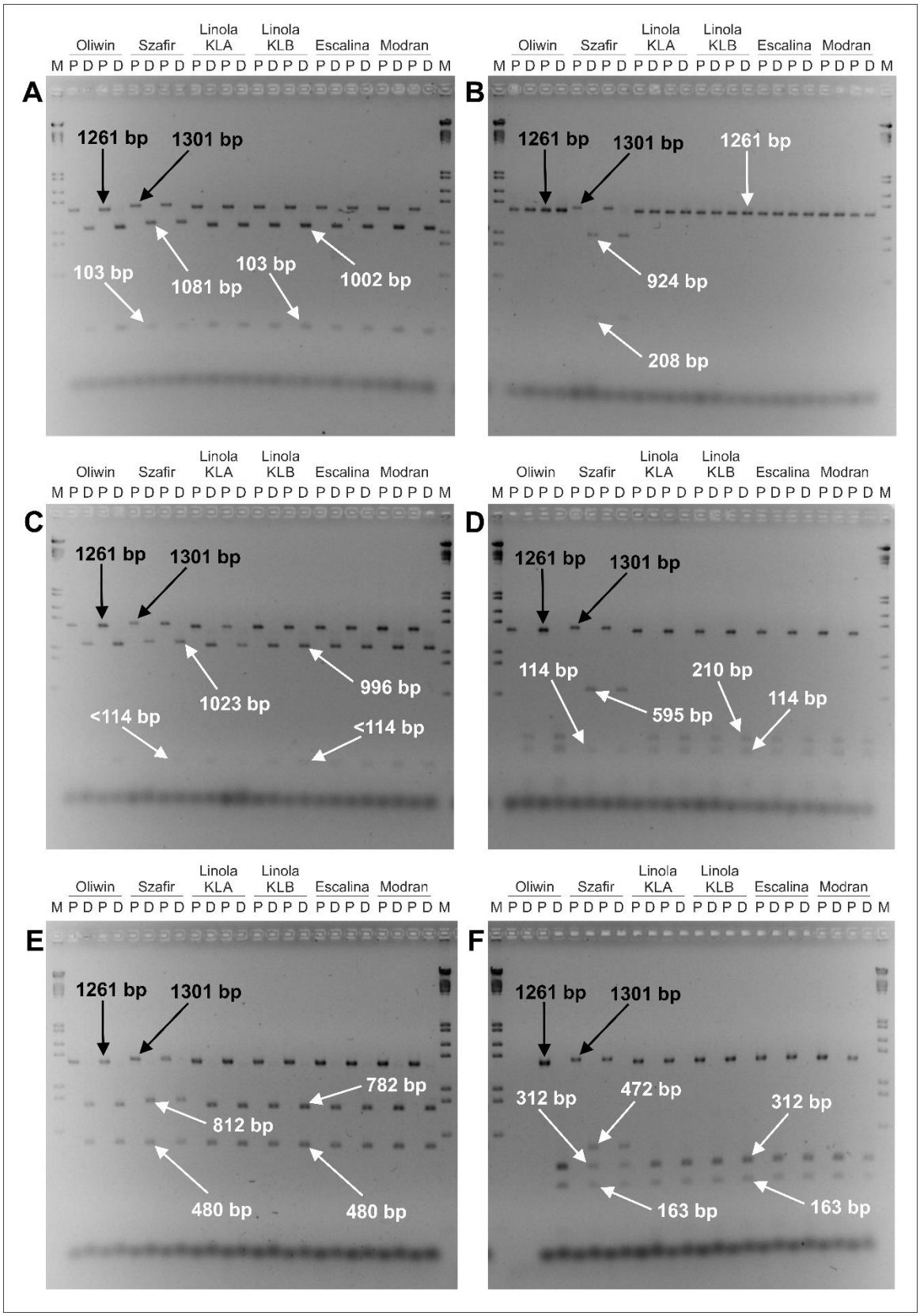


**Figure 3.** Results of analyses for twelve flax DNA samples using six CAPS markers obtained with the described primers and six various restriction enzymes. (**A**) *Pvu*I, (**B**) *Hin*dIII, (**C**) *Bsu*RI, (**D**) *Hin*1II, (**E**) *Vsp*I, (**F**) *Tru*1I. P—undigested (control) sample, D—digested sample, M—size marker; black and white arrows indicate obtained bands from the undigested (control) and digested samples, respectively.

**Table 3.** Band patterns of CAPS markers obtained using the described primers and six various restriction enzymes.

| Flax Cultivars | Estimated Size of the Amplified DNA Fragment (P Lane) | Restriction Enzyme | Estimated Sizes of DNA Fragments Obtained from the Digested Sample (D Lane) |
|---|---|---|---|
| Oliwin, Linola KLA, Linola KLB, Escalina, Modran | 1261 bp | *Pvu*I | 1002 bp, 103 bp |
| | | *Hin*dIII | 1261 bp |
| | | *Bsu*RI (=*Hae*III) | 996 bp, <114 bp |
| | | *Hin*1II (=*Nla*III) | 210 bp, 114 bp |
| | | *Vsp*I (=*Ase*I) | 782 bp, 480 bp |
| | | *Tru*1I (=*Mse*I) | 312 bp, 163 bp |
| Szafir | 1301 bp | *Pvu*I | 1081 bp, 103 bp |
| | | *Hin*dIII | 924 bp, 208 bp |
| | | *Bsu*RI (=*Hae*III) | 1023 bp, <114 bp |
| | | *Hin*1II (=*Nla*III) | 595 bp, 114 bp |
| | | *Vsp*I (=*Ase*I) | 812 bp, 480 bp |
| | | *Tru*1I (=*Mse*I) | 472 bp, 312 bp, 163 bp |

**Table 4.** The fatty acid compositions in half-seeds.

| Name | Oleic Acid C18:1 | Linoleic Acid C18:2 | α–Linolenic Acid C18:3 |
|---|---|---|---|
| Szafir | 24.7 | 11.0 | 54.6 |
| Oliwin | 18.8 | 14.4 | 57.6 |
| Linola KLA | 17.8 | 69.4 | 2.8 |
| Linola KLB | 18.4 | 68.7 | 2.7 |
| Escalina | 23.7 | 14.4 | 53.3 |
| Modran | 20.6 | 16.7 | 51.9 |

The previously described procedure for the CAPS marker [14] was proven as a suitable method for analyzing desaturase genes in flax. To obtain reliable results, it is recommended to follow the described procedure and to have the undigested (control) and digested samples from the same plant placed side by side on the agarose gel for better control of the reactions. We designed some specific primers and chose a set of restriction enzymes to focus on a specific region of the *LuFAD3A* gene. The designed CAPS markers were successfully used to identify the Szafir cultivar and distinguish this variety from the five other flax forms. This finding presented markers of potential interest for the breeders.

Many sequencing studies on the flax desaturase genes were previously conducted. Therefore, the structures and sequences of these genes and their products, as well as the levels of their expression, are already known [21–26]. It is of importance to characterize the flax *LuFAD3* genes in the particular plant of interest and to compare it with the existing known sequences. Based on the structure of these genes in the particular cultivar, and with the help of the previous findings, we can guess which mechanisms underly the changes in linolenic acid content. For example, some previous studies show that among the three loci found with the *LuFAD3* gene, only two (*LuFAD3A* and *LuFAD3B*, which were also studied by Vrinten et al. [8]) are supposed to be active and seem to be responsible for linolenic acid accumulation in flax. The expression patterns of these loci were highly correlated with linolenic acid accumulation during seed development. On the contrary, the role of the third *LuFAD3C* locus was rather small [21]. Based on these findings, we did not analyze the third locus at all, and finally, as was already mentioned, we focused only on the specific part of the *LuFAD3A* gene. This may of course limit the range of polymorphisms being checked. Many mutations correlating with linolenic acid content in seeds may be missing in our present analysis. However, the results of sequencing studies from other authors show that the *LuFAD3A* gene region that we analyzed was found to be the one where mutations resulting in the loss of desaturase activity occur

in many cultivars and lines. Apart from the mutants studied by Vrinten et al. [8], there are also some other examples of the specific mutations resulting in the appearance of the premature stop codon in various positions of the *LuFAD3A* gene and the production of the truncated protein [21–24,26]. This may also lead to the decrease of specific mRNA level via a mechanism termed nonsense-mediated mRNA decay (NMD) [21,23,27]. Both mechanisms, taken together, can cause the absence of the active enzyme coming from this locus and this may affect the final linolenic acid content in seeds. The same mechanism was revealed for the second locus (*LuFAD3B*) in the study made by Vrinten et al. [8] and was also reported by other authors [22]. However, another kind of mutation was also found in this locus, and the mechanism that underlies the inactivation of the resulting enzyme is different. The possible loss of enzyme activity is due to the mutation in the region of conserved His motifs (histidine box). The substitution of a histidine residue with other amino acids was found to disrupt the desaturase activity [21,22,25,26]. Additionally, the presence of possible complex regulatory pathways for the expression of the desaturase genes from flax was also reported [23,24]. Unfortunately, based on our results, we were unable to analyze such mechanisms in our flax cultivars, because we could not identify a specific mutation that correlated with low linolenic acid content in the studied Linola KLA and Linola KLB forms. We hope to find such mutations in the future and to compare it with the results of the described studies.

Our experiments were done mainly to characterize the flax material we are working with, and to make some initial comparisons of the *LuFAD3A* gene from our cultivars with the alleles of this gene from low linolenic flax forms cultivated elsewhere. The CAPS marker obtained using the *Pvu*I enzyme was previously used by Vrinten et al. [8] to recognize the presence of a mutation in the *LuFAD3A* gene allele from the low-linolenic solin line 593–708 (this mutation resulted in inactive desaturase in this flax line). Our CAPS marker, using a different primer pair with the same restriction enzyme, was also useful in detecting this mutation in flax. However, after testing our flax forms using this marker, we discovered that this mutation of the *LuFAD3A* gene is absent in both of the forms that we studied with low linolenic acid content (namely Linola KLA and Linola KLB), as well as in four other studied flax cultivars (the *Pvu*I restriction site is not lost, see Figure 3A and Table 3). Furthermore, the five new restriction enzymes used to obtain CAPS markers in our experiments did not recognize any sequence comprising mutations in the studied region of the *LuFAD3A* gene (from exon 5 to exon 6), resulting in the inactivation of desaturase. Moreover, the designed CAPS markers cannot differentiate between the two low linolenic lines (Linola KLA and Linola KLB) and the four other studied standard flax varieties. The only exception was the Szafir cultivar possessing some alternative sequences. This finding is probably due to the additional sequence inserted in the intron between exon 5 and exon 6 of the *LuFAD3A* gene, and cannot be linked to any specific mutation in the expressed region of this gene. We did not analyze the impact of this insertion on the expression of the *LuFAD3A* gene in the Szafir cultivar, however, the results of the biochemical analyses of fatty acids in seeds suggest that this has no effect on the phenotype. Our results confirmed that the Szafir cultivar is the standard oil flax with typical linolenic acid content in seeds (Table 4). Therefore, we think that the detected insertion must lie in the intron only, and the inserted fragment of genetic information is not expressed. Moreover, these results also suggest that the Szafir cultivar is genetically distant from the rest of the studied cultivars. Interestingly, in the group of five cultivars without the insertion we could observe strong phenotypic differences between the cultivars as they belong to various types of flax (Table 1). Such results may suggest that the insertion in the Szafir variety comes from some ancient event during the flax evolution. This also leads to the conclusion that the possible mutations in the alleles of *LuFAD3* genes from Linola KLA and Linola KLB lines, that are still to be found, probably come from some recent mutagenesis events that happened much later than the insertion in the intron of the Szafir variety.

Although the presented results lack the functional marker linked to low linolenic acid content in flax seeds, these results still have value as an experimental verification of the

*LuFAD3A* gene sequences from both the mRNA sequencing of this gene [8] and the whole flax genome sequencing project [13]. At this point we can only prove that the mutations in our low linolenic acid lines differ from the mutations identified by Vrinten et al. [8]. More investigations into the sequence of the *LuFAD3A* gene of Linola KLA and Linola KLB lines are needed to determine the real cause of low linolenic acid content in the seeds of these lines. At first, we will focus on the sequence analysis of the *LuFAD3A* gene, since in the present study we have already made some steps forward. We suppose that the mutations resulting in the appearance of the premature stop codon in the *LuFAD3A* gene, and the production of the truncated protein may be present in our low linolenic lines, similarly to the mutations revealed by other authors [8,21–24,26]. The positions of such mutations may be different than that detected by Vrinten et al. [8], and the sequencing of the studied gene should bring some results in this matter. Of course, some other mechanisms are also possible, and only the analysis of the whole gene sequence, including the sequence of the promotor region of the gene, can lead to the final results. At the same time, the analysis of other genes associated with the linolenic acid content in flax seeds, especially including the *LuFAD3B* gene from chromosome Lu12, is also needed. It is possible that some mutations affecting the histidine box of the *LuFAD3B* gene from our lines will be detected through the sequencing of this gene, as has already been revealed by other authors [21,22,25,26]. We plan to examine both *LuFAD3* genes to conduct a complete molecular test for that important trait. Nevertheless, we hope that the experiments presented here may be helpful in future studies aiming at the complete monitoring of alleles for all desaturase flax genes and the analyses of existing phenotype–genotype interactions.

## 4. Conclusions

1. Based on the present results, we can say that the previously described procedure for the CAPS marker can be used to analyze desaturase genes in flax.
2. The presented markers can be successfully used to identify the Szafir cultivar and to distinguish this variety from the other five flax forms. Therefore, breeders may potentially use these CAPS markers for assurance of the Szafir cultivar or its offspring's purity.
3. Our results suggest that the previously identified mutation in the *LuFAD3A* gene resulting in inactive desaturase was absent in both forms of low linolenic flax that we studied (namely Linola KLA and Linola KLB), as well as in four other flax cultivars. Therefore, it must be noted that none of the CAPS markers described in this article can be used as an effective selection tool for breeding flax with low linolenic acid content in seeds.

**Author Contributions:** S.S. conceived the project and was involved in funding acquisition and project administration; M.W. and S.S. provided the flax plants; M.M. carried out bioinformatics and planned the CAPS markers based on the gene sequences; A.L. prepared DNA samples for analysis; M.W. and M.M. carried out molecular analyses, analyzed experimental results and wrote the manuscript; M.M. provided visualization; S.S. and K.M. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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