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REGULAR ARTICLE

SCREENING OF COMMON FLAX *FAD* GENES BY PCR

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ABSTRACT

Currently, flax (*Linum usitatissimum* L.) is an important crop from commercial and economical aspects. In the spotlight is the linseed oil as a source of α-linolenic acid. The aim of presented study was to analyse fatty acid desaturase (*FAD*) genes in flax. Several genotypes of flax (*Hohenheim*, *La Plata 1938*, *Redwing USA* and *Escalina*) were used. The primers described by **Vrinten *et al.* (2005)** were used for PCR amplification reactions. Two *FAD3* genes, *LuFAD3A* and *LuFAD3B*, were identified in a genome of flax. Subsequently the nucleotide sequences between origins and genotypes of flax *FAD* genes were compared. Primarily were used the nucleotide sequences of *FAD2* and *FAD3C* genes available in NCBI database. Differences were found using BLAST program in nucleotide sequences of *FAD* genes and the specific primers were designed to amplify a specific target sequences in a genome of flax. These primers were used in PCR amplification reactions to identification of *FAD2* and *FAD3C* genes. The PCR products were separated by electrophoresis on agarose gel.

Keywords: Flax, PCR, *FAD* genes.

INTRODUCTION

Flax (*Linum usitatissimum* L., 2n = 2x = 30), is an annual self-pollinated crop **(Cloutier *et al.*, 2012)**. Flax belongs to the family *Linaceae*, order *Malpighiales*. The genus *Linum* consists of approximately 200 species, of which *L. angustifolium Huds*. is considered the wild progenitor of cultivated flax, *Linum usitatissimum L* **(Ragupathy *et al.*, 2011)**. Flax has been cultivated for around 9000 years and is therefore one of the oldest cultivated species. Flax – derived products have wide industrial use **(Fenart *et al.*, 2010)**.

Flax is commercially grown as a source of stem fibre and seed oil **(Cloutier *et al.*, 2012)**. In recent years *Linum usitatissimum* is economically very important fibre (flax) and oilseed (linseed) plant. Both flax and linseed are specialised developments of a single species, which originates from the Mediterranean and Southwest Asian regions and it was also among the earliest cultivated plants in Europe **(Millam *et al.*, 2005)**.

Flax seed oil is a rich source of *α*-linolenic acid (ALA), an essential dietary fatty acid of *ω*-3 class **(Khadake *et al.*, 2010)**. Linolenic acid is produced through the desaturation of linoleic acid by omega-3/delta-15 desaturases **(Vrinten *et al.*, 2005)**. Linseed crops are grown for the seed oil, which has a high oil content (over 50 % total) and the presence of polyunsatured fatty acid makes the oil higly susceptible to oxidation **(Millam *et al.*, 2005)**.

Desaturases are enzymes that drive the multi-step biosynthetic fatty acid pathway in a sequential manner, leading to synthesis of polyunsaturated fatty acids. Desaturase genes have been isolated and characterised in several plants, including flax **(Khadake *et al.*, 2010)**.

The fatty acid biosynthesis pathway is a primary metabolic pathway, because it is found in every cell of the plant and is essential to growth. In plants, fatty acid biosynthesis starts with acetyl-CoA, as the donor of 2-carbon units involved in a series of elongation steps catalyzed by four fatty acid elongase (FAE) enzymes, including the b-ketoacyl synthase (KAS) family **(Ohlrogge *et al.*, 1995)**.

The delta-15 desaturase *Fad3* gene in flax converts *ω*-6 linoleic acid (18 : 2) to linolenic acid (18 : 3). Two major isoforms of the delta-15 desaturase gene have previously been reported in flax, namely *Fad3A* and *Fad3B*, which control levels of ALA and share 95.4 % similarity at the amino acid level **(Khadake *et al.*, 2010)**.

Two independently inherited genes control the low-linolenic-acid trait in flax. Two genes, *LuFad3A* and *LuFad3B*, were identified. These genes encode microsomal desaturases capable of desaturating linoleic acid. Low-linolenic lines have 2–3 % linolenic acid,

a phenomenon that could be explained by the production of small amounts of *FAD3A* and/or *FAD3B* **(Vrinten *et al.*, 2005)**.

MATERIAL AND METHODS

Genomic DNA was isolated from flax using the optimised method described by **Rogers *et al.* (1994)**. The obtained DNA was quantified by NanoPhotometer IMPLEN.

Fatty acyl desaturase (*FAD*) genes were analysed in flax. Firstly, two *FAD3* genes (*LuFAD3A*, *LuFAD3B*) were identified using the primers described by **Vrinten *et al.* (2005)**. Secondly, *FAD2* and *FAD3C* genes were identified using the optimized protocol. The amount of DNA in reaction (10–50 ng in 15 μl total volume), the primer concentration (250–400 nmol.dm^{-3}) and $MgCl_2$ concentration (3–4,5 mmol.dm^{-3}) were optimized.

According to the optimization, PCR amplification reactions to identification of *LuFAD3A* and *LuFAD3B* genes were performed in a 10x Dream *Taq* Green Buffer solution – 20 mmol.dm^{-3} Tris-HCl (pH 8,8), 100 mmol.dm^{-3} KCl, 20 mmol.dm^{-3} $MgCl_2$, together with 0,8 mmol.dm^{-3} d NTP (Fermentas), 1U Dream *Taq* DNA Polymerase, 400 nmol.dm^{-3} of each primer (Microsynth) and 15 ng of template DNA. PCR water was added into 15 μl final volume of reaction.

The PCR cycling conditions were as follows: 94 °C for 4 minutes (initial denaturation), then followed by 25 cycles at 94 °C for 45 second (denaturation), 61 °C for 30 second (annealing), 72 °C for 2 minutes (polymerization) with a final 10 minutes extension at 72 °C and then cool down to 4 °C **(Vrinten *et al.*, 2005)**.

PCR reactions to identification of *FAD2* and *FAD3C* genes were performed in a Termo Scientific Dream *Taq* PCR Master Mix (2x); Dream *Taq* DNA polymerase is supplied in 2x Dream *Taq* buffer, dATP, dCTP, dGTP and dTTP (0,4 mmol.dm^{-3} each) and 4 mmol. dm^{-3} $MgCl_2$, together with 400 nmol.dm^{-3} of each primer (Microsynth) and 15 ng of the genomic DNA in a volume of 15 μl.

The PCR program consisted of a 5 minutes initial denaturation cycle at 95 °C, followed by 35 cycles of 95 °C for 30 second (denaturation), gradient of temperature 50 – 60 °C for 45 second (annealing), 72 °C for 2 minutes (polymerization) with a final 10 minutes extension at 72 °C and then cool down to 4 °C.

Primers used in PCR reactions for identification of *LuFAD3A* and *LuFAD3B* genes are listed in the tab 1. Designed primers for identification of *FAD2* and *FAD3C* genes in genome of flax are shown in the tab 2.

Table 1 Characterization of applied primers, **Vrinten *et al.* (2005)**

Name of primer		Sequence of primer
MutAF2	LuFAD3A, forward	5`-CAG TGA CCT GTT CGC ACC G-3`
MutAR2	LuFAD3A, reverse	5`-CCC GGC TAG GGT GAT CAT G-3`
Lu15BFLF	LuFAD3B, forward	5`-TTC AAA ACT GTG GCT CTG CAG-3`
NcDNAbEndR	LuFAD3B, reverse	5`-TCA CAT TGT TCA ACA ACC AGA-3`

Table 2 Characterization of designed primers

Name of primer		Sequence of primer
FAD2ID	Forward	5`-TTG ATC GTG ATG AGG TGT TT-3`
	Reverse	5`-GCG ATC ATT GTA AAT CGG AC-3`
FAD3CID	Forward	5`-TTC ACT ATC CCT CTC CCA AT-3`
	Reverse	5`-AGA AAC TTG ACA GGG AAC TC-3`

FAD2ID – the set of primers for identification of FAD2 gene in a genome of flax

FAD3CID – the set of primers for identification of FAD3C gene in a genome of flax

Amplified fragments were electrophoretically separated in 1,5 % agarose (Top-Bio) gel together with 1 × TBE and Gel Red 10 000 × (Biotium). Electrophoresis was conducted at a voltage of 65 V for 2 h. Electrophoreograms were processed with documentation system G:Box in GeneSnap program - Product version: 7.09 (Syngene) and GeneTools - Product version: 4.01 (Syngene).

The specific primers were designed for identification of *FAD2* and *FAD3C* genes from the comparison of known nucleotide sequences of flax *FAD2* and *FAD3C* genes available in NCBI database and these primers were used in PCR amplification reactions.

The primers for identification of *FAD3C* genes are marked in the figure 1:

```
   1 catgattcgt ctcggtcgac aatgaaaatt attcaatagt ataattagaa catgaatctt
  61 ttttacatct cgcaattatc tctacccatc cattcccaat ccggatccaa taatattcat
 121 tcttatagat ttctgctcat gtctaaatat ggcttaggtt taccttattc tcttaaataa
 181 tttaattctt attttctttt gaataaaaga aaataaaata ttttggtacc ataaatgagt
 241 ttttttggt tgtagaaact aaattagttt ccaccaattt ttctattaaa attaccatat
 301 tcgaatgggt attaattact gaactactcg ttgagacatc acttctcatt ccccattatt
 361 cctttgatct tgtttgttaa ttcacgtctc tctaataaat tggttgagtt tggtttggtc
   -
1501 tcaattggca tcagtttgtt ctgtttcaat gcttgatatg aattgggtga tataatgcag
1561 ctgccggaga aagtatacaa gaccctggac acaagcacca aattcatgag gttcactatc
1621 cctctcccaa tgtttgctta tcctatctac ttggtaagaa ccaccccaca ttcttacatc
1681 cttgcatgtt gtgtattggt ttctggttgg atttggattc agatttgggg aattgattgt
1741 gtgcctttga aatgaatgaa tggttacagt ggacgaggag cccagggaag aaagggtccc
1801 atttcaaccc ctacagtgac ctgtttgccc cacaagaaag gaaatcagtc ttaatctcta
1861 ccatctcttg gatttccatg gtcctaatcc tcctctacgc ctccttcctt tttggtttcc
1921 tcactgtctt caaagtctat accgtccctt acctggtaaa actactatct ccaaattcaa
1981 caatctgtaa gcttcatttc atttgctttg gctcctgagt tccctgtcaa gtttctccta
2041 gcagtgtaac ttgttatttg ataactttgt gccatctcac tattggctgc attttactat
2101 aatcaaactg ctacatacgt tattgcaact tgttaatcct tggtacgagg aatccaatga
2161 ataggatggt gattagcaca aacaaggctg aaactttgag caattttgca gatatttgtg
2221 gcgtggctgg acatggtgac atacctgcac caccacgggc acgaagagaa gctgccgtgg
2281 tacagaggtc aagagtggag ctacctacgt ggagggctga caaccgtaga tagagattac
2341 gggattatca acaacatcca ccacgacatt ggcactcacg tgattcacca tctgttccct
2401 caaatccctc actaccatct cgtagaagcg gtacagtagt agtaaacaaa accccatatt
   -
3541 ctttcacgag acaaattgga gttcaactca aacttcactc attaaaactt tcccgtgagt
3601 ccaatttgaa gtctgtaaac taaaaaaata tgaatacgac aagctatttt aaaacttatt
3661 tgggagtgtg agaggttcaa actctcaacc ttaagatcac tcatactagg ccaccaccgc
3721 ttcatgtctt ga
```

Figure 1 *Linum usitatissimum* fatty acid desaturase 3C (*FAD3C*) gene, complete cds
NCBI GenBank: HM991836.1 3732 bp linear DNA

The primers for identification of *FAD2* gene are marked in the figure 2:

```
  1 atgggtgcag gtggaagaat gtcagtgcct ccaccatcca gacctatgaa gaggtctcct
 61 tactcaaagc caccattcac gctcggtgag ctcaagaagg ccattcctcc acactgtttc
121 aaacgttcaa tcccccgatc gttcgcctac gtggcgtacg acctcaccat tgcagcaatc
181 ttctactaca tcgccaccac ttacttccac ctcctcccta gccctctcaa ctacctcgcc
241 tggccggtct actgggcctg ccagggctgc atcctcactg gagtatgggt gttggctcac
301 gaatgcggtc accatgcctt cagcgactac cagtggctcg acgacatggt tggcttcgtc
361 ctccattcgt ccctccttgt tccttacttc tcctggaagc acagccaccg ccgccaccat
421 tccaacacgg ggtcgcttga tcgtgatgag gtgtttgtcc ccaagcagaa ggccgaaatc
481 gggtggtact ccaagtacct taacaaccca cctggccgtg tgatcacatt ggccgtcaca
541 ttaacgctcg gttggcctct gtacttggca ttcaacgtct ccgggagacc atatgaccgg
```

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 601 ttcgcatgcc atttcgaccc tcatggtccg atttacaatg atcgcgagcg tatggagata
 661 tacctatccg acgcagggat attcaccgtg tgctacatcc tatacagact cgtcctcacg
 721 aaaggactcg tttgggtcgt gtccatttac ggagtcccac tattgatagt gaatggattc
 781 ctagtcctca tcactttctt gcagcacacg catccttctc ttccgcacta caagtcctcc
 841 gaatgggact ggctgcgagg cgccctctcg accgtggatc gagactacgg gttactcaac
 901 accgtgttcc acaacatcac cgacacacat gtcgcgcacc atctcttctc cacgatgcct
 961 cattaccacg cgatggaggc taccaaggcg atcaagccgg ttctcgggga gtattaccag
1021 ttcgatggga ctccctttgt gaaggccatg tggagggagg caaaggagtg catctatgtc
1081 gagccggatg aaggcgaccc cagccaaggc gtgttctggt acaacaataa gttatga
```

Figure 2 *Linum usitatissimum* cultivar NL 97 omega-6 desaturase (*FAD2*) gene, complete cds NCBI GenBank: EU660502.1 1137 bp linear DNA

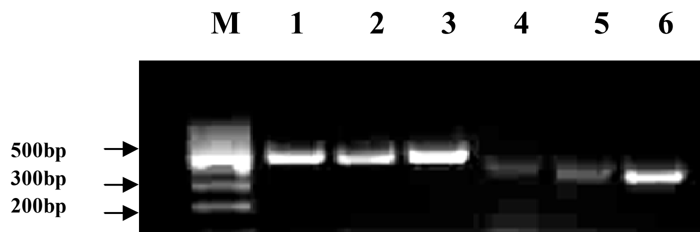
RESULTS AND DISCUSSION

In this study were used the primers described by **Vrinten *et al.* (2005)** for identification of *LuFAD3A* and *LuFAD3B* genes. Two pairs of primers were designed to amplify a specific target sequences in a genome of flax for identification of *FAD2* and *FAD3C* genes.

In the table 5 are shown genotypes of *FAD3* gene available in NCBI database. Based on the genotypes of *FAD3C* gene and their nucleotide sequences the pair of primers (forward and reverse) was designed for identification of *FAD3C* gene in flax.

Table 5 Genotypes of *FAD3* gene in flax available in NCBI database

	Genotypes	Accessions
FAD3C	SP2047	HM991839.1
	M5791	HM991837.1
	UGG5-5	HM991830.1
FAD3B		
	SP2047	HM991835.1
	M5791	HM991833.1
	UGG5-5	HM991834.1
FAD3A		
	SP2047	HM991831.1
	M5791	HM991829.1
	UGG5-5	HM991830.1



M – molecular marker

Samples 1–3: identification of *LuFAD3A* gene using the primers MutAF2, MutAR2 (genotypes *Hohenheim*, *La Plata*, *1938 Redwing USA*)

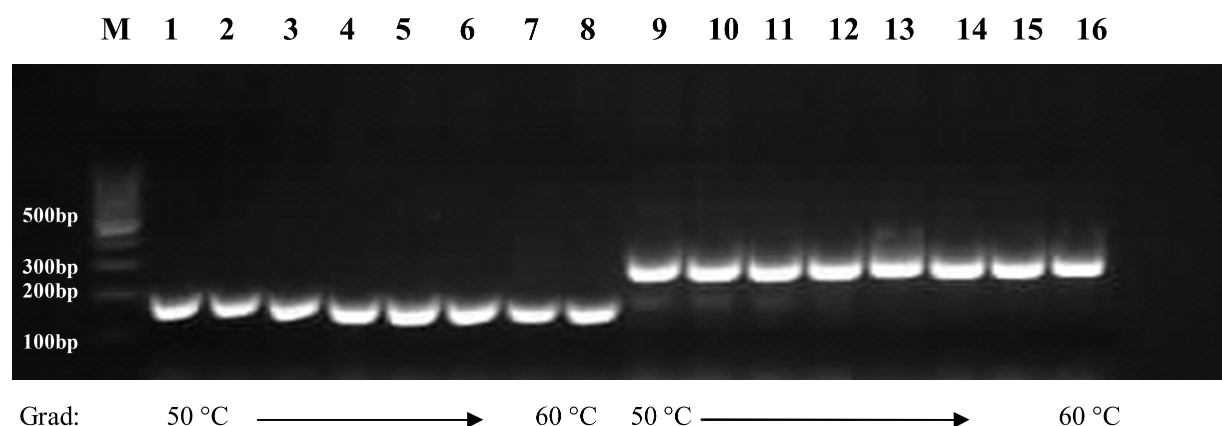
Samples 4–6: identification of *LuFAD3B* gene using the primers Lu15BFLF, NcDNAbEndR (genotypes *Hohenheim*, *La Plata*, *1938 Redwing USA*)

Figure 3 Identification of *LuFAD3A* and *LuFAD3B* genes using the primers described by **Vrinten *et al.* (2005)**

Using NCBI database by BLAST program were found different nucleotide sequences of *FAD* genes in flax. The nucleotide sequences of *FAD3C* gene were compared and the gaps or different nucleotides between origins and genotypes are shown in the figure 4. Gaps are indicated by dashes. Comparing of nucleotide sequences is very important for primers designing. Similarly were compared nucleotide sequences of *FAD2* gene and the specific primers were designed for identification of *FAD2* gene in flax.

```
Origin          2526 cggggggggggggggggg----ttgtttggatctttcgaacttataat 2570
Genotype SP2047 2525 cggggggggggggggggggggggttgtttggatctttcgaacttataat 2574
Origin          2526 cggggggggggggggggg---ttgtttggatctttcgaacttataata 2570
Genotype M5791  2580 cgggggggggggggggggggggttgtttggatctttcgaacttataata 2626
Origin          3248 gaaactaaatgatgtaaaataagcttcttcgtgacat-gacttt-atgg 3294
Genotype M5791  3304 ggaactaaatgatgtaaaataagcttcttcgtgacattgactttatgg 3352
Origin          2532 ggggggggggggg--ttgttttggatctttcgaacttataataatatga 2577
Genotype UGG5-5 2532 gggggggggggggggttgttttggatctttcgaacttataataatatga 2579
```

Figure 4 Comparison of selected nucleotide sequences of *FAD3C* gene



M – molecular marker

Samples 1–8: identification of *FAD2* gene using the primers FAD2ID (variety *Escalina*)

Samples 9–16: identification of *FAD3C* gene using the primers FAD3CID (variety *Escalina)*

Figure 5 Identification of *FAD2* and *FAD3C* genes in genome of flax

Flax seed oil is utilized for the fabrication of various biodegradable products. Flax oil is a rich source of omega-3 fatty acids used as nutraceuticals and also as a functional food for both humans and animals **(Cloutier *et al.*, 2012)**. Linseed oil is composed of main fatty acids, namely palmitic, stearic, linoleic and linolenic **(Cloutier *et al.*, 2011)**.

In our study we have considered with fatty acyl desaturase (*FAD*) genes. *FAD* genes were isolated and characterized in many plants, including flax. The presence of *FAD2* and *FAD3C* genes were confirmed by PCR. Two *FAD3* genes, *LuFAD3A* and *LuFAD3B*, were identified using the primers described by **Vrinten *et al.* (2005)**.

Nowadays, flax is widely screened for molecular markers (**Žiarovská, 2006; Kertészová et al., 2007; Ražná et al. 2007; Žiarovská et al, 2009, 2010; Smýkal et al, 2011**), as well as for *FAD* genes (**Vrinten *et al.*, 2005; Khadake *et al.*, 2010; Ražná, Žiarovská, 2012**).

The two *LuFAD3* genes have very similar amino acid sequences, and both are capable of desaturating linoleic acid. In the study of **Vrinten *et al.* (2005)** were provided further evidence that *LuFAD3A* and *LuFAD3B* are the major genes responsible for desaturation of linoleic acid in flax.

Khadake *et al.* (2010) in their study analysed the sequence variants of delta-15 desaturase from flax and predicted their structure. The *Fad3* gene variants were analysed in yeast for their functionality and compared on the basis of their rate of conversion of substrate fatty acids.

Banik *et al.* (2011) cloned three genes encoding fatty acid desaturase *FAD3* (*fad3a*, *fad3b*, and a novel *fad3c*) from four flax genotypes varying in linolenic acid content. Real-time PCR was used to quantify expression levels of the three *fad3* genes during seed development. High amounts of both *fad3a* and *fad3b* transcripts were observed and reached their peak levels at 20 days after anthesis. The fatty acid composition was analysed for all genotypes and stages of development and compared with the *fad3* gene expression patterns. Results showed that *fad3a* and *fad3b* are responsible for linolenic acid accumulation in flax seeds but did not support a major role for the novel *fad3c*.

In the present study were analysed and compared nucleotide sequences between origins and genotypes of *FAD2* and *FAD3C* genes by the NCBI BLAST program. Primers can be found by searching in highly conserved regions in genome of flax. Based on the comparing of nucleotide sequences were designed two pairs of primers for identification of *FAD* genes (FAD2ID, FAD3CID) in flax.

The microsomal delta-12 desaturase gene coding the enzyme fatty acid desaturase 2 (*FAD2*) is primarily responsible for more than 90% of the polyunsaturated fatty acid in non-photosynthetic tissues, such as roots and developing seeds of oilseed crops **(Miquel *et al.*, 1992)**.

Krasowska *et al.* (2007) cloned a putative delta-12 fatty acid desaturase *FAD2* gene encoding a 378 amino acid protein. Heterologous expression of this protein in yeast as an N-terminal fusion to green fluorescent protein (GFP) showed its localization within endoplasmic reticulum. Analysis of membrane lipids revealed the production of dienoic fatty acids, decreased levels of *FAD2* substrates and an increased concentration of longer fatty acids. In contrast to other *FAD2* genes expressed in yeast the extent of accumulation of dienoic acids was lower.

Fofana *et al.* (2004) have identified two copies of *FAD2* gene expressed in developing seeds of flax with closely matching sequences, but the sequences available are incomplete.

Khadake *et al.* (2009) in their study have cloned and characterized a new isoform of an intron-less *FAD2* gene from flax genomic DNA, which is 1.149 base pairs long, encoding a protein of 382 amino acids and revealing all the typical features of a membrane-bound desaturase.

CONCLUSION

By comparing the nucleotide sequences of *FAD2* and *FAD3C* genes available in NCBI database by BLAST program we can design the specific primers for amplification of target sequence in genome of flax. It is very important to know the differences and gaps in nucleotide sequences. Designed sets of primers, FAD2ID and FAD3ID, were used to identify the presence of *FAD2* and *FAD3C* genes. Results showed that these primers are suitable for identification of *FAD* genes. Two *FAD3* genes (*LuFAD3A*, *LuFAD3B*) were characterized using the sets of primers described by **Vrinten *et al.* (2005)**.

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