JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

EVALUATION OF THE FATTY ACID DESATURASE 3 GENES STABILITY IN FLAX SEEDS HARVESTED FROM RADIO-CONTAMINATED AREA NEAR CHERNOBYL

Veronika Lancíková[*1], *Jana Žiarovská*[2]

Address(es):
[1]Institute of Plant Genetics and Biotechnology, Plant Science and Biodiversity Center, Slovak Academy of Sciences, Nitra, Slovakia.
[2]Department of Genetics and Plant Breeding, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture, Nitra, Slovakia.

*Corresponding author: veronika.lancikova@savba.sk

doi: 10.15414/jmbfs.2020.9.4.816-819

ARTICLE INFO

Received 23. 5. 2019
Revised 16. 9. 2019
Accepted 22. 10. 2019
Published 3. 2. 2020

Regular article

OPEN ACCESS

ABSTRACT

Environment around Chernobyl recoveres from nuclear accident in 1986 and provides a base for plant adaptation research. Here, the initial platform was established for molecular screening of flax genome affected by radioactive contamination. Flax (*Linum usitatissimum* L.) cultivated in the radio-contaminated, and remediated experimental field near Chernobyl was investigated. Specifically, two gene isoforms of *FAD*3 gene, *FAD*3A and *FAD*3B, were evaluated using the restriction analysis, and direct sequencing. The aim of the presented study was to identify mutations, and single nucleotide polymorphisms within the *FAD*3A and *FAD*3B genes. The investigation was performed more than 25 years after Chernobyl accident. Therefore, the effect of chronic low dose rate irradiation on flax plants was considered.

Keywords: Chernobyl, *FAD*3A, *FAD*3B, restriction analysis, direct sequencing

INTRODUCTION

The accident at Chernobyl Nuclear Power Plant (CNPP) released a total of 7.4 x 10^{16} Bq of radionuclides into the environment (**Møller and Mousseau, 2006**). Over the years, the radiological situation in the affected areas was stabilized and the high-dose-rate acute radiation was replaced with persistent low-dose-rate chronic exposure (**Geraskin, 2003**). It is well known that the exposure of any organism to ionizing radiation is associated with the complex response that include increase in mutation rates, changes in the structure and functionality of the genome (**Evans and DeMarini, 1999; Kuchma *et al.*, 2011**). Previously it was also well documented that the life in radio-contaminated Chernobyl area is often associated with the increase in mutation rates (**Kovalchuk *et al.*, 2000; Kal'chenko and Fedorov, 2001; Kovalchuk *et al.*, 2003; Vornam *et al.*, 2004; Geras´kin *et al.*, 2005; Møller *et al.*, 2006; Kuchma *et al.*, 2011**). Despite that plants grow and successfully reproduce in radio-contaminated Chernobyl area (**Klubicova *et al.*, 2013**).

Importantly, flax (*Linum usitatissimum* L.) is the third largest natural fiber crop and one of the five major oil crops in the world (**Deng *et al.*, 2011**). Oil, extracted from flax seeds, is used as linoleum, varnish, paint, printer´s ink, and soap (**Cloutier *at al.*, 2009**). Moreover, flax oil is increasingly considered as edible oil due to high level of linolenic acid that is generally around 45 % to 65 % (Damude **and Kinney, 2007**). Linolenic acid is produced through the desaturation of linoleic acid by omega-3/delta-15 desaturases and in plants, this reaction occurs both in the plastids and in the endoplasmic reticulum (**Vrinten *et al.*, 2005**). Flax seed is naturally high in polyunsaturated fatty acids (PUFA) (Jhala, Hall, 2010) and is a rich source of α-linolenic acid (ALA), an essential dietary fatty acid of ω-3 class. Desaturases are enzymes that drive the multi-step fatty acid biosynthetic pathway and desaturase genes have been isolated and characterized in several plants, including flax. Membrane bound desaturases, especially delta-12 and delta-15 desaturases are currently of considerable interest as targets for manipulation of storage oils in plant seeds (**Khadake *et al.*, 2011**). In fatty acid biosynthesis pathway the delta-12 fatty acid converts oleic acid (18:1) to linoleic acid (18:2) and production of linoleic acid marks the synthesis of polyunsaturated fatty acid (PUFA) from monounsaturated oleic acid. Delta-12 desaturation is also an imperative prerequisite for the synthesis of omega-3 linolenic acid (18:3) (**Khadake *et al.*, 2009**) and the delta-15 desaturase ensures the conversion of ω-6 linoleic acid (18 : 2) to linolenic acid (18 : 3) (**Khadake *et al.*, 2011**). This process is the major factor in determining the quality of plant oils (**Khadake *et al.*, 2009**). Desaturases capable of desaturating linoleic acid are encoded by two independently inherited genes, *FAD3A* and *FAD3B, in flax*. The deduced proteins encoded by these genes share 95.4% identity (**Vrinten *et al.*, 2005**).

Molecular marker methods may be defined by the kind of DNA variation or polymorphism they detect and the way in which the polymorphism is detected or visualized. The first DNA-based molecular marker technique was restriction fragment length polymorphism (RFLP) (**Schulman *et al.*, 2012**). The ease of detection of DNA polymorphisms depends on both frequency and form of sequence variation (**Nakitandwe *et al.*, 2007**). Analysis of the extent and distribution of genetic diversity in crop plants is essential for optimizing sampling and breeding strategies (**Fu *et al.*, 2002**).

Chernobyl area is contaminated with long-lived radioisotopes including ^{90}Sr and ^{137}Cs (**Klubicová *et al.*, 2010**) nevertheless, in recent years there has been an increasing tendency toward remediation of contaminated areas for agricultural purposes (**Klubicová *et al.*, 2011**). The local ecosystem has been able to adapt to a constantly high level of radiation. For the reason to evaluate plant adaptation, seeds of a local flax genotype *Kyivskyi* were sown in experimental fields of the Chernobyl region (**Klubicová *et al.*, 2010**). Flax was selected because it is a crop of economic and historical importance, despite the relative paucity of molecular resources (**Klubicová *et al.*, 2011**). Evaluation of *FAD*3 gene isoforms was considered because a previous study observed changes in fatty acids composition (unpublished), especially increased alpha-linolenic acid content (data not shown). Two approaches, direct sequencing, and restriction analysis were employed to identify point mutations in *FAD*3A and *FAD*3B genes.

MATERIAL AND METHODS

Experimental fields and biological material

In 2007, cultivation of flax (*Linum usitatissimum* L.) of *Kyivskyi* genotype has began in experimental fields established near Chernobyl. Flax seeds used in this experiment were harvested in 2013, after six consecutive years of growing near Chernobyl. Flax seeds were harvested from radio-contaminated, and remediated experimental field in three biological replicates. Radio-contaminated experimental field is located 5 km from Chernobyl Nuclear Power Plant (CNPP), near the village Chistogalovka and have soil radioactivity 20650 ± 1050 Bq.kg^{-1} of ^{137}Cs, and 5180 ± 550 Bq.kg^{-1} of ^{90}Sr. Remediated field is located directly in Chernobyl town and have soil radioactivity 1414 ± 71 Bq.kg^{-1} of ^{137}Cs and 550 ± 55 Bq.kg^{-1}. Contents of aleurite (silt) and pelitic soil ranges from 20 to 30 % in both experimental fields. The soils contain 12 %

clay, 2 % organic material, and are sod-podzolic with a loamy-sand texture, which is derived from sandy fluvio-glacial deposits. Soil in remediated field has pH of 6.6, and in radio-contaminated field pH of 5.6. The electric conductivity of soils in both fields is 0.20 $dS.m^{-1}$.

DNA isolation

Genomic DNA was isolated using the method described by Rogers and Bendich (1994). In total 0.5 g (approximately 100 seeds) of mature flax seeds were ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was suspended in extraction solution (50% (v/v) phenol, 0.45 M sucrose, 5mM EDTA, 0.2% (v/v) 2-mercaptoethanol, 50 mM Tris-HCL, pH 8.8) and shaken for 30 min at 4 °C. After the centrifugation at 5000g for 10 min at 4 °C, the aquatic phase was transferred into new tube. The one volume of phenol-chloroform-isoamylalcohol solution (25:24:1) was added to aquatic phase, and mixed vigorously, then centrifuged at 9500g for 15 min at 4 °C. Phenol-chloroform extraction was repeated until clear aquatic phase without brown blur. DNA precipitation was carried out with one volume of isopropanol, incubation for 20 min at room temperature, and centrifugation at 5000g for 5 min and subsequent washing with one volume of 70% ethanol. Nucleic acid precipitate was transfer to new tube and dissolved in 300 µl TE buffer (pH 8, 10 mMTris, 1mM EDTA).

Restriction analysis

The restriction analysis of *FAD*3A (HM991828.1) and *FAD*3B (HM991832.1) genes was performed. The promoter, and coding regions of *FAD*3A, and *FAD*3B were amplified for further restriction digestion. Primers were designed using the NCBI primer designing tool (National Center for Biotechnology Information, Bethesda, USA). The primer sequences are shown in table 1. PCR reactions mixture contained Termo Scientific Dream *Taq* PCR Master Mix (2×); Dream *Taq* DNA polymerase is supplied in 2× Dream *Taq* buffer; dATP, dCTP, dGTP and dTTP (0,4 $mmol.dm^{-3}$ each); 4 $mmol.dm^{-3}$ $MgCl_2$ and 400 $nmol.dm^{-3}$ of each primer (table 1). Amplification was performed in C1000 Thermal Cycler under following conditions: 94 °C 4 min; 94 °C 45 sec, 61 °C 30 sec, 72 °C 2 min; 72 °C 10 min. PCR products were verified on 1% agarose gel.

Table 1 Nucleotide sequences of primers applied for PCR amplification of *FAD*3A and *FAD*3B genes in flax

Gene		Primer sequence 5'-3'	PCR product (bp)
***AD*3A**	1_For	ATCGCCACAACTTCGAACCA	1179
	1_Rev	AATGCAGGATGCCAAGTGGA	
	2_For	ATCCCTCTGCCCGAAACTTG	754
	2_Rev	ATCTTGAAAGGGGGAGCAGC	
	3_For	GAAGAACCTAGCAGCCAGCA	765
	3_Rev	TGCATGCAACAGCCCAGATA	
	4_For	CTGGACAGCTGGACTGTCTG	933
	4_Rev	GCTGCCCAATTCCGTTTTGT	
	5_For	GCCACTCAGTCTATTCATTA	1201
	5_Rev	CTAGAATTGAGGAAAAAACATG	
	6_For	ATCTGGCGATGGGTGTGCGG	1500
	6_Rev	CAAGAAGGAAATCCCCTGACTCC	
	7_For	TGACGGGTGTCGTGGATGGAT	1924
	7_Rev	ATGACAATGGAACCAGAGAGC	
***FAD*3B**	1_For	AAGACCGATGCCCACTTGAG	1992
	1_Rev	AGCGTGCAGCACCTAATCTT	
	2_For	ATACTGTGAGCCTCAAAGCA	1244
	2_Rev	GACAGAATTACCCAATGTCAG	
	3_For	GTAAGTAAAGAGACTGATAAG	1330
	3_Rev	ATTGACCAGGGATCGGATTT	
	4_For	CGATTTCGGCGGAATTGGAC	1789
	4_Rev	ACTGCTGGTACGGAACTTGG	
	5_For	AGCGGTAAACAATTTGATTAT	1279
	5_Rev	AACACAGTCGAAACCCTATA	

For restriction analysis, ten microliters of PCR product was digested with 0.5 - 5 U of the appropriate enzyme in a total volume of 30 µl for 3 hours. Digests were evaluated on 1.5 % agarose gels. Electrophoresis was conducted at a voltage of 60 V for 2h. Electrophoreograms were processed with documentation system G: Box in GeneSnap program - Product version: 7.09 (Syngene). Restriction endonucleases employed for *FAD*3 genes analysis, and their recognized restriction sites are shown in table 2. Restriction enzymes for the study were selected according the software NEBcutter V2.0 (**Vincze *et al*., 2003**). Selected enzymes were identified as the restriction endonucleases with the highest coverage of cutting sites within analysed genes.

Table 2 Characterization of restriction endonucleases selected for restriction analysis of *FAD*3A and *FAD*3B genes

Gene	Restriction endonuclease	Recognized restriction site	No. of cutting sites
***FAD*3A**	*Mnl*I	CCTC(N)$_6$ ▲N	32
	*Nla*III	CATG▼	33
	*Aci*I	C▼CGC	16
	*Alu*I	AG▼CT	25
	*Scr*FI	CC▼NGG	12
***FAD*3B**	*Mnl*I	CCTC(N)$_6$ ▲N	27
	*Aci*I	C▼CG▲C	20
	*Nla*III	CATG▼	26
	*Bsa*JI	C▲CNNGG	11
	*Fat*I	▼CATG	26
	*Hpy*188I	TC▲N▼GA	23

Direct sequencing

Direct sequencing of *FAD*3A, and *FAD*3B PCR products was performed. PCR products were purified using QIAquick PCR purification kit (Qiagen, Venlo, Netherlands), and commercially sequenced. Microsynth AG, Austria provided sequencing services. The raw chromatograms were processed using Chromas Lite 2.6.6 software (Technelysium Pty Ltd., South Brisbane, QLD, and Australia). Nucleotide sequences of *FAD*3A, and *FAD*3B genes in flax seeds cultivated in radio-contaminated, and remediated fields were compared against original sequences from NCBI database to identify the presence of mutations. For evaluation of nucleotide sequences ClustalX 2.1 (**http://www.clustal.org/clustal2/**) software was used.

RESULTS AND DISCUSSION

Herein, the flax seeds harvested from radio-contaminated, and remediated fields near Chernobyl with certain level of radioactive contamination were analysed. Previously obtained data (unpublished) indicates the increasing in alpha-linolenic acid content in flax seeds cultivated on the radio-contaminated experimental field. Therefore, two essential isoforms of *FAD*3 gene responsible for the conversion of linoleic acid to alpha-linolenic acid, *FAD*3A and *FAD*3B, were evaluated. Combination of restriction analysis to screen specific cleavage sites and direct sequencing of PCR products was applied. The major aim of the study was to identify point mutations caused by irradiation stress in the Chernobyl environment. The *FAD*3A gene was digested using the following enzymes *Mnl*I, *Nla*III, *Aci*I, *Alu*I, *Scr*FI, and the *FAD*3B using the *Mnl*I, *Aci*I, *Nla*III, *Bsa*JI, *Fat*I, and *Hpy*188I restriction endonucleases. Restriction profiles obtained by analysis were aligned against restriction sites virtually predicted by NebCutter V2.0 (**Vincze *et al*. 2003**).The analysis resulted in the monomorphic restriction profiles in both investigated genes. The polymorphism was not detected between flax seeds harvested from radio-contaminated, and remediated experimental field near Chernobyl.

In order to confirm the absence of mutations, and single nucleotide polymorphisms (SNPs) in sequences of *FAD*3A, and *FAD*3B, direct sequencing of PCR products was employed. Sequencing results were in agreement with restriction analysis, and no changes were found at the nucleotide level of analysed *FAD*3 genes.

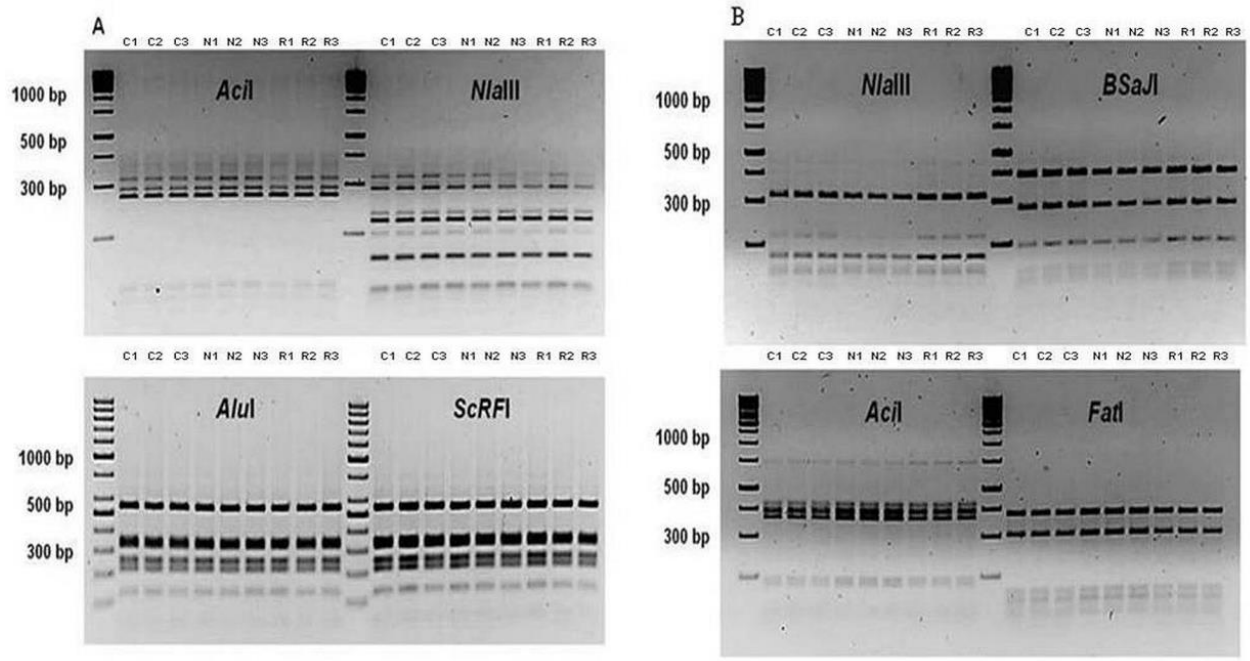

Fig.1 Fragments of restriction patterns determined for two *FAD*3 gene isoforms; three groups of flax seeds were analysed in biological triplicate, group 1 are flax seeds used to establish experimental fields in 2007 with no previous contact with Chernobyl environment, these seeds were used as controls (restriction profiles are labeled as C1, C2, C3); group 2 are flax seeds harvested from nonradioactive (remediated) experimental field (N1, N2, N3); group 3 are flax seeds harvested from radio-contaminated experimental field near Chernobyl (R1, R2, R3); A shows restriction profiles of *FAD*3A gene using the *Aci*I, *Nla*III, *Alu*I, and *ScR*FI enzymes; B shows restriction profiles of *FAD*3B gene using the *Nla*III, *BSa*JI, *Aci*I, and *Fat*I enzymes.

Currently, no genomic data are available for common flax affected by long-term chronic exposure to radiation. While the area in near proximity to the Chernobyl still remains substantially contaminated with long-lived radioisotopes such as ^{90}Sr and ^{137}Cs, local ecosystems are reportedly adapted to radio-contamination (**Vornam *et al*., 2004; Klubicová *et al*., 2010; Abramov *et al*., 2011**). The following defence mechanisms allows plants to survive under the harsh environmental conditions a) ability to repair DNA damage after the low-dose irradiation (**Abramov *et al*., 2011**), b) stimulated repopulation, i.e the replacement of damaged cells with undamaged (**Serebryanyi and Zoz, 2002**), c) activation of plants antioxidant system (**Abramov *et al*., 2011**), d) epigenetic changes manifested through the genome hypermethylation (**Kovalchuk *et al*., 2003**).

Flax is a promising candidate for investigation of molecular alterations because of a high level of genetic diversity and active genomic responses to abiotic stress (**Cloutier *et al*., 2012**). The effects of ionizing radiation on genetic material of higher plants, and its involvement in both adaptive processes and species evolution was investigated by **Esnault *et al*. (2010)**. Chronic, and long-term irradiation may affect the genetic structure of populations, and genetic variability might be reduced. The effect of long-term chronic exposure to radionuclides was discussed in the study presented by **Geras'kin *et al*. (2013)**. Ionizing radiation causes primary damage at the molecular level, however plants are able to increase a resistance of population to exposure under the long-term radioactive contamination. This may explain the stability of investigated *FAD*3 genes. Plants may respond to the chronic low-dose radiation stress differently than to the acute high-dose radiation. Chronic low-dose irradiation may lead to the adjustment of adaptation mechanisms. Hence, plant can be able to cope with environmental radiation stress with only minor genomic rearrangements.

CONCLUSION

In conclusion, flax of local ukrainian genotype *Kyivskyi* cultivated in the experimental fields near Chernobyl was investigated at the molecular level. Specifically, two gene isoforms, *FAD*3A and *FAD*3B, essential for synthesis of alpha-linolenic acid were evaluated. The presence of mutations, and SNPs in genomic DNA of *FAD*3 genes was investigated using the restriction analysis, and direct sequencing. Polymorphism was not revealed between flax seeds harvested from radio-contaminated, and remediated field. It can be concluded that exposure to the ionizing radiation has no significant effect on the stability of *FAD*3A and *FAD*3B genes.

Acknowledgments: This work was co-funded by a grant from the Slovak Grant Agency VEGA 2/0075/17 and Research Centre AgroBioTech built in the framework of European Community project Building Research Centre "AgroBioTech" ITMS 26220220180. The authors thank to Dr. Martin Hajduch for project coordination, prof. Namik M Rashydov for coordination of experimental fields and sample collection, Mrs. Valentyna V. Berezhna and Mr. Volodymyr Sakada for technical help with maintenance of the Chernobyl fields.

REFERENCES

ABRAMOV, V.I., STEPANOVA, A.A., FAMELIS, S.A. (2011). Some radiobiological effects in higher plants growing on the territory of the East Ural Radioactive Trace. *Biophysics*, 56,129-143.

CLOUTIER, S., MIRANDA, E., WARD, K., RADOVANOVIC, N., REIMER, E., WALICHNOWSKI, A., DATLA, R., ROWLAND, G., DUGUID, S., RAGUPATHY, R. (2012). Simple sequence repeat marker development from bacterial artificial chromosome end sequences and expressed sequence tags of flax (*Linum usitatissimum* L.). *Theoretical and Applied Genetics*, 125(4), 685-694. https://dx.doi.org/10.1007/s00122-012-1860-4

DENG, X., LONG, S., HE, D., LI, X., WANG, Y., HAO, D., QIU, C., CHEN, X. (2011). Isolation and characterization of polymorphic microsatellite markers from flax (*Linum usitatissimum* L.). *African Journal of Biotechnology*, 10(5), 734-739.

ESNAULT, M., LEGUE, F., CHENAL, C. (2010). Ionizing radiation: Advances in plant response. *Environmental and Experimental Botany*, 68, 231-237. https://dx.doi.org/10.1016/j.envexpbot.2010.01.007

FU, B.Y., DIEDERICHSEN, A., RICHARDS, K.W., PETERSON, G. (2002). Genetic diversity within a range of cultivars and landraces of flax (*Linum usitatissimum* L.) as revealed by RAPDs. *Genetic Resources and Crop Evolution*, 49(2), 167-174. https://dx.doi.org/10.1023/A:10147160

GERAS'KIN, S., EVSEEVA, T., OUDALOVA, A. (2013). Effects of long-term chronic exposure to radionuclides in plant populations. *Journal of Environmental Radioactivity*, 121, 22-32. https://dx.doi.org/10.1016/j.jenvrad.2012.03.007

JHALA, J.A., HALL, M.L. (2010). Flax (*Linum usitatissimum* L.): Current Uses and Future Applications. *Australian Journal of basic and Applied Sciences*, 4(9), 4304-4312.

KHADAKE, R., KHONDE, V., MHASKE, V., RANJEKAR, P., HARSULKAR, A. (2011). Functional and bioinformatic characterisation of sequence variants of *Fad3* gene from flax. *Journal of the Science of Food and Agriculture*, 91, 2689-2696. https://dx.doi.org/10.1002/jsfa.4515

KHADAKE, R., RANJEKAR, P., HARSULKAR, A. (2009). Cloning of a Novel Omega-6 Desaturase from Flax (*Linum usitatissimum* L.) and Its Functional Analysis in *Saccharomyces cerevisiae*. *Molecular Biotechnology*, 42, 168-174. https://dx.doi.org/10.1007/s12033-009-9150-3

KLUBICOVÁ, K., BERČÁK, M., DANCHENKO, M., SKULTETY, L., RASHYDOV, N.M., BEREZHNA, V.V., MIERNYK, J.A., HAJDUCH, M. (2011). Agricultural recovery of a formerly radioactive area: I. Establishment of high-resolution quantitative protein map of mature flax seeds harvested from the remediated Chernobyl area. *Phytochemistry*, 72(10), 1308-1315. https://dx.doi.org/ 10.1016/j.phytochem.2010.11.010

KLUBICOVÁ, K., DANCHENKO, M., SKULTETY, L., MIERNYK, A.J., RASHYDOV, N.M., BEREZHNA, V.V., PREŤOVÁ, A., HAJDUCH, M. 2010.

Proteomics Analysis of Flax Grown in Chernobyl Area Suggests Limited Effect of Contaminated Environment on Seed Proteome. *Environmental Science and Technology*, 44(18), 6940-6946. **https://dx.doi.org/10.1021/es100895s**

NAKITANDWE, J., TROGNITZ, F., TROGNITZ, B. (2007). Reliable allele detection using SNP-based PCR primers containing Locked Nucleic Acid: application in genetic mapping. *Plant Methods*, 3(2). **https://dx.doi.org/10.1186/1746-4811-3-2**

OH, J.T., GORMAN, M., CULLIS, A.C. (2000). RFLP and RAPD mapping in flax (*Linum usitatissimum*). *Theoretical and Applied Genetics*, 101, 590-593. **https://dx.doi.org/10.1007/s001220051520**

ROGERS, S., BENDICH, A. (1994). Extraction of total cellular DNA from plants, algae and fungi. *Plant molecular biology manual*, 8(1). **https://dx.doi.org/10.1007/978-94-011-0511-8_12**

SCHULMAN, H.A., FLAVEL, J.A., PAUX, E., ELLIS, N. (2012). The Application of LTR Retrotransposons as Molecular Markers in Plants. *Methods in Molecular Biology*, 859, 115-153. **https://dx.doi.org/10.1007/978-1-61779-603-6_7**

SEREBRYANYI, A.M., ZOZ, N.N. (2002). Stimulated Repopulation as a Basis of Antimutagenesis and the Adaptive Response in Plants. *Genetics*, 38, 264-268.

VINCZE, T., POSFAI, J., ROBERTS, R.J. (2003). NEBcutter: a program to cleave DNA with restriction enzymes.
Nucleic Acids Res, 31, 3688-3691. **https://dx.doi.org/10.1093/nar/gkg526**

VORNAM, B., KUCHMA, O., KUCHMA, N., ARKHIPOV, A., FINKELDEY, R. (2004). SSR markers as tools to reveal
mutation events in Scots pine (*Pinus sylvestris* L.) from Chernobyl. *Eur. J. Forest Res*, 123, 245-248.
https://dx.doi.org/10.1007/s103420040026-6

VRINTEN, P., HU, Z., MUNCHINSKY, A.M., ROWLAND, G., QIU, X. (2005). Two *FAD3* Desaturase Genes Control the Level of Linolenic Acid in Flax Seed. *Plant Physiology*, 139(1), 79-87. **http://dx.doi.org/10.1104/pp.105.064451**