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IN SILICO BASED DEVELOPMENT OF dLUTE LENGTH POLYMORPHISM MARKER FOR COMMON FLAX GERMPLASM EVALUATION

JANA ŽIAROVSKÁ*, MILAN BEŽO, VERONIKA LANCÍKOVÁ AND KATARÍNA RAŽNÁ

Slovak University of Agriculture in Nitra, Department of Genetics and Plant Breeding, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovak Republic
**Corresponding author e-mail: jana.ziarovska@uniag.sk; Ph: +421376414244; fax +421376415599*

Abstract

The first study of the pattern of dLUTE insertions as detected by PCR length polymorphism technique was performed. *In silico* approach was used for developing the dLUTE based marker system. Alignment analysis were performed to find a region that is dLUTE specific and primers designed withing this region not corresponding to any of sequences of L-genes. Realized length polymorphism analysis of common flax accessions confirmed the results of *In silico* analysis of polymorphic potential of designed marker system. The PCR analyse based of flax dLUTE transposon resulted in ten different alleles amplified in PCR withing the length range from 250 up to the 980 bp. In total, twenty-four common flax accessions were evaluated using the developed approach and the grouping of them according the oil/fiber type was obtained in constructed dendrogram.

Key words: *Linum usitatissimum*, L.; dLUTE; Length polymorphism; DNA marker.

Introduction

Flax (*Linum usitatissimum*, L.) was domesticated in central Asia and the area of Mediterranean Sea and still used for seed oil and stem fibers. Nowadays, this two basic types of use is supplemented by using of common flax as a functional food (Bassett *et al*., 2009; Ragupathy *et al*., 2011). In the field of molecular markers research of common flax, many different techniques were reported to be used, such as RFLP (Oh *et al*., 2000), RAPD (Chen *et al*., 1998; Fu & Diederichsen, 2002), ISSR (Rajwade *et al*., 2010; Uysal *et al*., 2010), EST-SSR (Cloutier *et al*., 2009), IRAP (Žiarovská *et al*., 2012), iPBS (Smýkal *et al*., 2011), BAC-end sequence analysis (Ragupathy *et al*., 2011) or coding region analysis (Allaby *et al*., 2005). DNA based markers were succesfully used in common flax genetic variability analysis (Cullis *et al*., 1999; Fu *et al*., 2002) as well as in identification of individual genotypes (Oh *et al*., 2000).

In flax genome, 15 known transposable elements are identified and stored in public nucleotide databases. In total, 12 partial sequences for FL1-12 and Cassandra retrotransposons are reported and a sequence of dLUTE transposon as well (Ragupathy *et al*., 2011). Actually, dLUTE is the only known and confirmed transposon in common flax. It was described and analysed by Luck *et al*. (1998). The same authors named it as *defective Linum usitatissimum* transposable element-dLUTE and they have identified it in two spontaneous mutant alleles of the L6 flax rust resistance gene. dLUTE possess no ORF what indicates its nonautonomy. It seems to be similar to the Ac group of plant transposons as it has unperfect long terminal repeats and cause insertion site duplications (Cullis, 2005; Luck *et al*., 1998).

Lawrence *et al*. (1993, 1995) has used the similarity of dLUTE and Ac/Ds transposons in the transposon marker system for flax rust resistance gene localization. Beside dLUTE itself, dLUTE transposon similar sequences were reported in flax genome as abundant by Luck *et al*. (1998).

The aim of the study was to develop the dLUTE insertions based marker system for flax germplasm analysis using the *In silico* approach combined with the sequence characteristics of the flax transposon dLUTE.

Materials and Methods

Plant material: Common flax (*Linum ussitatissimum*, L.) accessions were obtained from The Research Institute of Plant Production in Piešťany, Slovak Republic and AGRITEC, Research, Breeding & Services, Ltd., Czech Republic (Table 1). Genomic DNA was extracted from *In vitro* regenerated plants using the Rogers & Bendich, (1994) protocol. Extracted DNA was quantified spectrophotometrically by measuring absorbance at 260 nm and stock DNA was dilluted to make a working solution of 30 ng/µl for PCR analysis.

Table 1. List of *Linum usitatissimum*, L. accessions used in analysis.

Variety name	Origin	Type
Albidum[a]	IND	Oil
Albocoeruleum[e]	Data not available	Fibre
Deubgrc 28197	Data not available	Data not available
Deubgrc 28198	Data not available	Data not available
Svaloef[e]	SWE	Fibre
Flanders[b]	CAN	Oil
Pskow II.[b]	RUS	Fibre
Red Wing[d]	USA	Oil
Rembrant[b]	NDL	Fibre
Renodlat oljelin[e]	SWE	Oil
Daero[b]	HUN	Data not available
Lilas[c]	FRA	Fibre
Gisa[e]	Egypt	Oil
Hor Nr 048[e]	DNK	Fibre
Krasnoder[e]	SUN	Fibre
La Plata[b]	ARG	Oil
Krasnokutsk[b]	SUN	Data not available
Norfolk Princess[e]	GBR	Fibre
Otofte 15/47[e]	DNK	Fibre
Rekord[e]	CZE	Fibre
Horan[b]	CZE	Fibre
Marina[b]	NLD	Fibre
Escalina[d]	NLD	Fibre
PRFGL 93[e]	DEU	Fibre

PCR performing and electrophoreotic separation: dLUTE based length polymorphism analysis were performed in a total volume of 15 µl reaction volume contained 1× PCR buffer (ThermoScientific); 1,5 mmol l^{-1} $MgCl_2$ (Invitrogen); 0,3 mmol l^{-1} each dNTP (Promega); 1U Dream*Taq* polymerase (ThermoScientific), 30 ng of template DNA and 4 µM primers. *In silico* analysis of dLUTE resulted in the following primers used in PCRs: forward primer 5´gccctgtgctgaaatctga 3´ and reverse primer 5´cagcacaggttattgggcgg 3´. Time and temperature profile of PCRs was as follows: 94°C 2 min.; 35 cycles of 1 min. at 94°C, 1 min. at 54°C and 2 min. at 72°C. Final extention was performed for 10 min. at 72°C. PCR products were separated in 2% (w/v) agarose gels in 1 × TBE buffer. Gels were stained by GelRed™ and digitally photographed. All the accessions were growth and sampled through the two seasons to ensure the stability of the markers and all the PCR amplifications were repeated at least twice to establish reproducibility of polymorphic fragments and scored independently by KODAK EDAS software.

Results and Discussion

***In silico* based analysis of dLUTE sequence:** As Luck *et al*. (1998) reported in their study, at minimum 17 fragments were identified in the flax genome, that contained multiple copies of dLUTE sequences. Based on this, the first step in our study were bioinformatic analysis of dLUTE for the purpose of designation of functional primers for length polymorphism analysis, not only dLUTE PCR identification, as was reported by Luck *et al*. (1998).

In total, 12 flax rust resistance L allelas sequences are reported in NCBI database (National Centre for Biotechnology Information) together with the flax rust resistance protein gene. Their comparison using genomic BLAST software (Basic Logical Alignment Search Tool) shows the similarity of their sequences ranged from 96% up to the 99%. This high consensus of flax rust resistance genes sequences results into the high coefficients of genetic similarity (Fig. 1).

Results of found identities of flax resistance L-genes became a base of *In silico* approach aimed to the development of dLUTE primers for length polymorphism analysis. Following alignment analysis were performed to find a region that was specific for dLUTE. Then primers were designed within the region that would not correspond to any of sequences of L-genes. Subsequent analysis was performed to find a conservative region of all known *Linum usitatissimum* flax rust resistance protein genes. Found conservative region strength from the nucleotide 1294 up to the 3750 of the LH allele (accession code in NCBI AF093649). Important for further analysis was, that the insertion site of dLUTE transposon is localized withing this region, concretelly in 3182 downstream (Luck *et al*., 1998). In following step, analysis of the possible matching between conserved region of L-genes and dLUTE longer than 20 bp was done to exclude the designation of primers that would not posses the specifity. Using BLAST software, such matching was excluded. Avoiding the annealing of *In silico* developed primers to any of non-conservative regions of L-genes, BLAST for more than 10 nucleotides similarity was performed for dLUTE transposon. In this analysis, regions that were complementar or compliant among dLUTE and L-genes were found (Fig. 2 and Table 2).

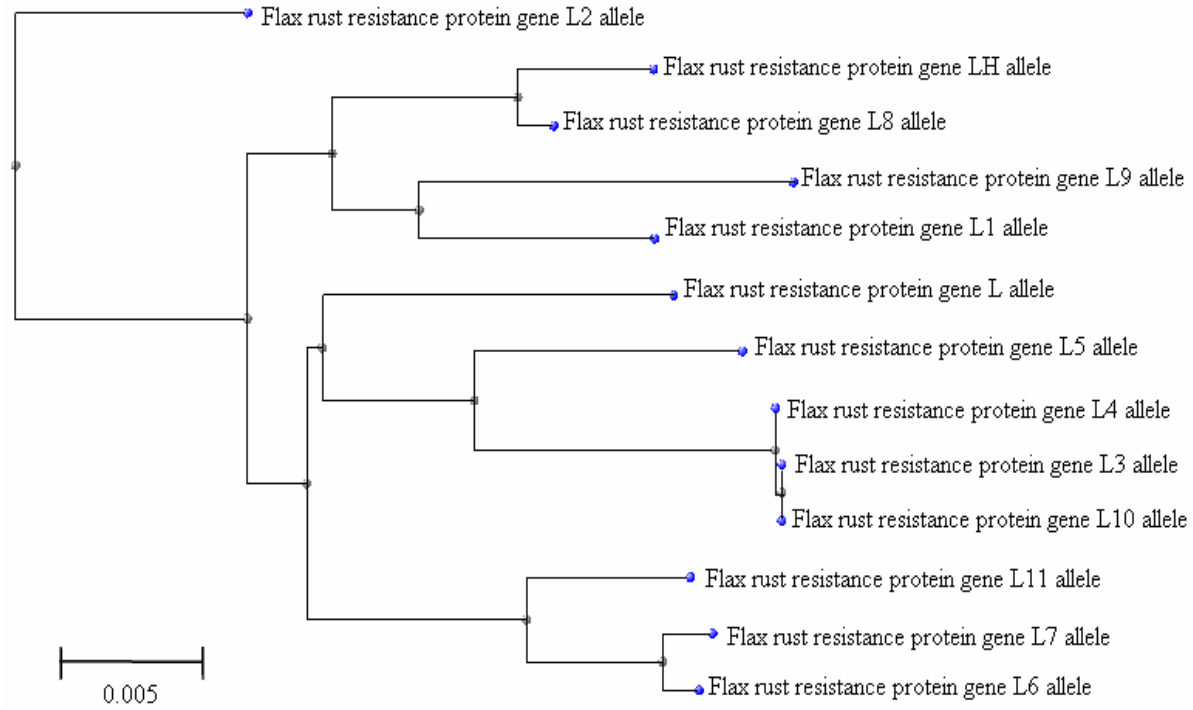

Fig. 1. Similarity of sequences of common flax L genes.

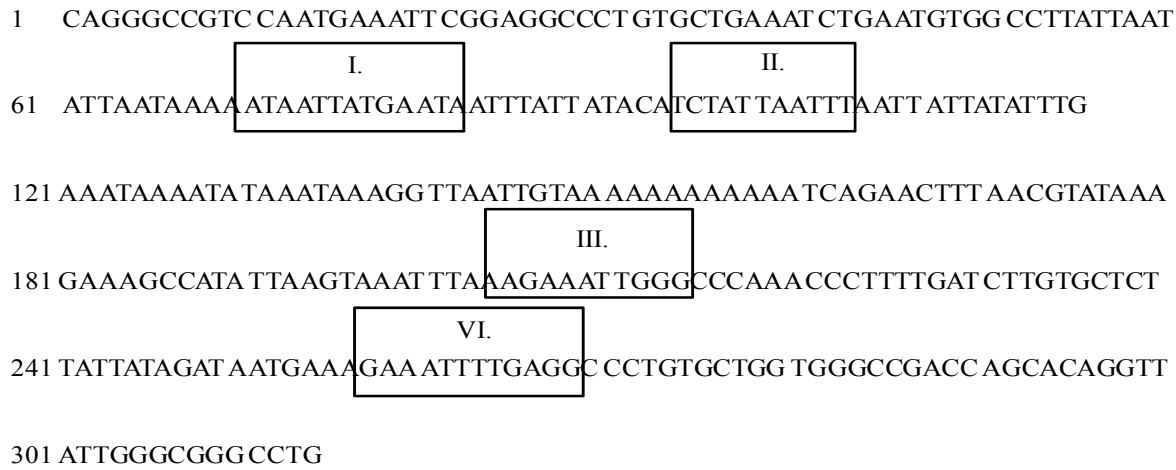

Fig. 2. Regions of dLUTE that possess the similarity with non-conservative regions of L-genes analysed as unsuitable for primer designing.

Table 2. Similarity of dLUTE nucleotides to the non-conservative regions of flax rust resistance L-genes.

Region	Type	Sequences accession codes* found as similar
I.	Complementar	AF093649; AF093647; U27081
II.	Concordant	AF093649; AF093648; AF093647; AF093646; AF093645; AF093644; AF093643; AF093642; AF093641; AF093640; AF093639; AF093638; U27081
III.	Concordant	AF093646
IV.	Concordant	AF093649; AF093647

* Accession codes as in NCBI database; numbers of regions as in the figure 2

After this final alignment analyse primers for length polymorphism analysis among dLUTE transposon were designed using Primer3 software (http://frodo.wi.mit.edu) as forward primer 5´gccctgtgctgaaatctga 3´ and reverse primer 5´cagcacaggttattgggcgg 3´.

Intra-specific analysis of common flax length polymorphism using dLUTE marker: Polymorphic potential of designed marker system was prooved by the results of *In silico* analysis realized by PCR analysis of common flax accessions. The length polymorphism analyse based of flax dLUTE transposon resulted in ten different alleles amplified in PCR withing the length range from 250 up to the 980 bp (Fig. 3).

In the case of length variability of amplified fragments based on dLUTE insertions, 80% polymorphism was detected among accessions and the average Nei and Li index of genetic similarity was 0.67. Grouping of accessions in constructed dendrogram was realized in nine levels (Fig. 4).

A high discrimination ability of DNA marker systems based on transponable elements was confirmed for dLUTE in this study. The analysis of obtained length polymorphism data, when used only single marker, resulted into 80% polymorphism among 23 tested accessions. Using this single marker, dicrimination of all analysed acessions was at the level of 72%. The high number of polymorphic markers based on transposable elements are reported in literature, as they are abundant in plant genomes. The replication and activity of transposable elements result in high genome variability, what provided them as an excellent molecular markers (Schulman *et al*., 2004).

In constructed dendrogram, fibre accessions were in separate groups, based on alleles obtained in analysis (fig. 4).

Oil varietes of common flax was not grouped together in one separated branch of dendrogram, but all of them are localized as more similar among themselves then to the fibre ones. This finding is in concordance with previously reported data, when after RAPD analysis of 2800 flax accessions performed by Diederichsen & Fu (2006, 2008). The authors concluded, that molecular genetic variability of common flax was relatively low, but oil genotypes possess higher genetic variability when compared to fibre genotypes. In this study, the average genetic coefficient of dissimilarity was 0.38 for fibre and 0.5 for oil genotypes. When evaluating the genetic variabily, result of RAPD and dLUTE based analysis are comparable, but in the case of RAPD analysis, no specific fibre and oil varieties grouping was obtained.

Discrimination of oil and fibre accession of common flax was not achieved when marker system based on retrotransposons was used (Smýkal *et al*., 2011). The author has used LTR regions of retrotransposons as markers, but no clear grouping was obtained after dendrogram construction. Smýkal *et al*. (2011) assume, that nonnot existing grouping of fibre and oil morphotypes can be a consequence of the used IRAP marker system. IRAP markers display the length polymorphism not only, but almost in intergenic space of genome. That is, why it is not expecting any linkage of IRAP markers to coding regions or concrete characteristisc. Luck *et al*. (1998) reported dLUTE as to be presented in many copies inserted into the genes in the flax genome. In the case of dLUTE transposon length polymorphism marker system, there can be assumed the utilization of it towards the polymorphism analysis where coding region is displayed and linked, too.

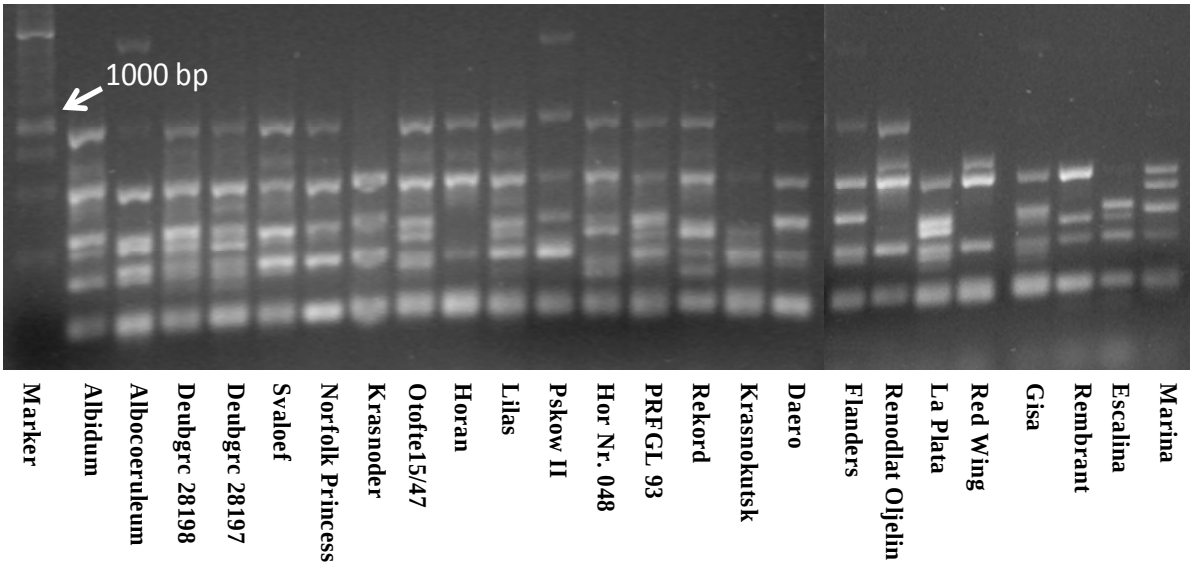

Fig. 3. Electrophoreogram of amplified fragments of dLUTE based polymorphic marker technique.

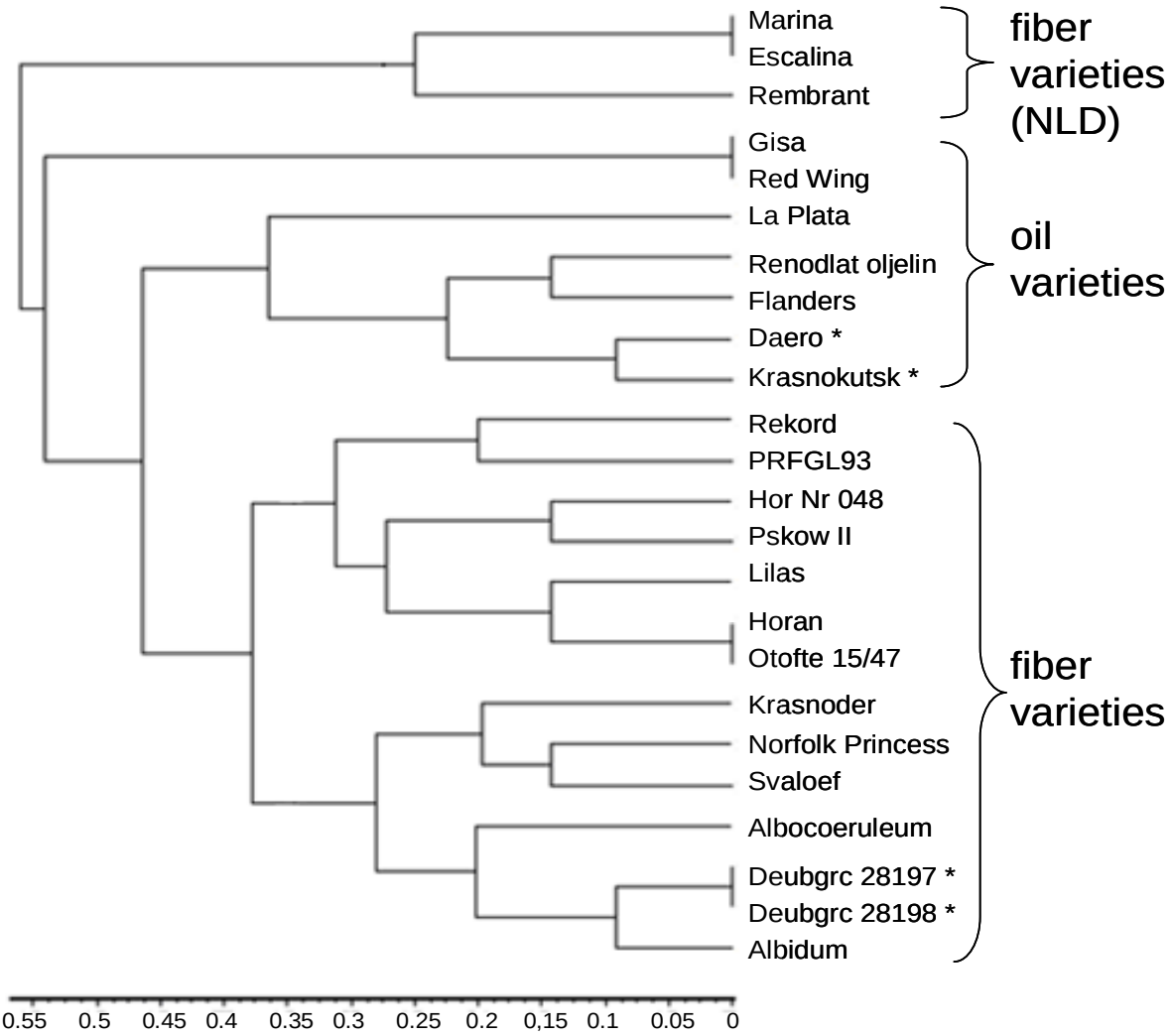

Fig. 4. Dendrogram of common flax varieties constructed with the usage of dLUTE based polymorphic marker; *unknown data about the type of usage for fibre/oil.

Transposable elements are reported as active parts of genomes with the transposition caused by different stresses and the ability to display the genome changes (Belyayev *et al*., 2010; Petit *et al*., 2010; Trebichalský *et al*., 2013). That is, why the stability of obtained dLUTE length polymorphism pattern was tested not only by repeating the analysis, but for different growth phases, too. The same accessions were analysed in the phase of tillering and for full matured plants, too. The dLUTE length polymorphism profile was in all cases the same.

As transposable elements (TEs) are reported to be widespread in plant genomes and played a key component of their evolution, all the methods based on TEs are very useful when the diversity of them is the object of the study (Poczai *et al*., 2013). The availability of a genome assembly of flax (*Linum usitatissimum* L.) affords new opportunities to explore the diversity of TEs and their relationship to genes and gene expression (González & Deyholos, 2012).

Conclusion

DNA marker analysis are still actual and in many applications in plant genetic resources management provide very effective tool. Here, the combination of bioinformatic and PCR based analysis are reported to develop a new dLUTE based marker technique for common flax genetic resources evaluation. It is applicable for routine analysis of common flax genome for individual varietes or landraces.

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