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USING RETROTRANSPOSON-BASED MOLECULAR MARKERS FOR ANALYSIS OF GENETIC VARIABILITY IN LATVIAN POPULATIONS OF ALFALFA

Abstract
Study of genetic diversity is very important to understand ecological adaptation of natural plant populations and to estimate of it potential for breeding. The goal of our work was to reveal most effective retrotransposon-based primers for analysis of alfalfa accessions. Twenty nine specific PCR primers were tested to find out those showing highest level of polymorphism in samples involved in the investigation. Selected primers can be applied to Latvian alfalfa genetic resources consisting from breeding varieties and semi-wild populations.

Key words: Medicago sativa, genetic diversity, retrotransposons

Introduction

Alfalfa (*Medicago sativa*) is a complex of perennial, allogamous species divided in several subspecies. It includes diploid (2n=2x=16) subspecies *M. sativa* ssp. *coerulea* and *M. sativa* ssp. *falcata* and tetraploid (2n=2x=32) ssp. *sativa* and ssp. *falcata*. Diploid *M. sativa* ssp. *coerulea* and tetraploid ssp. *sativa* are characterized by violet flowers with coiled pods, in they turn subspecies *falcata* is characterized by yellow flowers with straight to C-shape pods (Bolton, 1962).

Molecular marker technology is playing a vital role in plant biology including genetic diversity investigation, genetic linkage mapping, phylogenetic relationships and molecular breeding. To study genetic diversity of alfalfa different markers have been used – RAPD (Randomly Amplified Polimorphic DNA), AFLP (Amplified Fragment Length Polymorphism), but most commonly SSR (Simple Sequence Repeats or microsatelites). SSR markers in *Medicago sativa* have been first developed by Diwan et al. (1997). Later another set of SSRs from *M. truncatula* also have been used to amplify and detect polymorphism in alfalfa (Julier at al., 2003).

Retrotransposons belongs to the mobile genetic elements and are ubiquitous in the genomes of many eukaryotic organisms and they comprise the long terminal repeat (LTR) and non-LTR retrotransposons (Kumar et al., 1997; Kumar and Bennetzen, 1999). There are several marker systems based on the analysis of distribution of different retrotransposons in genome. Sequences of LTR retrotransposons are suitable to identify polymorphism of analyzed forms belonging to a single species by differnt PCR-fingerprinting techniques: IRAP (Inter-Retrotransposon Amplified

Polymorphism), REMAP (REtrotransposon-Microsatellite Amplified Polymorphism) and SSAP (Sequence-Specific Amplyfication Polymorphism) methods (Kalendar et al., 1999). The limiting factor of most of them is a necessity of development the own molecular marker systems for each plant species depending on specific retrotransposon sequences. Till now, they are not available markers for all crops of the interest. Recently Kalendar and Schulman (2010) developed a new universal method for DNA fingerprinting which is based on location of specific inter primer binding sites (iPBS) of the LTR retrotransposons.

In this work several specific PCR primers were tested to select those which recover most of polymorphism of alfalfa samples involved in the investigation.

Materials and methods

Alfalfa accessions used in experiments were received from the Latvian Research Institute of Agriculture (RIA). Two semi-wild populations and Latvian alfalfa variety 'Skrīveru' (Jansone, 2008) were used to detect best suited (polymorphic) primers.

Table 1. Alfalfa accessions used for detection of polymorphic primers

Accession	Species	Accession type
Aizkraukle	*M. varia*	semiwild from locality Aizkraukle
Dzelmes	*M. falcata*	semiwild from locality Dzelmes
'Skrīveru'	*M. sativa*	Latvian commercial variety

Plant DNA was isolated from 2-3 weeks old alfalfa seedlings using CTAB procedure (Saghai-Maroof et al., 1984). Isolated DNA was quantified using spectrophotometric analyses (Thermo scientific Nanodrop 1000) and quality was assessed by visualization of gel electrophoresis. PCR reactions were performed in a 25 µl reaction mixture (Table 2). Twenty nine primers (Kalendar et al., 2010) that show high PCR efficiency both in plants and animals were selected for screening: 2076, 2077, 2079, 2080, 2081, 2083, 2087, 2221, 2239, 2240, 2242, 2243, 2251, 2270, 2272, 2273, 2277, 2295, 2298, 2373, 2376, 2378, 2380, 2384, 2391, 2392, 2393, 2394, 2415.

Table 2. PCR reaction mixture

Component	Stock solution	Amount for 1 reaction (µl)
H_2O		14.3
10x Dream Taq Buffer*	10x	2.5
dNTPs*	10 mM	0.5
Praimer**	4 µM	2.5
DreamTaq polymerase*	5 u/µl	0.2
Pfu DNA polymerase*	2.5 u/µl	0.025
Total without DNA		**23.0**

DNA dilution	10-20 ng	5.0
Total for reaction		**25.0**

* - Fermentas; ** - Invitrogen

Amplification was performed in Gene Amp® PCR System 9700 thermocycler under following conditions:

95 °C 3 min
95 °C 5 sec
50 °C 60 sec } 31 cycle
72 °C 90 sec
72 °C 10 min
4 °C soaking

Segregation of PCR product was done in 1.7 % agarose gel by electrophoresis with 20x20 gel track, at 100 V, 3 hour and visualized by staining with ethidium bromide. Fingerprints were captured by a digital camera and processed by the „Totallab 1D" software.

Results

From 29 tested primers, 18 revealing polymorphism between alfalfa individuals (Table 3).

Table 3. Primers revealed polymorphism in alfalfa populations

Primer	Nucleotide Sequence (5'→3')
2081	GCAACGGCGCCA
2083	CTTCTAGCGCCA
2221	ACCTAGCTCACGATGCCA
2240	AACCTGGCTCAGATGCCA
2242	GCCCCATGGTGGGCGCCA
2243	AGTCAGGCTCTGTTACCA
2251	GAACAGGCGATGATACCA
2272	GGCTCAGATGCCA
2273	GCTCATCATGCCA
2277	GGCGATGATACCA
2295	AGAACGGCTCTGATACCA
2298	AGAAGAGCTCTGATACCA
2373	GAACTTGCTCCGATGCCA
2378	GGTCCTCATCCA
2380	CAACCTGATCCA
2392	TAGATGGTGCCA
2393	TACGGTACGCCA
2394	GAGCCTAGGCCA

Ten primers gave monomorphic products and for one primer (2087) amplification was not occurred. The Figure 1 shows obtained fingerprints using primers 2087, 2240, 2243 and 2391. Primers 2240 and 2243 produced high number of clear and easy countable bands.

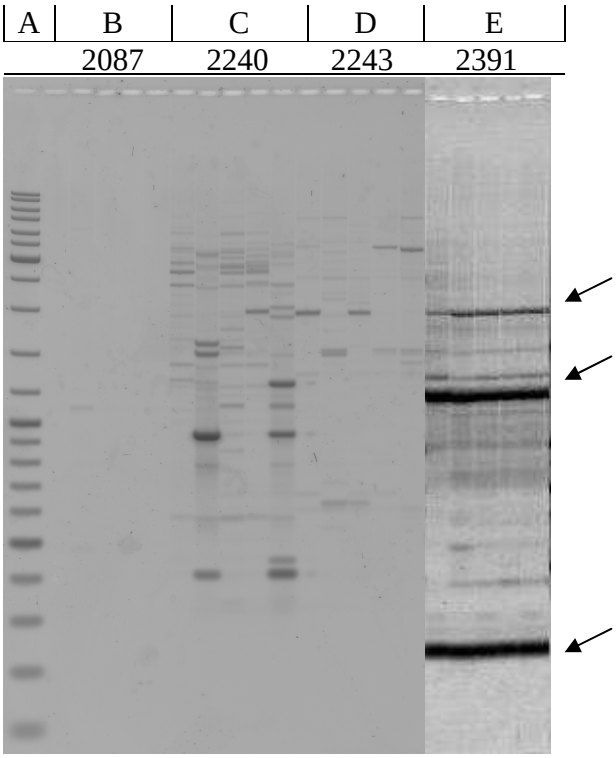

Fig. 1. Gel fingerprints: (A) GeneRuller Ladder Mix, (B) primer without amplification products, (C; D) polymorphic primers, (E) monomorphic primer. Black arrows indicate non-polymorphic bands for primer 2391.

Conclusions

From 29 tested primers, 18 revealing polymorphism between alfalfa individuals. Ten primers gave monomorphic products, after using of one of primers there was no amplification. DNA fingerprinting technique developed by Kalendar and Schulman proved to be successful for genetic diversity detection in alfalfa and therefore can be applied for evaluation of genetic diversity within and between populations of alfalfa species.

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