GENETICA, BIOLOGIA MOLECULARĂ ŞI AMELIORAREA

SCREENING OF THE R2 RUST RESISTANCE GENE IN DIFFERENT SUNFLOWER GENOTYPES USING SSR MARKERS

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Introduction

MAS (marker-assisted selection) is widely used molecular biology technique, which considerably facilitates selection of valuable genotypes increasing rates and efficiency of breeding process. It suggests application of molecular markers linked with genes of interest [3, 7]. Availability of many different types of molecular markers gives possibilities to choose the most reliable types of them according to the aims of investigation.

One of the most widely used type of markers is SSR (simple sequence repeat) markers or microsatellites, which represents mono-, di-, tri-, tetra- or pentanucleotide tandem repeats accidentally distributed within eukaryotic genomes [13].

High level of polymorphism, reproducibility, low price and simple implementation of these markers contribute for development and implementation of SSR method for different plant species such as soybean *Glycine max* [1, 9], rice *Oryza sativa* [10, 16], maise *Zea mays* [15], grape *Vitis vinifera* [14] and other.

Creation of first integrated and a single reference map, unification of sunflower SSR marker nomenclature enlarges possibilities of application of SSR markers for sunflower individual gene identification [12].

There are SSR markers linked with sunflower genes such as stearoyl-acyl carrier protein desaturase, resulting in high oleic acid concentration [6]; *ms9*, causing nuclear sterility [2]; *Tph2* associated with high γ-tocoferol levels [5]; *Or5* causing broomrape resistance [11] and other.

Recently linkage of three SSR markers with *R2* gene, ensuring rust resistance, has been demonstrated. Three microsatellite markers ORS795, ORS882 and ORS333 were used for reliable selection of resistant genotypes [8].

Hence, the aim of our investigation was screening of sunflower genotypes cultivated in Republic of Moldova using SSR markers linked with *R2* rust resistance gene.

Materials and methods

Investigations included 21 sunflower genotypes: 11 F_1 hybrids (Drofa F_1, LC 40 CMS x LC Raus Rf, Drofa CMS x LC Raus Rf, Drofa CMS x LC 637 Rf, Drofa CMS x LC 7 Rf, LC SW 38 CMS x LC 637 Rf, LC SW 38 CMS x LC 4 Rf, Drofa CMS x LC 39 Rf, Xenia CMS x LC 39 Rf, LC 40 CMS x Xenia Rf and Xenia F_1), 5 CMS (Drofa, LC 40, LC SW 38, LC 391A and Xenia) and 5 Rf lines (Drofa, LC Raus, LC 637, LC 7 and LC 39). DNA was extracted from etiolated seedlings using standard CTAB extraction protocol [4] with some modifications.

Amplification was performed in final volume of 10 µl containing: 50 ng DNA, 1x DreamTaq™ Green Buffer (Fermentas), 200 µM each of dNTPs, 2,5 mM $MgCl_2$, 0,4 µM each primer, 0,5 units of DreamTaq™ Green DNA Polymerase (Fermentas). PCR was performed at GeneAmp® PCR System 9700 (Applied Biosystems) using Touchdown PCR program: 94°C for 3 min; 8 cycles at 94°C – 30 s, 62°C – 30 s (-1°C/ cycle) and 72°C - 45 s; followed by 30 cycles at 94°C - 30 s, 54°C - 30 s and 72°C - 45s; with final extension at 72°C – 3 min. The amplified fragments were detected using 6 % non-denaturing polyacrylamide TBE gel electrophoresis, stained with ethidium bromide and visualized by UV fluorescence.

Results and discussions

Recent literature data shows strong association of SSR markers ORS333 with rust susceptibility and combination of ORS795 and ORS882 with rust resistance [8].

According to these data it was expected to obtain different amplified fragments characteristic for: homozygous susceptible lines - 150 bp (ORS333), homozygous resistant lines - 300 bp (ORS795) and 380 bp (ORS882) and combination of these three amplicons for heterozygous individuals [8].

Table 1. Characteristics of primers used in investigation

Marker	Repeat	Tm	Length, bp			Allele number	
			Expected [8]	In literature	In analysed genotypes aprox.	In litera-ture	In analysed genotypes
ORS333	$(GT)_{13}$	57	150	141/152/ 157/471	150/161/174/ 197/410/422	4	6
ORS795	$(CT)_{17}GG(CA)_{10}$	57	300	304/null	300	1	1
ORS882	$(GT)_{14}$	59	380	159/169/ 173/180	107/162/177/ 194/230/240	4	6

However, obtained results differ very much from the expected; only two markers ORS333 and ORS795 gave us fragments of expected length (table 1).

Analysis of SSR profiles generated by ORS333 primer pair revealed presence of fragment of 150 bp associated with rust susceptibility in all of investigated genotypes. Also other 5 alleles were identified (table 1).

Polymorphism between genotypes was manifested in region upwards 400 bp (fig. 1).

So there are 3 genotypes possessing alleles 422 and 410 (Drofa CMS x LC 637 Rf, LC SW 38 CMS x LC 637 Rf and LC 7 Rf), 14 genotypes which have 422 bp allele (7 F_1 hybrids Drofa F_1, Drofa CMS x LC Raus Rf, Drofa CMS x LC 7 Rf, LC SW 38 CMS x LC 4 Rf, Drofa CMS x LC 39 Rf, Xenia CMS x LC 39 Rf, Xenia F_1, 5 analysed CMS lines and 2 Rf lines LC 637 Rf, LC 39 Rf), 2 genotypes possessing 410 bp (LC 40 CMS x Xenia Rf and LC 637 Rf) and 2 genotypes which have no amplification products in this region (table 2).

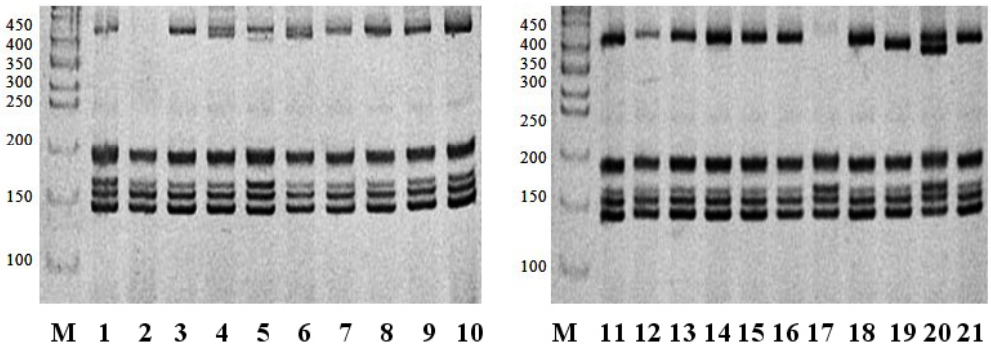

Figure 1. Results of the SSR-PCR amplification with ORS333 visualized in 6 % polyacrylamyde gels.

M – molecular mass ladder Step Ladder, 50 bp (Sigma), 1-Drofa F_1, 2-LC 40 CMS x LC Raus Rf, 3-Drofa CMS x LC Raus Rf, 4-Drofa CMS x LC 637 Rf, 5-Drofa CMS x LC 7 Rf, 6-LC SW 38 CMS x LC 637 Rf, 7-LC SW 38 CMS x LC 4 Rf, 8-Drofa CMS x LC 39 Rf, 9-Xenia CMS x LC 39 Rf, 10-Xenia F_1, 11-LC 40 CMS x Xenia Rf, 12-Drofa CMS, 13-LC 40 CMS, 14-LC SW 38 CMS, 15-LC 391A CMS, 16-Xenia CMS, 17-Drofa Rf, 18-LC Raus Rf, 19-LC 637 Rf, 20-LC 7 Rf, 21-LC 39 Rf.

Table 2. Genotypes classification according to amplification profiles in 400-450 bp region obtained using primer pair ORS333

Allele length	422 bp	410 bp
Genotypes	**F_1 hybrids:** Drofa F_1, Drofa CMS x LC Raus Rf, Drofa CMS x LC 7 Rf, LC SW 38 CMS x LC 4 Rf, Drofa CMS x LC 39 Rf, Xenia CMS x LC 39 Rf, Xenia F_1 **Rf lines:** LC 637 Rf, LC 39 Rf **CMS lines:** Drofa, LC 40, LC SW 38, LC 391A and Xenia	**F_1 hybrids:** LC 40 CMS x Xenia Rf, **Rf lines:** LC 637 Rf
	Rf lines: Drofa CMS x LC 637 Rf, LC SW 38 CMS x LC 637 Rf, LC 7 Rf	

ORS795 primer pair was generated one PCR product with aprox. 300 bp length in 11 sunflower genotypes (Drofa F_1, Drofa CMS x LC Raus Rf, Drofa CMS x LC 637 Rf, LC SW 38 CMS x LC 4 Rf, Drofa CMS x LC 39 Rf, LC 40 CMS x Xenia Rf, Drofa CMS, LC 40 CMS, LC SW 38 CMS, LC 391A CMS and LC 637 Rf) from 21 investigated. There are 6 F_1 hybrids, 4 CMS lines and 1 Rf line from 11 sunflower genotypes which possesses fragment of expected length (fig. 2).

Other genotypes have not given any amplification profiles with ORS795 primers, which indicate presence of null allele in these genotypes (fig.2).

Amplification with primer pair ORS882 have not create fragment of expected length associated with resistance among the set of investigated sunflower genotypes (fig. 3).

This primer pair is monomorphic, have not create any profile differences within investigated genotypes. Approximate alleles lengths are 240, 230, 194, 177, 162 and 107 bp (table 1).

Obtained results demonstrate the necessity to confirm molecular data by phenotypic screening of resistance within the set of investigated genotypes.

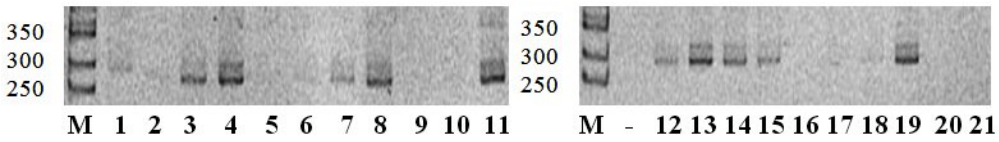

Figure 2. Results of the SSR-PCR amplification with ORS795 visualized in 6 % polyacrylamyde gels.

M – molecular mass ladder Step Ladder, 50 bp (Sigma), 1-Drofa F_1, 2-LC 40 CMS x LC Raus Rf, 3-Drofa CMS x LC Raus Rf, 4-Drofa CMS x LC 637 Rf, 5-Drofa CMS x LC 7 Rf, 6-LC SW 38 CMS x LC 637 Rf, 7-LC SW 38 CMS x LC 4 Rf, 8-Drofa CMS x LC 39 Rf, 9-Xenia CMS x LC 39 Rf, 10-Xenia F_1, 11-LC 40 CMS x Xenia Rf, 12-Drofa CMS, 13-LC 40 CMS, 14-LC SW 38 CMS, 15-LC 391A CMS, 16-Xenia CMS, 17-Drofa Rf, 18-LC Raus Rf, 19-LC 637 Rf, 20-LC 7 Rf, 21-LC 39 Rf.

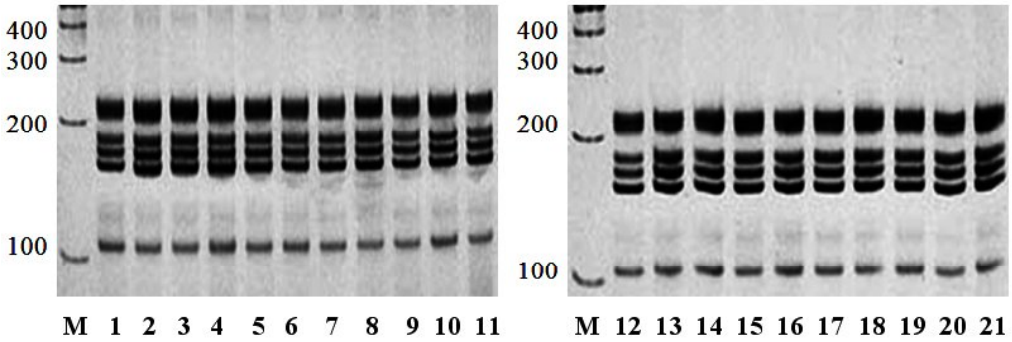

Figure 3. Results of the SSR-PCR amplification with ORS882 visualized in 6 % polyacrylamyde gels.

M – molecular mass ladder GeneRuler™ 100 bp Plus DNA Ladder (Fermentas), 1-Drofa F_1, 2-LC 40 CMS x LC Raus Rf, 3-Drofa CMS x LC Raus Rf, 4-Drofa CMS x LC 637 Rf, 5-Drofa CMS x LC 7 Rf, 6-LC SW 38 CMS x LC 637 Rf, 7-LC SW 38 CMS x LC 4 Rf, 8-Drofa CMS x LC 39 Rf, 9-Xenia CMS x LC 39 Rf, 10-Xenia F_1, 11-LC 40 CMS x Xenia Rf, 12-Drofa CMS, 13-LC 40 CMS, 14-LC SW 38 CMS, 15-LC 391A CMS, 16-Xenia CMS, 17-Drofa Rf, 18-LC Raus Rf, 19-LC 637 Rf, 20-LC 7 Rf, 21-LC 39 Rf.

Conclusions

ORS333 was the most polymorphic from three investigated markers and it can be used for polymorphism identification among investigated genotypes.

Polymorphism between genotypes was manifested in region upwards 400 bp. So there are 3 genotypes possessing alleles 422 and 410 (Drofa CMS x LC 637 Rf, LC SW 38 CMS x LC 637 Rf and LC 7 Rf), 14 genotypes which have 422 bp allele (7 F_1 hybrids Drofa F_1, Drofa CMS x LC Raus Rf, Drofa CMS x LC 7 Rf, LC SW 38 CMS x LC 4 Rf, Drofa CMS x LC 39 Rf, Xenia CMS x LC 39 Rf, Xenia F_1, 5 analysed CMS lines and 2 Rf lines LC 637 Rf, LC 39 Rf), 2 genotypes possessing 410 bp (LC 40 CMS x Xenia Rf and LC 637 Rf) and 2 genotypes which have no amplification products in this region.

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