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Study of Informative DNA Markers of the *Rf1* Gene in Sunflower for Breeding Practice

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Abstract

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The investigation of DNA markers associated with the pollen fertility restoration gene (*Rf1*) was conducted in *Helianthus*. Two sequence-characterized amplified region (SCAR) markers – HRG01 and HRG02 were informative for the identification of *Rf1* gene in selections of sunflower plants. The codominant character of the HRG01 marker and HRG01 amplicon polymorphism has been determined. Five annual and twenty-six perennial species of sunflower were tested for the presence of *Rf* genotypes. HRG02 proved to be a more appropriate marker for *Rf1* determination in perennial species, and HRG01 was more informative for annual species. We have also developed the multiplex RT-PCR test system, which allows simultaneously detecting the dominant allele of *Rf1* and CMS-PET1 associated mitotype.

Keywords: cytoplasmic male sterility (CMS); DNA-based markers; fertility restorer genes; *Helianthus*; testing system

Sunflower is one of the most popular oil crops all over the world. Presently, the industrial production of sunflower seeds is based upon use of highly productive interlinear hybrids. The production of such hybrids involves a genetic system of plant pollination control, which includes maternal lines carrying genes of cytoplasmic male sterility (CMS) and paternal lines having the fertility restorer (*Rf*) genes (Horn *et al.* 2003). Marking of the fertility recovering genes is important for precise selection of the carriers of dominant and recessive alleles of the *Rf* genes among separated populations. This significantly accelerates the obtainment of parental forms of hybrids. Thus, the investigations of markers tightly linked with *Rf* genes are held worldwide, for example, several *Rf* genes markers were identified in maize (Zhang *et al.* 2006), sunflower (Horn *et al.* 2003), soya (Dong *et al.* 2012), barley (Ui *et al.* 2015) etc.

Currently, virtually all sunflower hybrids in the world have a single CMS source – PET1, which was derived from *H. petiolaris* (Pratap & Kumar 2014). It was shown that CMS in sunflower is caused by 5000 bp insertion, which occurs on the border with the *atpA* gene and forms a new open reading frame *orfH522* (Horn *et al.* 1991). Nuclear *Rf* genes are responsible for polyadenylation of the *atpA-orfH522* transcript that makes it accessible for RNase II and eventually leads to the restoration of pollen fertility (Horn *et al.* 2003).

In most of the cultivated sunflowers, two dominant nuclear alleles, *Rf1* and *Rf2*, are necessary to restore male fertility, and *Rf2* is present in nearly all

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sunflower lines (Yue *et al.* 2010). According to the published data, a large number of DNA markers of the 13th linkage group were linked with *Rf1* loci of sunflower: ORS 511, ORS 224, ORS 317, ORS 630, ORS 799, ORS 1030 (Tang *et al.* 2002), HRG01, HRG02 (Horn *et al.* 2003) and STS-115 (Yue *et al.* 2010). Thus the *Rf1* gene was mapped on the 13th linkage group (Tang *et al.* 2002). However, the *Rf2* localization is still unclear (Yue *et al.* 2010). Therefore, the goal of the present study was to identify the informative DNA markers of *Rf1* gene both for cultivated sunflower lines and wild sunflower species and to develop on their basis a PCR based testing system for applied selection tasks.

MATERIAL AND METHODS

Research was aimed at 29 Rf lines and 17 CMS lines of sunflower which were obtained from L.A. Zhdanov Don Experimental Station of the All-Russian Institute of Oil Plants (Russia). Rf lines were homozygous for the *Rf1* gene (*Rf1Rf1*). In genomes of CMS lines only recessive alleles of *Rf1* gene were present (*rf1rf1*). The genotypes of Rf and CMS lines were confirmed by the parent-offspring analysis. In each line (Rf or CMS) 5 plants were investigated. Other objects of study were 5 annual and 26 perennial sunflower species which were obtained from N.I. Vavilov Institute of Plant Genetic Resources (St. Petersburg, Russia). The lists of investigated annual and perennial species are presented in Tables 1 and 2, respectively.

Twelve interspecific hybrids were also used for the study. Parental forms of hybrids were 12 perennial species and 4 CMS-PET1 lines, which are presented in Table 3. The hybrids were obtained from the Kuban Experimental Station of N.I. Vavilov Institute of Plant Genetic Resources. The research of pollen fertility was conducted in hybrids of the 10th generation, obtained by self-pollination. The pollen fertility was investigated using an aceto-carmine method (Stanley & Linskens 1974).

Total DNA isolation and PCR were carried out as it was described earlier (Markin *et al.* 2016). Primers designed by Horn (Horn *et al.* 2003) were used for the amplification of SCAR markers (HRG01 and HRG02)

Sanger sequencing of HRG01 amplicons was carried out on a 3130 Genetic Analyser (Applied Biosystems, USA) using the BigDye Terminator Ver. 3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Nucleotide sequences were edited and aligned by BioEdit Software Version 7.2.5 (Hall 1999).

RT-PCR primers and TaqMan probes were designed using Primer Quest℠ (http://eu.idtdna.com/PrimerQuest) on the basis of nucleotide sequences of HRG01 marker and *orfH522* gene (NCBI GenBank registration No. X55963.1). Primer sequences are as follow: for HRG01 forward – GGCATGATCAAGTACATAAGCACAGTC, reverse – TATGTACGGGAATGAGCTCCGGTT; for *orfH522* forward – TGAGTTTACTCCGGCAACTCGTTC,

Table 1. The results of amplification of the HRG01 and HRG02 markers of the *Rf1* gene in annual sunflower species

Species	Accession No. in the N. I. Vavilov Institute of Plant Genetic Resources	No. of investigated plants	Presence of the marker (%)	
			HRG01	HRG02
Helianthus annuus L.	441183	20	100	25
	441236	16	75	50
	441245	20	50	50
H. praecox Englem. & Gray	560400	16	75	0
H. debilis Nutt.	560388	15	100	0
	560395	13	100	0
	545666	18	100	0
H. petiolaris Nutt.	440560	20	100	25
	503232	19	100	0
	–	18	100	0
H. argophyllus T. & G.	1805	16	100	0
	1000	17	100	0

– without a number

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Table 2. The results of amplification of the HRG01 and HRG02 markers of the *Rf1* gene in perennial sunflower species

Species	Accession No. in the N. I. Vavilov Institute of Plant Genetic Resources	No. of investigated plants	Presence of the marker (%)	
			HRG01	HRG02
Helianthus ciliaris DC.	–	15	0	100
H. tracheifolius Mill.	–	15	0	100
H. tuberosus L.	441026	15	0	100
H. multiflorus L.	–	15	0	100
H. tomentosus Michx.	2107	15	0	100
H. nuttallii T. & G.	442735	15	0	100
H. hirsutus Raf.	560389	15	0	100
H. laevigatus T. & G.	–	15	0	100
H. microcephalus T. & G.	–	15	0	100
H. floridanus A.Gray ex Chapm.	–	15	0	100
H. simulans E.Watson	545659	15	0	100
H. salicifolius Dietr.	440074	17	0	100
	–	17	0	100
H. giganteus L.	2100	15	0	100
	489253	15	0	100
H. grosseserratus M.Martens	545698	18	0	100
	545711	15	0	100
H. decapetalus L.	545682	14	100	100
H. laetiflorus Heiser & D.M. Sm.	1886	19	100	100
H. smithii Heiser	–	16	100	100
H. eggertii Small	–	15	100	100
H. angustifolius L.	1889	20	30	100
	–	20	30	100
H. divaricatus L.	2099	15	60	100
	545674	18	100	100
H. mollis Lam.	2102	15	100	100
	530453	20	100	100
	5088	17	0	100
H. occidentalis Riddel	441062	16	0	100
subsp. *plantagineus*	–	16	0	100
(Torr. & A.Gray) Heiser	–	19	100	100
H. occidentalis Riddel subsp. *occidentalis*	–	20	30	100
H. strumosus L.	440679	17	100	100
	440683	16	0	100
	–	18	50	100
H. rigidus Desf.	2106	18	100	100
	545658	13	0	100
	1886	15	0	100
	545660	15	0	100
	545681	15	0	100
	545646	15	0	100
H. californicus DC.	530447	14	0	100
H. maximiliani Schrad.	440553	16	50	100

– without a number

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Table 3. The fertility of pollen of perennial species and interspecific hybrids of sunflower

Parental species	Pollen fertility (%)	Hybrid combination	Pollen fertility (%)
Helianthus ciliaris	96.5 ± 1.3		
H. decapetalus	89.7 ± 7.4		
H. divaricatus	90.6 ± 3.4		
H. giganteus	96.6 ± 1.1	HA 232 × *H. giganteus*	93.3 ± 0.9
H. hirsutus	91.9 ± 3.8	VIR 114 × *H. hirsutus*	75.3 ± 3.7
H. maximiliani	94.0 ± 2.4	HA 232 × *H. maximalliani*	84.0 ± 3.7
H. mollis	87.0 ± 1.5	HA 232 × *H. mollis*	92.4 ± 3.2
H. strumosus	95.8 ± 3.2	HA 232 × *H. strumosus*	97.8 ± 0.6
H. occidentalis	98.7 ± 0.4	VIR 151 × *H. occidentalis*	95.2 ± 2.3
H. rigidus	99.2 ± 0.3		
H. californicus	94.5 ± 0.5	HA 232 × *H. californicus*	92.3 ± 2.2
H. nuttalii	92.2 ± 3.9		
H. laetiflorus	87.6 ± 0.4	HA 232 × *H. laetiflorus*	87.5 ± 3.5
H. angustifolius	84.9 ± 2.6	HA 232 × *H. angustifolius*	90.6 ± 5.3
H. floridanus	71.6 ± 3.1	VIR 129 × *H. floridanus*	96.2 ± 1.1
H. grosseserratus	95.7 ± 1.3	HA 232 × *H. grosseserratus*	76.7 ± 1.8
H. tracheifolius	87.0 ± 1.5	VIR 151 × *H. tracheifolius*	94.7 ± 1.3

reverse – TGCTCTTGAATGGCAGTGGTGATG. TaqMan probes were (FAM)-TGTCACGCATG-CAAGTACTCCCACTT-(RTQ1) and (R6G)-ACA-GATCACGCCCTATAAAGGCCGAA-(BHQ1) for HRG01 and *orfH522*, respectively.

RESULTS AND DISCUSSION

First of all, it should be noted that in our investigation HRG01 (about 450 bp) and HRG02 (about 740 bp) SCAR (sequence characterized amplified region) markers were informative for the *Rf1* dominant allele identification (Figure 1). The obtained data of *Rf1* genotyping fully coincided with data of the parent-offspring analysis. Thus, it confirms the efficiency of HRG01 and HRG02 markers for sunflower selection in order to develop the parental lines of the CMS PET1-Rf system. Although these markers have been developed by Horn *et al*. (2003), in our study we detected the codominant nature of HRG01

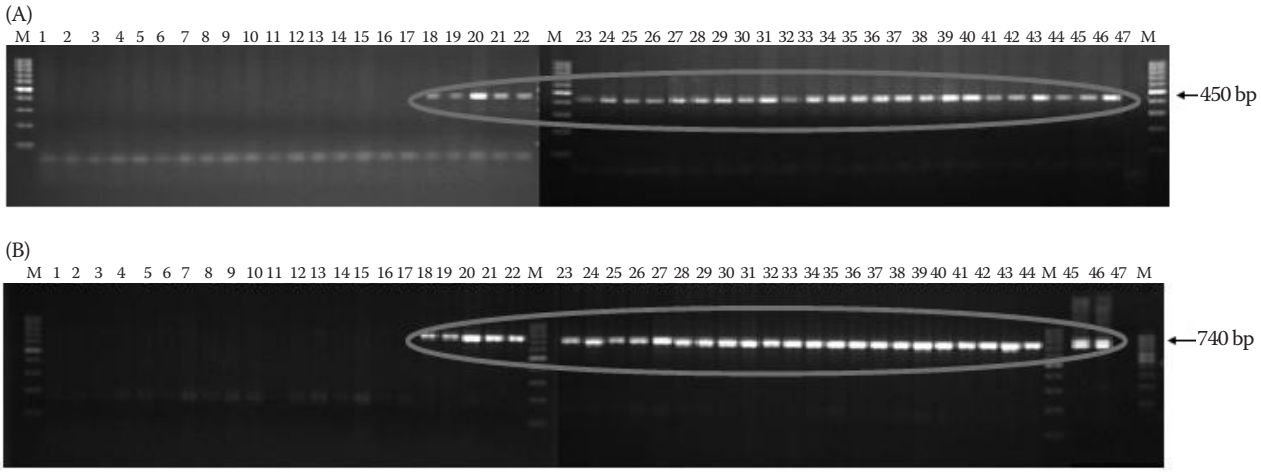

Figure 1. PCR patterns of cytoplasmic male sterility (CMS) and Rf lines which were obtained using HRG01 (A) and HRG02 (B) markers: 1–17 – electrophoretic spectra of the CMS line (*rf1rf1*); 18–46 – electrophoretic spectra of the fertility restorer line (*Rf1Rf1*); 47 – negative control; M – molecular weight standard (100 bp DNA ladder, Evrogen); fragments informative for the Rf line identification are marked

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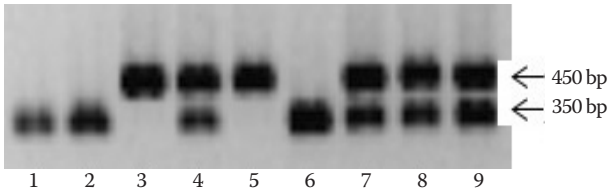

Figure 2. PCR products of the HRG01 marker obtained at an annealing temperature of 54°C
1, 2, 6 – CMS lines (*rf1rf1*); 3, 4, 5 – the fertility restorer lines (*Rf1Rf1*); 7, 8, 9 – F_1 hybrids (*Rf1rf1*)

marker. A decrease of the HRG01 primer annealing temperature from 58°C to 54°C resulted in the second amplicon appearance (Figure 2). Plants of Rf lines (*Rf1Rf1*) had only 450 bp amplicons. However, in plants of all CMS lines (*rf1rf1)* a new amplicon with 350 bp length was detected. Accordingly, the F1 hybrids (*Rf1rf1*) had both amplicons (Figure 2). Therefore, the sites of HRG01 primer annealing in CMS and Rf lines are polymorphic, however, the difference is low, likely one to a few single nucleotide polymorphisms. It is known that new alleles occur due to single nucleotide rearrangements. High density and evolutionary stability make such rearrangements the most effective genetic markers (Galeano *et al*. 2012). To prove the codominant nature of HRG01 marker and reveal the polymorphism of CMS and Rf lines sequencing of specific amplicons (350 and 450) was carried out. The direct sequencing of the HRG01 amplicons of three samples with *Rf1Rf1* genotype and three samples with *rf1rf* genotype was done. The alignment of these sequences on the 247 bp motif (from 108 to 355 position) revealed 223 identical and 24 polymorphic sites. Polymorphic sites are presented in Table 4. The level of similarity between samples with the same genotype was 99.6–100%, whereas similarity between different genotypes was 90.7–91.1%. Therefore, it may be concluded that 350 bp and 450 bp HRG01 PCR products are uniquely amplifying from the same locus. So a decrease in the HRG01 primer annealing temperature leads to a change from the dominant type of marker to a more informative codominant marker.

Secondly, the analysis of the *Rf1* gene presence in the annual and perennial species was done using HRG01 and HRG02 markers. Wild species of sunflower have a great genetic potential for improving agriculturally valuable traits of cultivated sunflower. Among them fertility restoring genes are of particular interest for developing the original selective material and hybrid breeding (Horn 2006). The investigation of annual sunflower species with HRG01 and HRG02 markers revealed ambiguous results (Table 1). PCR products of HRG01 (about 450 bp) were observed in all five studied species. However, the absence of HRG01 marker was detected in some parts of the investigated plants of *H. praecox* and *H. annuus*. So the specific amplicons were found in 75% of *H. praecox* (560400) and *H. annuus* (441236) samples, and in 50% of *H. annuus* (441245) investigated plants. HRG02 amplicons (about 740 bp) were observed in *H. annuus* and *H. petiolaris* only. The HRG02 marker was detected in 50% of *H. annuus* (441236, 441245) samples, in 25% of *H. annuus* (441183) and *H. petiolaris* (440560) samples. Perhaps, this is so because annual species of sunflower have more variable nucleotide sites of the HRG02 primer annealing sequence compared to HRG01. However, this is only an assumption.

Table 4. Polymorphic sites of the codominant marker HRG01 of the *Rf1* gene

No.	Polymorphic sites																							
	108	109	110	114	128	153	167	169	171	174	179	184	199	200	209	210	224	229	230	243	278	292	327	333
1	T	C	A	T	T	T	A	T	A	T	G	A	G	A	A	C	C	G	A	G	G	–	A	G
2	T	C	A	T	T	T	A	T	A	T	G	A	G	A	A	C	C	G	A	G	G	–	A	G
3	T	C	A	T	T	T	A	T	A	T	G	A	G	A	A	G	C	G	A	G	G	–	A	G
4	A	T	C	C	G	C	T	A	G	C	A	T	A	G	G	C	T	A	C	T	A	C	G	A
5	A	T	C	C	G	C	T	A	G	C	A	T	A	G	G	C	T	A	C	T	A	C	G	A
6	A	T	C	C	G	C	T	A	G	C	A	T	A	G	G	C	T	A	C	T	A	C	G	A

1, 2, 3 – polymorphic sites of the SCAR marker of the *Rf1* allele amplified on the template of genomic DNA of the fertility restorer lines (temperature of primer annealing 58°C); 4, 5, 6 – polymorphic sites of the SCAR marker of the *rf1* allele amplified on the template of genomic DNA of the CMS lines (temperature of primer annealing 54°C)

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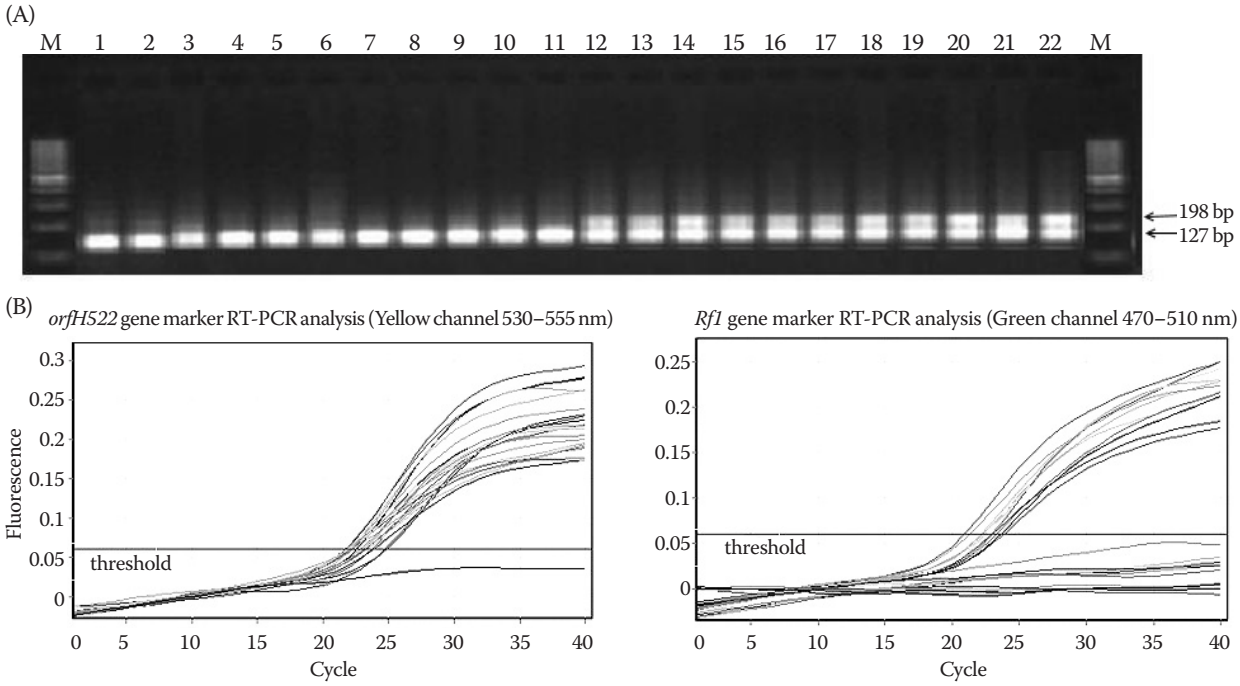

Figure 3. Multiplex amplification products of the HRG01 locus linked to the *Rf1* gene and marker of the mitochondrial *orfH522* gene associated with CMS-PET1: (A) PCR, 1–11 – CMS lines; 12–22 – Rf lines; M – molecular weight standard (100 bp DNA ladder, Evrogen), (B) real-time PCR

The data of HRG01 and HRG02 marker analysis in perennial sunflower species are presented in Table 2. Unlike the results of annual species research, the HRG02 (about 740 bp) marker was identified in all perennial samples studied, whereas the amplification of HRG01 was observed in 12 out of 26 species only. It is interesting to note that in some species the presence of the HRG01 (about 450 bp) marker varied greatly. For instance, the marker was detected only in one out of six *H. rigidus* (2106) populations. The marker was found nearly in 30% of *H. angustifolius* and *H. occidentalis* investigated plants, in half of *H. strumosus* and *H. maximiliani* samples. More than half of *H. divaricatus*, *H. mollis* investigated plants also demonstrated the presence of HRG01 amplicons (Table 2). According to the data obtained an opposite assumption could be made – perennial species had more conservative nucleotide sites of the HRG02 primer annealing sequence compared to HRG01.

Thirdly, the association of SCAR marker, defined in wild species, with fertility restoration has been proved by test crosses. Some of perennial sunflower species, carrying *Rf1* gene, according to genotyping by HRG02, were used for hybridization with CMS-PET1 cultivated sunflower lines (Table 3). Actually we predominantly used perennial sunflower species characterized by the same periods of blossoming as cultivated sunflower lines. However, survivability of pollen had also been the criterion for choosing paternal lines. The pollen fertility of hybrids varied from 75.3% to 97.8% (Table 3) and all hybrids carried the HRG02 marker, this confirms the introgression of *Rf1* gene. Thus, the HRG02 marker, which has been detected in all the wild perennial species, can be considered as a highly informative identifier of *Rf1* gene.

We have also designed a multiplex RT-PCR system for simultaneous detection of *Rf1* gene and mitochondrial marker associated with CMS-PET1 (*orfH522*). Approbations of the test system on CMS and Rf lines were successful. It is interesting to note that all studied lines had a 127 bp amplicon (Figure 3), which indicates the presence of the CMS-PET1 mutation and the same mitotype in CMS and Rf lines, respectively. The second amplicon (198 bp) has been detected only in plants of Rf lines (Figure 3). Such a test system is expected to increase the efficacy of selection of parental forms in order to obtain heterotic hybrids.

Therefore, the informative SCAR markers HRG01 and HRG02, as well as the developed RT-PCR testing system may be used for the marker-assisted selection of sunflower. The discovered codominant nature of

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HRG01 allows identifying allelic variants of *Rf1* loci (*Rf1* or *rf1*) in varieties of domesticated sunflower. As well as the HRG02 marker application is useful for searching the fertility restorer gene (*Rf1*) among wild type species.

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