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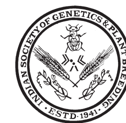

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RESEARCH ARTICLE

The use of molecular markers for acceleration of the selection process while developing sunflower (*Helianthus annuus* L.) maintainer lines

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Abstract

Sunflower hybrid production is based on cytoplasmic male sterility (CMS). The CMS system includes three main components: male sterile lines (Srfrf), maintainer lines (Nrfrf), and fertility restorer lines (NRfRf). An experiment was conducted to analyze the lines that are the candidates for maintainer lines for identifying fertility restoration gene (Rf_1), using molecular markers to facilitate hybrid breeding. Out of 477 lines, the application of fertility restorer-linked molecular marker(s) helped identify 365 lines as maintainers, but test cross analysis revealed that 371 lines functioned as maintainers.

Keywords: CMS, fertility restorers, maintainer, SCAR marker, sunflower

Introduction

Sunflower (*Helianthus annuus* L.) is one of the world's most important oilseed crops. The main focus of the sunflower breeding program is the selection of genotypes, resistance to pathogens to the parasitic plant broomrape (Orobanche cumana Wallr.), and resistance to the herbicides of the imidazoline and sulfonylurea groups and drought resistance, etc. All these attributes must be combined in breeding the material to realise the genetic potential of sunflower in the form of high productivity in the different agro-climatic zones of Ukraine. In recent years, the focus on creating genotypes with the help of molecular markers (marker-assisted breeding) is being used efficiently and quickly to combine several genes of basic agronomic traits, which has become relevant. Resistance to downy mildew (Pl) and broomrape (Or), herbicide tolerance (Ahas) genes, and fertility restoration (Rf) genes are currently important for sunflower breeding with integration into modern genotypes/hybrids (Dimitrijevic and Horn 2018). Sunflower hybrids are mainly produced using cytoplasmic male sterility (CMS) and fertility restoration (Seiler *et al.*, 2017). The hybrid breeding system is based on the use of cytoplasmic male sterile lines (S*rfrf*), maintainer (N*rfrf*), and fertility restorer lines (N*RfRf*) (Letian et al. 2014). The widely used CMS is PET1 type originated from an interspecific hybridization of *H. petiolaris,* sub-species *petiolaris,* and *H. annuus* (Leclercq 1969). An alternative source of cytoplasmic male sterility is PET2 type, which is also orinates from the crosses between *H. petiolaris* with *H. annuus*, but leads to a different mechanism of CMS (Reddemann and Horn 2018). Another CMS system, i.e., PEF1 is also obtained from the crossing of *H. petiolaris,* sub-species *fallax* with *H. annuus* (Schnabel et al. 2008).

Currently, about 70 sources of CMS in sunflower have been described, but fertility restoration genes have been identified only for some of them (Serieys 2005). Among the

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described fertility restorer genes are named as Rf_1, Rf_2, Rf_3, Rf_4, and others (Serieys 2005; Horn et al. 2003; Popov et al. 2014; Markin et al. 2017; Yue et al. 2017; Leclerq and Philippon 1984; Liu et al. 2011; Feng and Jan 2008; Qi et al. 2012). Molecular markers for these genes have also been identified, and sunflower genotypes carrying these genes have been developed. The Rf_1 gene is a pollen fertility restoration gene discovered in the 1970s in the T66006–2–1 line, which was obtained by crossing the wild sunflower with the cultivated sunflower (Serieys 2005). Different types of DNA markers, such as RAPD, ALFP, and SSR, etc., were involved in the study of the Rf_1 gene. Two SCAR markers were developed from two linked RAPD loci (OPK13_454 and OPY10_740). They were marked as HRG01 and HRG02. The gene Rf_1 is located on the 13th linking group (LG13) (Horn et al. 2003). Based on the F_2 generation analysis, it was found that the SCAR markers, HRG01 and HRG02 are closely linked to the Rf_1 gene. In the same study, it was shown that the markers HRG01 and HRG02 in F_2 have a cleavage with a ratio of phenotypic classes of 3:1 (Popov 1 et al. 2014). The analysis of five annual and 26 perennial sunflower species and characterization SCAR–markers HRG01 and HRG02 linked to Rf_1 gene revealed that SCAR–marker HRG02 is better used for perennial sunflower species while HRG01for annuals (Markin et al. 2017). The analysis of the population obtained from the public sunflower lines, RHA439 and CCS HA441, the SSR marker ORS511 was created, which is also closely linked to the Rf_1 gene and located at a distance of 3.7 cM (Yue et al. 2017); they also used TRAP marker, K11F05Sa12–160, which is located at a distance of 0.4 cM from the Rf_1 gene

The molecular markers closely linked to the traits allow breeders to select lines in early generations effectively. Preferably, the markers linked to the Rf_1 gene are used in hybrid breeding and identify the lines carrying the gene for fertility restoration. In some of the cases, the use of only one marker system does not give a full guarantee for complete restoration. Therefore, to confirm the trait, a field assessment is done. Since conventional breeding takes about 12–15 years to create a highly productive and competitive hybrid, molecular markers are recommended for an effective tool to accelerate hybrid breeding. Further, to use DNA markers effectively, they must be validated on various source materials. Keeping in view the above, a study was carried out to identify true maintainers using a molecular marker HRG01linked to fertility restoration.

Materials and methods

Plant materials

The maintainer lines namely, BH320, BH039 and BH3978 used were taken from the working collection of the Ukrainian Scientific Institute of Plant Breeding (VNIS). Three hybrids viz., NK Neoma, ES Artimis, and Dragan were used in crosses involving the above-mentioned maintainer lines. In the first stage of the study, the maintainers (*Nrfrf*) were crossed with all three hybrids (*SRfrf*). The F1 hybrids involving these lines, BH320/NK Neoma, BH039/EC Artimis, and BH3978/Dragan were self-pollinated. Based on the morphological characters, the selection was practiced in F2 populations. Suitable single plants were advanced to F3 and F4 populations. From the first group of lines originating from the cross, BH320/NK Neoma,130 lines were taken for testing. One hundred and fifty-six lines were selected from the population derived from BH039/Artimis cross, whereas 191 lines were chosen from a cross between BH3978 and Dragan. Thus a set of 477 lines of sunflower was composed and tested for the presence of Rf_1 gene. Molecular analysis for the presence of a linked marker to the Rf_1 gene and analytical cross-breeding to control the maintaining and restoration characteristics in the sunflower lines was done.

Polymerase Chain Reaction (PCR)

The molecular analysis was done to find out the presence of the SCAR marker HRG01 linked to the *Rf1* gene among the lines. Genomic DNA was isolated from sunflower leaves using a protocol developed by Rogers and Bendich in 1985. The SCAR marker was identified by PCR with a pair of primers that flank certain areas of sunflower genomic DNA. The nucleotide sequence of the primer, HRG01 locus is: F: 5′–TATGCATAATTAGTTATACCC–3′ and R: 5′–ACATAAGGATTATGTACGGG–3′ (Horn 2003). A pair of primers to the ORS510 microsatellite locus (F: 5′–CATCGCGTCCCTCTCTCTAA–3′; R: 5′–CCAACCATCACAGCAATCAG–3′) was used as a reference marker (Tang et al. 2002). These primers were combined into a multiplex. GenePak PCR Core reagent kits manufactured by Izogen (Russia) were used. The final volume of the reaction mixture was 20 μl and contained 20 ng of genomic DNA with the addition of 0.2 μM of each primer. 20 μL of mineral oil were added to the tubes with the reaction mixture. PCR was performed on a thermal cycler Tertsyk (Russia) according to the program: 1 cycle of initial denaturation at 94°C for 10 minutes, 35 cycles of 94°C for 45 seconds, 58°C for 45 seconds, 72°C for 60 seconds, and 1 cycle of final elongation at 72° C for 6 minutes. T e amplification products were separated by high resolution 2% agarose gel electrophoresis and ethidium bromide in low ionic buffer (Horn 2003) and subsequent photography in UV light with a Nikon D50 photosystem. A fragment length marker of 50 bp was used as a marker of fragment length.

The field assessment

A field assessment of the presence or absence of the fertility restoration gene was performed on all the three F_1 hybrids obtained by crossing maintainer lines and hybrids. Phenotypic evaluation of F_1 hybrids was also done during active flowering.

Results and discussion

The main objective of the present study was to differentiate the maintainer lines. The present study was carried out the molecular analysis and field assessment of the lines selected from three crosses in sunflower. The selected SCAR marker HRG01 linked to the *Rf_1* gene met the criteria of identification of fertility restoration in the desired material. About 477 derivatives from the three crosses were subjected to analysis out of which 365 lines were identified as candidates for maintainer lines as there was no amplification of marker linked to fertility restorer gene (Fig. 1 representing only 29 lines). A total of 112 lines showed an amplification product of 426bp, indicating the presence of fertility restorer gene Rf_1, which was confirmed by the location of a marker at a distance of 0.4cM from the target gene (Fig. 2 representing only 11 lines). The remaining 365 lines did not contain the amplified product of 426bp hence the presence of Rf_1 gene was ruled out.

Horn et al. (2003) mapped the *Rf_1* gene restoring pollen fertility in PET1 based F1 hybrid in sunflower. They used two RADP markers (ORK13_454 AND OPY10_740) converted into SCAR markers (HRG01 and HRG02), differentiated by the fertility restoration gene in the inbred lines. After analyzing five annual and 26 perennial sunflower species, Markin *et al.* (2017) concluded that it is better to use the SCAR marker HRG-02 for the perennial species, and HRG-01 for the annuals. Goryunov et al. (2019) also used PET1 based lines and the associated mapping of the *Rf_1* fertility restoration gene was performed. A 7.72 MB segment on the 13th chromosome was isolated, which was used to identify 21 fertility restorers.

The present study has demonstrated the efficiency of the SCAR marker HRG01 in the F_3/F_4 generation. The selected lines of mutant origin and samples of interspecific sunflower hybrids were analyzed by Popov (2015), who validated the SCAR marker HRG01 linked to gene for fertility resoration in sunflower. The present study has confirmed that this marker effectively differentiates the lines of different origins. In the second stage of this study, the evaluation of F_1 hybrids obtained through the test cross was carried out. The evaluation test based on pollen fertility involved crossing sterile lines (*Srfrf*) with 477 sunflower lines (*NRfRf* or *NRfrf* or *Nrfrf*) derived from the three crosses. Based on the presence fertility restoration (characterized by the presence of *Rf_1* gene), the genotypes were classified into: 1 –fertile (*SRfrf*), indicating that the tested lines are not the maintainers; 2- sterile (*Srfrf*), all plants were male sterile indicating that the tested lines are the maintainers of sterility. As a result of the field assessment, it was found that out of 477 lines, 371 lines were maintainers (*Srfrf*) and the remaining 106 lines were fertile indicating that these lines contain the restorer gene of fertility (*SRfrf*).

During the analysis of F_1 hybrids after test crossing, no discrepancies were found in the results obtained both after molecular analysis and after field assessment in the first group of lines derived from BH320/NK Neoma. This suggested that the exchange of segment (Rf1 gene) between homologous chromosomes containing fertility restorer gene was normal. Because recombination is an unpredictable phenomenon during meiosis, the ability to identify certain traits is an effective tool when the selection is performed in the early stages of breeding programs. However, the discrepancy among the lines derived from BH039/EC Artimis was revealed as 101 lines were identified as maintainers in test cross-evaluation among the tested 156 lines, while only 107 lines were candidates for maintainer

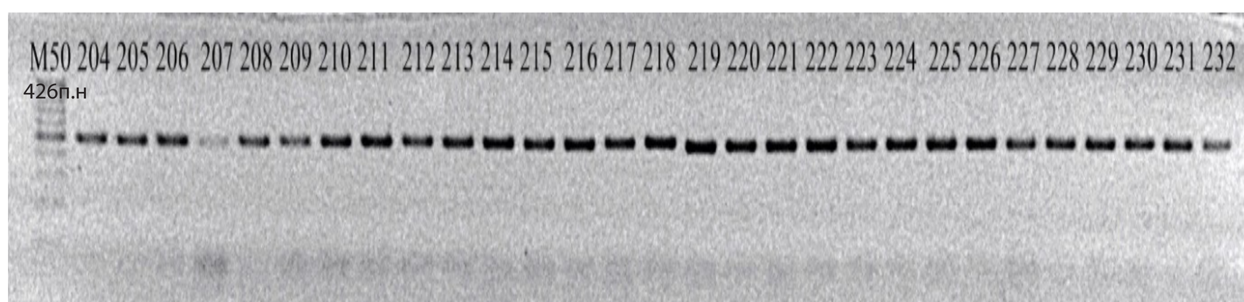

Fig. 1. Electrophoregram of the SCAR marker HRG01 separation. M50 – marker DNA ladders 50 bp, 204 – 232 individual plants of the studied lines, in which there is no amplicon in the amount of 426 bp

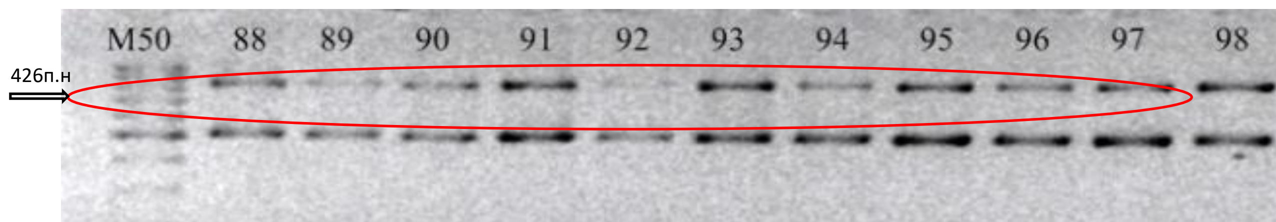

Fig. 2. Electrophoregram of the SCAR–marker HRG01 separation. M50 – marker DNA ladders 50 bp. There is an amplicon in 426 bp in the samples 88 – 98

based on molecular analysis. In the lines derived from third cross (BH3978/Dragan), 128 lines were identified as the maintainers as a result of molecular analysis whereas in analytical crossing, the 140 maintainers were identified. The differences in the number of identification of maintainer lines are reflected in the study; may be due to the errors caused in field evaluation and the size of the population from different crosses subjected for molecular and field analysis. Based on the molecular markers, 365 line were characterized as maintainers, while in the field evaluation, 371 lines were identified as maintainers. There may be differences in recombination frequency among the selected crosses involving diverse materials. Therefore, the use of molecular markers is an effective tool as done in the present study (SCAR marker HRG01 linked to the Rf_1 gene) to select maintainers and fertility restorers in diverse early generation populations is preferred.

Authors' Contribution

Conceptualization of research (PY, SY); Designing of the experiments (PY, KM, SY, MP); Contribution of experimental materials (PY); Execution of field/lab experiments and data collection (BV, PY); Analysis of data and interpretation (BV, PY, PV); Preparation of manuscript (BV, PV, SY).

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