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Molecular Marker Aided Selection of Restorer of Fertility (Rf/rf) Alleles to Develop Maintainer Lines in Sunflower

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ABSTRACT

Sunflower (*Helianthus annuus* L.), one of the main oil crops in the world, is also increasingly used for its ornamental value. Marker-assisted selection (MAS) helps identify desired traits through molecular markers linked to specific genes, speeding up the breeding process. In sunflower breeding, dominant and codominant markers are used to detect Rf1/rf1 alleles for the selection of plants carrying maintainer and/or restorer (Rf) nuclei. In a breeding program, the status of Rf/rf alleles in the nucleus of the maintainer (B) lines cannot be ascertained without a test-cross due to the presence of fertile cytoplasm. The aim of this study was to test the utilization of the SCAR markers linked to Rf/rf alleles in sunflower to test if the markers can be integrated into a breeding program to develop maintainer lines. The plant materials included restorer and maintainer lines, as well as backcross and test-cross populations segregating for Rf/rf alleles. The markers (PPR621.5) have proven to be valuable for MAS in sunflower breeding. The Rf/rf specific dominant markers were used with the atp9 internal control marker to differentiate PCR failure from the absence of an allele. The markers were also phenotypically confirmed using advanced lines, BC_1F_1, BC_2F_1, and BC_1F_2, as well as test-cross populations. The application of SCAR markers in developing new restorer and maintainer lines can enable efficient determination of Rf/rf allele status, thereby eliminating the need for test crosses in maintainer nuclei and significantly enhancing breeding efficiency. Detection of Rf/rf alleles by molecular markers in maintainer populations with fertile cytoplasm may help reduce cost and breeding time to develop maintainer lines.

1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the world's fourth most important oil crop, primarily cultivated in temperate and semi-arid regions. In addition to its primary purpose for human consumption, sunflower oil has many applications and can be utilized as a supplement in the chemical and pharmaceutical industries. Sunflower originated in North America and was probably first introduced to Europe through Spain in the 16th century as a garden ornamental flower and gradually moved eastward and northward in the European continent reaching Russia in the 18th century (Azarin et al. 2023). Sunflower as oil crop and ornamental use is mainly hybrids developed via use of male sterility.

The techniques of cytoplasmic male sterility (CMS) and fertility restoration (Rf) have been extensively utilized for hybrid seed production in sunflower, and in some other major field crops. Leclerq (1969) discovered CMS in sunflower through an interspecific hybridization between a wild sunflower species, Helianthus petiolaris, and a cultivated sunflower, *H. annuus*. Although multiple types of CMS have been identified, the current production of commercial hybrids utilizes the male-sterile cytoplasm (PET1) discovered by Leclerq (1969). Numerous PET1 fertility restoration lines have been created, and one to four dominant fertility restoration genes have been identified (Serieys, 1996). Rf2 is present in nearly all cultivated sunflower lines (Reddy and Thammiraju 1977, Leclerq and Philippon 1984, Miller and Fick 1997). Two dominant nuclear alleles, Rf1 and Rf2, are required to restore male fertility in most cultivated sunflower lines.

Sunflower is the second largest commodity grown from hybrid seed after maize (Dimitrijevic and Horn, 2018). Commercial hybrid seed production relies on cytoplasmic male sterility (CMS) and male fertility restorer (RF) lines. Fertility restorer genes are nuclear-based and tend to surmount the F1 generation's cytoplasmic male sterility. A high grain filling percentage requires a satisfactory fertility restoration. Rauf (2019) reviewed a selection of the more than 70 cytoplasmic male sterility sources developed for sunflower. These CMS sources can only be tapped using appropriate restorer genes. Hybrid sunflower breeding generally relies on the Rf1 gene obtained from the T66006-2-1-B line (Kinman, 1970). Diversification of cytoplasmic and fertility restorer genes is an essential breeding goal to reduce genetic susceptibility to diseases and pathogens. Talukder et al. (2019) have identified a variety of fertility restorer genes (Rf1–Rf7, Rf-PEF1) compatible with diverse CMS sources. A selection of the validated markers used to identify various Rf genes, and their corresponding linkage group is presented in Table 2. Molecular markers are used to identify and differentiate the alleles. These markers are particularly important for plant breeding and genetic studies (Akkose Baytar et al. 2021). Marker-assisted selection (MAS) allows for rapid and efficient selection of plants carrying Rf genes. These markers save time and cost in seed production, ensure that genetic purity is maintained, and prevent contamination in hybrid seed production. The use of molecular markers to determine whether Rf1 and Rf2 genes are homozygous or heterozygous in sunflower maintainer lines is important in plant breeding and genetic studies. These markers aim to detect the presence or absence of cytoplasmic male sterility (CMS) fertility restorer genes. It is a critical step in maintainer line development that would nullify the need for cumbersome test crossing.

The study by Horn et al. (2003) showed fine mapping of the Rf1 gene. Anashchenko and Duka (1985, 1986) identified Rf genes from different sources of fertility restoration. Liu et al. (2003) performed fine mapping of the fertility-restoring Rf1 gene for cytoplasmic male sterility in cotton (*Gossypium hirsutum* L.). Similarly, Wu et al. (2014) developed an InDel marker linked to the Rf1 gene in cotton and evaluated its use in breeding programs.

The development and use of these markers contribute to the acceleration of genetic studies in sunflowers and other crops and the establishment of more effective breeding programs. The aim of this study was to evaluate the utility of the molecular markers to identify plants carrying restorer of fertility alleles at homozygote or heterozygous (RfRf, rfrf, or Rfrf) states in segregating maintainer populations in sunflower breeding.

2. MATERIALS AND METHODS

Plant Material

The plant materials were grown at Akdeniz University, Antalya, Türkiye, during the spring and fall growing seasons in 2023 and 2024.

A homozygous restorer line resistant to herbicide sulfonylurea (SU) obtained from Osterras seed company (Antalya, Türkiye), and was crossed with a commercial male-sterile ornamental sunflower hybrid, Sunrich Orange F_1 (SOF_1). Resulting F_1s were backcrossed both to SOF_1 and to Predovik, a commonly used public maintainer line. The BC_1F_1 population of SOF_1 segregated 1:1 for both male sterility/fertility and SU herbicide resistance. Fertile and SU resistant BC_1F_1 plants were backcrossed to SOF_1 once more to create the BC_2F_1 population; the

same plants were also used as male parents and backcrossed to the F_1s originating from the Sunrich Orange F_1 x Peredovik cross. As expected, the resulting BC_2F_1 population of SOF_1 segregated 1:1 for both male sterility/fertility and SU herbicide resistance, while the BC_1F_1 population derived from Peredovik segregated for SU herbicide resistance (1:1) and was all fertile due to Peredovik fertile cytoplasm. The SU-resistant BC_1F_1-Peredovik plants were selfed to generate the BC_1F_2 population. In the BC_1F_1 and BC_2F_1 populations of SOF_1, the Rf allele was expected to segregate 1:1 (Rfrf:rfrf) in line with the observation of male fertile/male sterile phenotypes. In the Peredovik BC_1F_1 population, Rf/rf allele was expected to segregate 1:1 (Rfrf:rfrf) as well in the BC_1F_2 population; the ratio was 3:1 (Rf:rfrf) for Rfrf plants, and all rfrf for the other half of the population.

Test cross populations: Two different test cross populations were created. The first one was heterozygous fertile BC_1F_1 plants from the SOF_1 population that was crossed with a CMS line to generate a population segregating 1:1 for male sterility/fertility due to Rfrf / rfrf genotypes in the nucleus. The second one was the same heterozygous fertile BC_1F_1 plants from the SOF_1 population that were crossed with the maintainer line Peredovik, which is expected to segregate 1:1 for Rfrf:rfrf allele in the nucleus, but all plants were expected to be fertile due to the Peredovik cytoplasm.

Table 1. The pedigree, generations, and properties of plant materials used in the study.

Population / Genotype	**Pedigree**	**Generation**	**Properties**
Restorer	Osterras	Advance line	Sulfonylurea (SU) resistant
Maintainer (Peredovik)	Public	Advance line	Maintainer
Sunrich Orange F_1 (SOF_1)	Commercial	Hybrid	Male-sterile (CMS) ornamental hybrid
F_1	SOF_1 x Restorer	F_1s	SU resistant-sterile cytoplasm, fertile hybrid
F_1s x Peredovik	F_1 x Peredovik	F_1p	Maintainer (fertile) cytoplasm
F_1s x SOF_1	F_1s x SOF_1	BC_1F_1s	SU resistance and male fertility segregate (1:1), sterile cytoplasm
BC_1F_1s (fertil +SU) x F_1p	BC_1F_1s x F_1p	BC_1F_1p	SU resistance (1:1), restorer of fertility in nucleus 3:1 (Rf:rfrf)
BC_1F_1s (fertil +SU) x SOF_1	BC_1F_1s x SOF_1	BC_2F_1s	Sterile cytoplasm, 1:1 segregation for male sterility and SU resistance
BC_1F_2p	BC_1F_1p x BC_1F_1p	BC_1F_2p	Fertile cytoplasm, restorer of fertility in nucleus 3:1 (Rf:rfrf) for half, and all rfrf for the other half
Test-cross	BC_1F_1p (Rfrf) x CMS	BC_1F_1	Sterile cytoplasm, segregated 1:1 for fertility/sterility
Test-cross	BC_1F_1s (rfrf) x CMS	BC_1F_1	Sterile cytoplasm, all sterile,

DNA Extraction

Genomic DNA was isolated according to the standard Cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). The youngest leaf materials were incubated with 15 mL extraction buffer (100 mM of Tris/HCl pH 8.0, 1.4 M of NaCl, 20 mM EDTA, 2% CTAB, 1% Na2S2O3) at 65ºC for 1 hour. After chloroform–isoamyl alcohol (24:1) solution was added to each tube and the samples were obtained by centrifugation. Approximately 300 µL of the supernatant was transferred into new tubes, and 300 µL of isopropanol was added to each sample. After mixing, the tubes were stored overnight at –20 °C. The samples were centrifuged for 10 min at 14,000 × g and washed with 70% ethanol. After adding 100 µL of sterile pure water, the tubes were stored at 4 °C for short-term and at –20 °C for long-term use.

Polymerase Chain Reaction

SCAR markers used for analysis, relevant primers, expected sizes of PCR amplification products, and literature are presented in Table 2.

Table 2. Sequences of SCAR primers, lengths of PCR products, and related literature

Marker	Primer sequence (5'-3')	Expected size of the SCAR marker and allele (Rf/rf)	Reference
67N04_F1a	TGCAAGATAGGCGACTGAG	170 (Rf)	Horn et al. 2019
	GGCTCATCTCCAATTA		
67N04_F2b	TGAGGGCTCATCTCCAGCTG	155 (rf)	Horn et al. 2019
67N04_R	GGCTGCCATTAGTGAAGGAG	Common (Rf/rf)	
PPR621.5 F1	CAGTAATCTCCACATGAACATTG	164 (rf)	Horn et al. 2019
PPR621.5 F2	CAATAATCTCCACATGAACATTC	164 (Rf)	Horn et al. 2019
PPR621.5 Rev	CCGGATTGTGTTCCGATTAG	Common (164 Rf/rf)	
HRG02 F	AAA CGT GGG AGA GAG GTG G	738 (Rf)	Horn et al. 2003
HRG02 R	AAA CGT GGG CTG AAG AAC TA		
atp9_for	GGTGCAAAATCAATAGGGGCCG	474 (internal control)	Accession X51895
atp9_rev	ACCGAATGAATGCGTCACAAGG		

For the Rf1/rf1 restorer gene, 67NO4 is a codominant SCAR marker. One of the three primers is common to both alleles of Rf/rf, one is specific to the Rf allele, and the other to the rf allele. If the triple primer set creates only a 170 bp long band, it indicates that the plant is RfRf homozygous restorer, if only a 155 bp long fragment is present, then it is rfrf homozygous maintainer, and a heterozygous genotype yields both fragments.

Again, two different dominant markers designed from a gene developed for Rf1 and shown as a candidate gene for Rf1; the primer combination PPR621.5_F1/PPR621.5_Rev creates a 164 bp long band specific to the rf (maintainer) allele only, while the primer combination PPR621.5_F2/PPR621.5_Rev creates a 164 bp long band specific to the Rf (restorer) allele only. Dominant markers are used in combination with the atp9 primers as an internal PCR control.

The HRG02 dominant SCAR marker was developed specifically for the Rf1 restorer allele by Horn et al. (2003). This dominant SCAR marker is utilized in conjunction with the atp9 marker as well to figure out if the absence of the expected 738 bp long marker band is due to a PCR failure or to a homozygous rfrf genotype.

For the PCR reaction, 2 µl of DNA was mixed with 13 µl of master mix. The master mix contained 1.5 µl dNTP (5 mM), 4.7 µl ddH2O, 1.5 µl 10x PCR buffer with MgCl2, 1.8 µl forward primer (10 mM) and 1.8 µl reverse primer (10 mM), and 0.2 µl Taq polymerase. PCR was performed on a thermal cycler according to the program denaturation at 94ºC for 3 min, followed by 35 cycles of 94ºC for 45 seconds, 45 seconds annealing depending on the primer combination, 30 seconds polymerization at 72ºC. The PCR program was finalized with elongation at 72ºC for 6 minutes. SCAR markers were separated in a 1.5% agarose gel electrophoresis and ethidium bromide and subsequent photography in a UV light system.

Data Analysis

In this study, the Chi-square (χ^2) test was used to test deviation between observed and expected phenotypic ratios. Observed (O) and expected (E) frequencies were calculated, and the Chi-square value was determined with the formula. The p-value was calculated using the degrees of freedom and Chi-square distribution table, and statistical significance was evaluated (Hartl and Cochrane, 2005; NCBI, 2010).

3. RESULTS

67NO4 marker

PCR screening was performed with 67N04_F1a/Rev and 67N04_F2b/Rev primers on 32 different sunflower plant samples representing maintainer, CMS, and Restorer x CMS hybrids. With the codominant marker (67N04_F1a/Rev) developed for the Rf1 (Rf/rf) restorer gene, a 170 bp long Rf homozygous restorer band was obtained in hybrids, 17 to 23, having a heterozygous (Rfrf) genotype. The homozygous rfrf nucleus genotype was evident on the maintainer and CMS samples with a 155 bp long rf allele fragment (Figure 1).

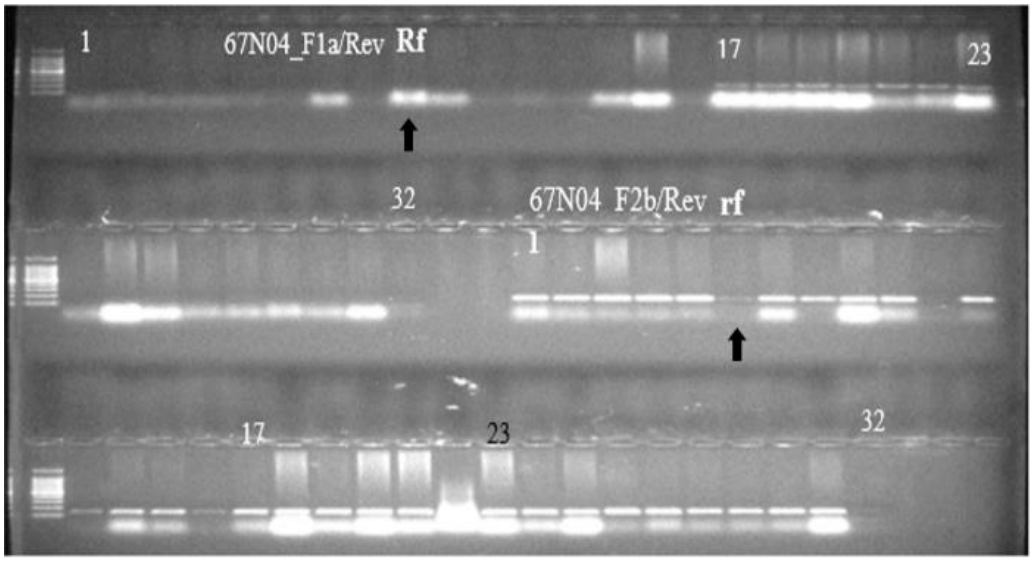

Figure 1. Primers 67N04_F1a/Rev (170 bp (Rf)) and 67N04_F2b/Rev (155 bp (rf)). 1-16 and 24-32 maintainer and CMS lines, respectively; 17-23 hybrids (restorer x CMS).

PPR621.5 marker

Primer screening was performed on the same 32 plant samples using PPR621.5_F1/Rev and PPR621.5_F2/Rev primers. As expected, the rf allele-specific primer combination PPR621.5_F1/Rev yielded 164 bp long fragments on maintainer, CMS, and hybrid plant samples. Rf1 specific primer combination (PPR621.5_F2/Rev) yielded the expected 164 bp long allele-specific band on the hybrids (Figure 2).

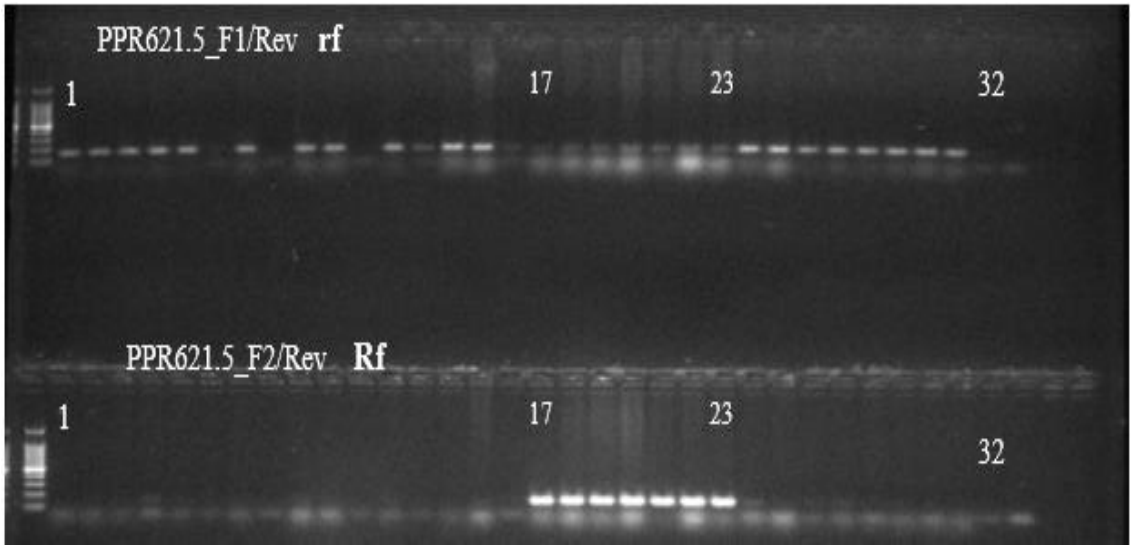

Figure 2. Marker PPR621.5_F1/Rev for the maintainer lines. Marker PPR621.5_F2/Rev for the restorer lines. 1-16 and 24-32 maintainer and CMS lines; 17-23 hybrids (restorer x CMS).

Because the marker is dominant (allele specific), atp9 internal PCR control primers were also added into the reactions while using PPR621.5_F2/Rev primers. In a segregating maintainer population (BC_1F_2p in Table 1) of 105 plant samples, the expected 164 bp for the Rf1 allele and the 474 bp long atp9 fragments were formed, where 72 plants showed the presence of the Rf1 allele (RfRf+Rfrf) while 33 plants had homozygous (rfrf) maintainer nuclei (Figure 3).

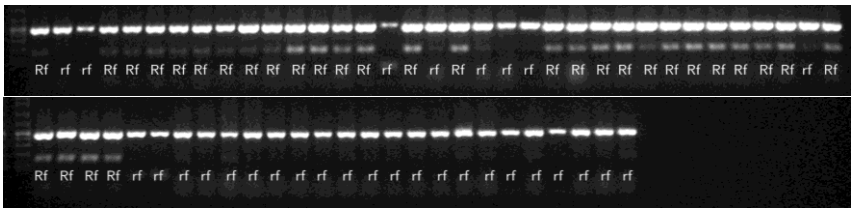

Figure 3. Dominant Rf1 specific PPR621.5_F2/Rev marker and atp9 internal PCR control in an F_2 segregating maintainer population (BC_1F_2p).

The primer PPR621.5_F2/Rev and atp9 were also tested in a BC_1F_1 population (BC_1F_1p in Table 1) consisting of 70 plants segregating for Rf/rf alleles in a fertile cytoplasm. We further confirmed the Rf1 allele-specific dominant marker, where 45 plants yielded the 164 bp Rf1 allele (Rf1rf1) while the rest lacked that allele (rf1rf1) (Figure 4).

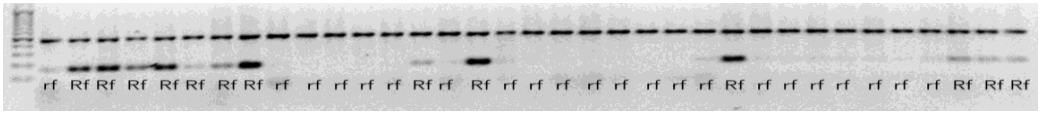

Figure 4. Marker PPR621.5_F2/Rev for Rf allele (164 bp) for the restorer lines and internal control marker atp9 (474 bp).

The 288 plants made up of segregating maintainer populations at BC_2F_1 and BC_1F_2 were genotyped using the marker. Maintainer lines homozygous for rf1rf1 in the nucleus were determined. PPR621.5 F2/Rev marker was also used for Rfs allele detection along with the atp9 primers (Figure 5). The marker showed expected segregation ratios in both populations, 1:1 and 3:1, respectively.

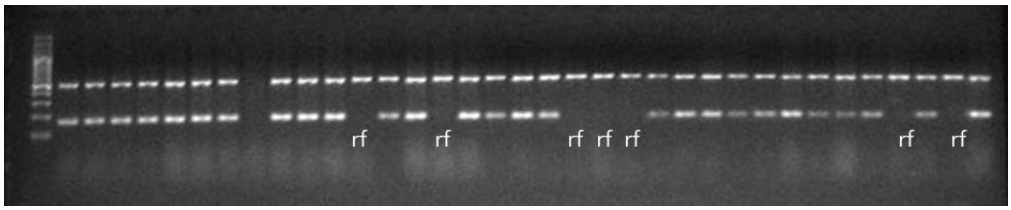

Figure 5. Marker PPR621.5_F2/Rev and atp9 for the Rf1 allele. The plant material consisted of both BC_1F_2 and BC_2F_1 maintainer populations.

In order to test the result of marker screening, a test cross was carried out. A few of the 76 plants either homozygous (rfrf) or heterozygous (Rfrf) with maintainer cytoplasm at the BC_1F_1 population, segregating for the Rf/rf allele, were crossed with a CMS line. The Rf allele-specific SCAR marker PPR621.5_F2/Rev indicated that 42 plants were homozygous (rfrf) and 34 heterozygous (Rfrf) for the restorer of fertility alleles in their nucleuses (Figure 6).

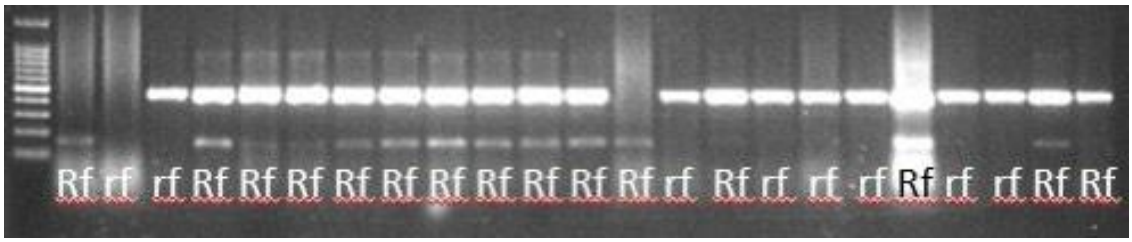

Figure 6. Marker PPR621.5_F2/Rev for the Rf allele segregating 1:1 in A BC_1F_1-B population of 76 samples.

The heterozygous (Rfrf) fertile BC_1F_1p plants were used as donor and crossed to a CMS line to create test-cross populations. The 16 plants obtained from the test cross (Rfrf x rfrf) were planted for phenotyping where 8 of them were fertile and 8 sterile (Figure 7).

Figure 7. Testcross result of BC_1F_1p line with heterozygous (Rfrf) nucleus was crossed to a CMS line to create the test-cross population where fertility/sterility segregated 1:1.

The homozygous (rfrf) fertile BC_1F_1p plants were used as donor and crossed to a CMS line to create test-cross populations. The 30 plants obtained from the test cross (rfrf-B x rfrf-A) were also planted, where all 30 showed complete sterility (Figure 8).

Figure 8. The BC_1F_1p line with homozygous (rfrf) nucleus was crossed to a CMS line to create the test-cross population, where all plants were sterile.

HRG02 marker

The dominant SCAR marker HRG02 linked to the Rf1 allele was also used in combination with primers for the atp9 as an internal control. The screening was performed on 32 plant samples consisting of maintainers, CMS, and hybrids (Restorer x CMS) with primers HRG02 F/R/atp9. The HRG02 F/R amplified a 738 bp band only in hybrid (17-23) samples (Figure 9).

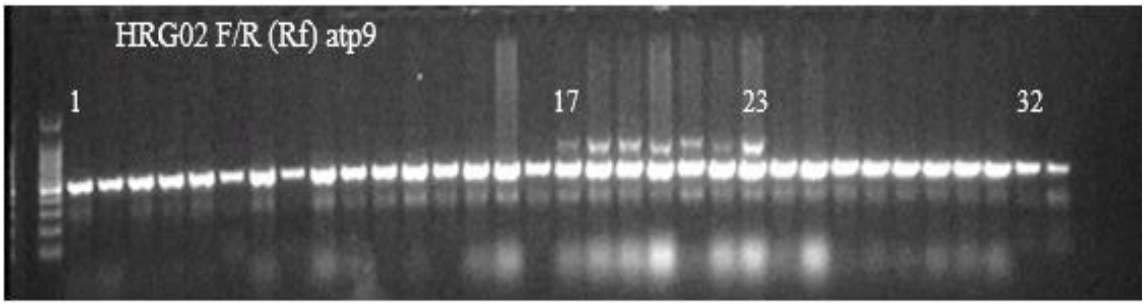

Figure 9. Duplex PCR reaction with the marker HRG02 and atp9, using maintainer (1-16), hybrids (17-23), and CMS (24-33) plant samples.

In Figure 10, the 8 BC_1F_1s plants exhibited a genotypic segregation ratio of 1:1, with 50% fertile and 50% sterile individuals, as expected. These plants were analyzed using the markers HRG02/Rev (Rf), PPR621.5 F1/Rev (rf), and PPR621.5 F2/Rev (Rf) with atp9 internal control primers. Upon examination of the marker results, it was determined that the outcomes of HRG02 were consistent with those of PPR621.5 F1 and PPR621.5 F2 markers.

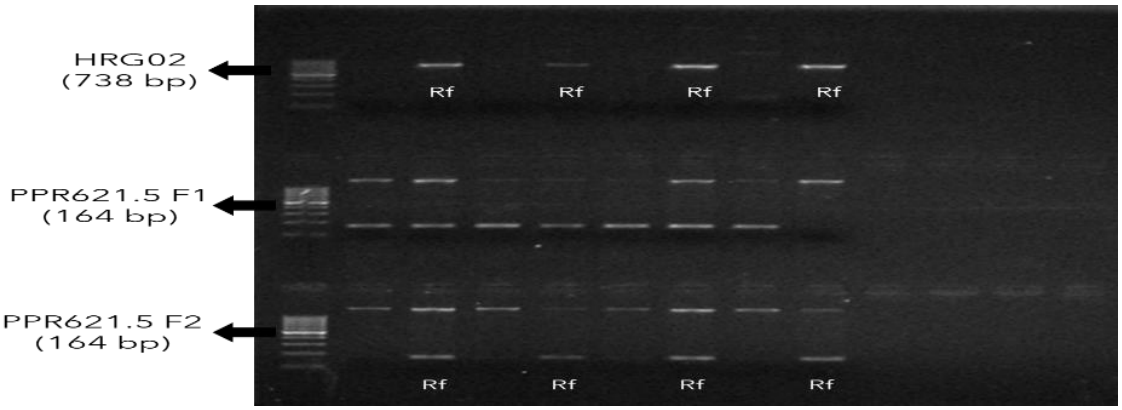

Figure 10. Duplex PCR reaction with the marker HRG02, PPR621.5 F1, PPR621.5 F2, and atp9, using BC_1F_1s plant samples segregating for Rf allele.

4. DISCUSSION

The tightly linked or allele-specific SCAR markers are highly effective for utilization in MAS. In this study, SCAR markers were exploited to detect the Rf/rf (restorer of fertility) allele at CMS, maintainer, and restorer populations. We tested six markers linked to the Rf/rf alleles to determine and confirm the reliable markers. All six developed SCAR markers enabled discrimination between restorer lines, maintainer lines, and CMS lines. However, PPR621.5 F2/Rev primers with atp9 internal control primers can be successfully and reliablly used for selection of Rf/rf alleles at various backgrounds.

The presence of Rf genotypes was examined in 26 perennial and 5 annual sunflower species using SCAR markers by Markin et al. (2017). Their analysis of annual sunflower species using HRG01 and HRG02 markers produced ambiguous findings. They stated that all five of the species under study had HRG01 PCR products (about 450 bp). HRG02 amplification products (about 740 bp) were observed only in *H. annuus* and *H. petiolaris*. HRG02 marker was detected in 50% of the *H. annuus* (441236, 441245) samples and in 25% of the *H. annuus* (441183) and *H. petiolaris* (440560) samples (Markin et al. 2017). In contrast to the findings of annual species research, only 12 out of 26 species showed HRG01 amplification, but the marker HRG02 (about 740 bp) was identified in all perennial species examined. Therefore, the HRG02 marker detected in perennial species can be considered a highly informative identifier of the Rf1 gene.

Figures 3, 4, 5, 6, 9, and 10 demonstrate that duplex PCR reactions worked very well and therefore atp9 represents a reliable internal control. The markers HRG02 and HRG01 developed by Horn et al. (2003) have proven to be almost universally usable in hybrids between restorer and maintainer lines.

The 67N04 primer, developed as a codominant SCAR marker for the Rf1/rf1 restorer gene in the study by Horn et al. (2019), is highly valuable as it enables the identification of heterozygous individuals. Additionally, in

the same study, two SCAR markers (PPR621.5R and PPR621.5M) were successfully developed to identify restorer and maintainer lines in PCR reactions. These markers serve as effective tools to distinguish between the cytoplasmic restoration and maintenance pools of PET1.

Four markers were developed in Reddemann and Horn (2018) study: HRO_ATP9-PET2, which differentiates CMS PET2 from normal, fertile cytoplasm; HRO_PET1, which is specific for CMS PET1 and indicates that orfH522 is absent in CMS PET2; HRO_ATP1-PET1, which detects CMS PET1 in conjunction with the internal atp1 control; and HRO_ATP1, which serves as an internal PCR control that is present in all cytoplasms. Application of these four diagnostic markers allowed for a clear differentiation between CMS PET1, CMS PET2, and the fertile cytoplasm in sunflower. Results for CMS PET2 indicated that the molecular mechanisms behind PET2 male sterility in sunflower depend on recombination events involving the atp9 gene, which leads to two new open reading frames, orf288 and orf231. Furthermore, no PCR signal for orfH522 was visible in CMS PET2, clearly differentiating it from the CMS PET1 mechanism.

In the study by Babych et al. (2021), the SCAR marker HRG01 linked to the Rf1 gene met the criteria for identifying fertility restoration in the desired material. The use of molecular markers, particularly the SCAR marker HRG01 linked to the Rf1 gene, was preferred for selecting maintainers and fertility restorers in diverse early-generation populations.

5. CONCLUSION

In this study, sunflower plant materials that belong to CMS, maintainer or restorer populations, segregating for the Rf/rf alleles, were tested using the Rf/rf specific co-dominant and dominant SCAR markers. Using plant populations at various backgrounds and generations, we showed that markers offer a reliable selection tool for Rf/rf alleles in breeding populations. The PPR621.5 F2 and PPR621.5Rev markers in particular represent valuable tools for MAS in sunflower breeding. The development of new restorer and maintainer lines for sunflower breeding with SCAR markers can now be carried out much more efficiently, and there will be no need for test crossing to understand the status of Rf/rf alleles in the nucleus of maintainer lines. This finding, as also shown in Figure 10, suggests that PPR621.5 F1, PPR621.5 F2, and HRG02 markers can be reliably used for the detection of Rf1/rf1 alleles.

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