TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2022) 46: 955-965

doi:10.55730/1300-011X.3056

Development of DNA markers associated with sunburn resistance in pomegranate (Punica granatum L.) using bulk segregant analysis

Keziban YAZICI[1,*], Mehtap ŞAHİN-ÇEVİK[2]

[1]Department of Horticulture, Faculty of Agriculture, Recep Tayyip Erdoğan University, Rize, Turkey
[2]Department of Agricultural Biotechnology, Faculty of Agriculture, Isparta University of Applied Sciences, Isparta, Turkey

Received: 16.05.2022 • **Accepted/Published Online:** 09.11.2022 • **Final Version:** 05.12.2022

Abstract: Production and consumption of pomegranate have been increasing in the world in recent years due to its health benefits to humans. Sunburn is an important physiological disorder caused by high temperature, light, and radiation. With the changes in climatic conditions that have occurred in recent years, the effect of sunburn is increasing and limiting the production and quality of pomegranate. Therefore, development of pomegranate cultivars resistance to sunburn is one of the main objectives of pomegranate breeding program. However, the development of a new variety takes a very long time. In order to shorten this time, molecular markers have been used in plant breeding in recent years. In this study, molecular markers associated with sunburn resistance in pomegranate fruit were developed using bulked segregant analysis technique (BSA) in a pomegranate progeny population whose morphological, pomological, and phenological evaluations were completed. Two RAPD fragments (OPAI-08-650 and OPPP-14-900) associated with sunburn resistance or sensitivity were determined and used as markers. One of these RAPD fragments was purified, cloned and sequenced to produce a more specific sequence characterized amplified regions (SCAR) marker, designated as PgSCARGYH1. The markers developed in this study will shorten the breeding process of sunburn-resistant pomegranate cultivars. In addition, it will also contribute to the increase in pomegranate production in terms of quality and quantity.

Key words: Sunburn, pomegranate, molecular markers, bulk segregant analysis, marker-assisted selection

1. Introduction

Pomegranate (*Punica granatum* L.) is an economically important fruit species grown in tropical and subtropical regions of the world. The demand for the production and consumption of pomegranate have been increasing in recent years because of many health benefits (Lansky and Newman, 2007; Diaz-Mula et al., 2019). Although pomegranate can adapt to different soil and climatic conditions, sunburn appears to be the most important factor that causes serious economic losses in pomegranate production areas in some years (Narjesi, 2021). Sunburn reduces the quality of pomegranate fruits by turning the colors of fruit peels from brown to black and decreasing the water content and drying of the fruits. These irreversible changes reduce marketable quality and attractiveness of the fruit and lead to significant economic losses (Yazıcı and Ercişli, 2017). It is expected that damages of sunburn will continue to increase in the coming years because of increasing temperatures and radiation caused by climate change.

Sunburn is a physiological disorder that occurs as a result of environmental factors such as high temperature, light, and excessive solar radiation (Schrader et al., 2003). Besides these factors, genetic factors also have a significant effect on the degree of sunburn damage in pomegranate fruit. The damages caused by sunburn can be reduced using evaporative cooling, shading material (Narjesi, 2021), wrapping fruit with special cloths or paper bags (Sarkomi et al., 2019), and application of reflective materials and chemicals (Sharma et al., 2018). However, the effect of these methods for reducing sunburn is low and they also have adverse effects on fruit quality. At the same time, application of these methods requires a lot of effort, and they are not very economical either. Therefore, more effective methods against sunburn should be applied or new pomegranate varieties resistant to sunburn should be developed through the breeding (Yazıcı and Ercişli, 2017). It was observed that pomegranate genotypes grown under the same climatic, soil, and cultural conditions had different sunburn ratios and the level of resistance to sunburn was also different in some pomegranate types (Abu-bakar et al., 2013). Therefore, developing high-quality varieties with sunburn resistance gains more importance in pomegranate productions.

* Correspondence: keziban.yazici@erdogan.edu.tr

Developing a new variety through the classical fruit breeding methods takes quite a long time. During the last 2 decades, molecular markers associated with important traits have been developed and integrated to breeding program of many fruit crops to shorten this long breeding period (Laurens et al., 2012). The use of these molecular markers in breeding allows selection of individuals with desired characteristics in a shorter time period. This technique used in plant breeding is called marker-assisted selection (MAS) and forms the basis of molecular breeding studies (Mercati and Sunseri et al., 2020). Application of MAS requires development of molecular markers with related traits of interest.

Molecular markers express the differences between the DNA of different individuals in the form of nucleotide sequences (Nadeem et al., 2018). Since the early 1980s, different DNA markers have been developed in different plants in order to facilitate the plant breeding process (Xu and Crouch, 2008). Among these molecular markers, the RAPD marker developed by Welsh and McClelland (1990) and Williams et al. (1990) has been a preferred marker technique due to the fact that it does not require sequence information, requires very little DNA, is an easy technique in terms of ease of application and produces lots of markers distributed to all over the genome (Grover and Sharma, 2016). Because of these advantages, RAPD marker has been used in many different studies such as identification and phylogenetic analysis of plant species, genetic mapping, and applications of MAS (Mehlenbacher et al., 2006; Zargar et al., 2016; Sirijan et al., 2020). Although the RAPD markers have so many advantages, they may also have reproducibility problems in the absence of very good amplification. In order to eliminate the disadvantage of the RAPD markers, Sequence-characterized amplified region (SCAR) marker has been developed (Paran and Michelmore, 1993) and used in breeding studies of many plants (Al-Qurainy et al., 2018; Sowa and Paczos-Grzęda, 2020).

Molecular markers associated with trait of interest can be developed through genetic and/or quantitative trait loci (QTL) mapping (Leite et al., 2016; Nantawan et al., 2019). These markers can also be developed using BSA analysis. In recent years, DNA markers related to different characteristics have been identified with the combination of BSA analysis and next-generation sequencing techniques (Nguyen et al., 2019). BSA is a technique for identifying the genetic loci that is responsible for the trait of interest using two pools of individuals from the opposing tails of the phenotypic distribution (Li and Xu et al., 2021; Shen and Messer, 2021). It was first developed by Michelmore et al. (1991) and has been used successfully in many plants to find molecular markers linked with desired traits up to date (Chavez and Chaparro, 2011; Farooqi et al., 2016; Dong et al., 2017; Lim et al., 2021).

The use of molecular markers in pomegranate breeding has remained at a very limited level. They have been mostly used to distinguish different pomegranate genotypes (Sarkhosh et al., 2009; Ercisli et al., 2011a; Ercisli et al., 2011b; Alamuti et al., 2012; Parveresh et al., 2012; Zhang et al., 2012; Al-Sadi et al., 2015; Madadi et al., 2017; Luo et al., 2018; Zarei and Sahraroo, 2018; Patil et al., 2020). Molecular markers have also been used to elucidate the genetic structure of the pomegranate fruit and to detect molecular markers associated with some important traits including different fruit quality and tree traits and bacterial blight (Harel-Beja et al., 2015; Singh et al., 2015). However, to our knowledge, no marker trait association study has been conducted for sunburn and no markers associated with sunburn resistance or sensitivity in pomegranate has been reported to date.

In this study, a hybrid progeny population evaluated for many years of individuals' sunburn resistance or susceptibility were analyzed by bulked segregant analysis (BSA) using RAPD and SSR markers. Two RAPD markers associated with sunburn resistance or sensitivity were identified and one of these markers was converted to SCAR markers useful for screening pomegranate genotypes for sunburn resistance.

2. Materials and methods

2.1. Plant material

Ninety-four pomegranate progenies obtained from the reciprocal crosses involved in Ernar, which is moderately resistant to sunburn, Hicaznar, which is highly sensitive to sunburn, and Fellahyemez, which is very resistant to sunburn, pomegranate cultivars and four new cultivars developed in 1994 in Western Mediterranean Agricultural Research Institute (BATEM) were used. Morphological, pomological, and phenological characteristics of these plants were completed previously (Onur et al., 1999; Yazıcı and Şahin, 2016). In addition, sunburn resistance and/or susceptibility of these genotypes were evaluated in another study (Yazıcı and Ercişli, 2017) and it was revealed that the resistance levels of these hybrid plants have different levels of sunburn resistance. Genotypes, previously scored as sunburn resistant or sensitive, were observed again in terms of resistance to sunburn for another 2 years in this study according to Yazici and Ercişli (2017). Based on these 2-year evaluations, the genotypes were divided into three groups, individuals most resistant to sunburn, most sensitive to sunburn, and moderately resistant to sunburn, and used in bulk analysis and validation of the markers (Figure 1, Table 1).

2.2. DNA isolation and bulking

Leaf samples were collected from plants that were resistant, sensitive, and moderately resistant to sunburn. Genomic DNA was isolated from these leaves using CTAB DNA

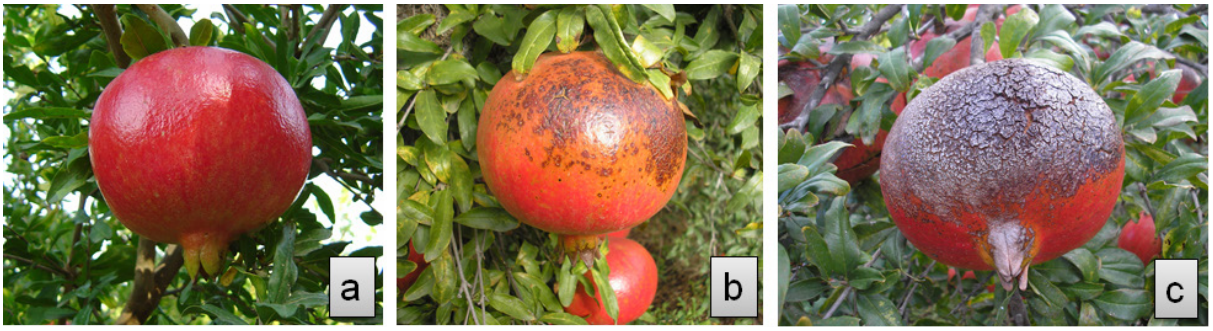

Figure 1. Images of sunburn-resistant (a: 47-52), moderately sunburn-resistant (b: 20-35), and susceptible (c: Hicaznar) pomegranate genotypes and cultivar used in this study.

Table 1. Pomegranate genotypes selected and used in this study grouped again as resistant or sensitive to sunburn.

Sunburn-resistant	Moderately resistant to sunburn	Susceptible to sunburn
16-145	18-111	19-81
20-138	16-179	59-64
16-174	17-48	17-22
16-42	20-45	18-20
17-95	16-147	19-93
17-21	16-149	19-66
17-35	16-182	19-61
47-52	16-169	17-07
19-147	16-58	19-71
17-34	18-131	20-154
17-21	20-35	18-19
16-125	19-112	16-99
21-55	16-77	16-95
21-154		18-100
16-102		17-64
16-88		16-33
17-06		

isolation protocol developed by Doyle and Doyle (1990). The quantity and quality of isolated DNAs were measured using a NonoDrop spectrophotometer (Thermo, USA) and 1.5% agarose gel electrophoresis, respectively. Equal amounts of genomic DNAs from 4 to 6 individuals that were most resistant to sunburn (17-06, 16-145, 20-138, 16-174, 16-42) and most susceptible to sunburn (19-81, 59 -64, 17-22, 18-20, 19-93) were pooled from the individual plant belonging to the same phenotypic class and mixed.

2.3. Polymorphisms analysis by RAPD and SSR primers

The bulked genomic DNAs of opposite characters were analyzed by polymerase chain reaction (PCR) using a total of 260 random (Operon Technologies Inc., Alameda, CA, USA) and 24 SSR primers. The SSR primers previously developed in pomegranate by different researchers were randomly chosen and used in this study. On the other hand, the RAPD primers used in this study were firstly chosen using primers known to give polymorphism in pomegranate (Zamani et al., 2007; Sarkhosh et al., 2009; Ercişli et al., 2011a; Ercişli et al., 2011b; Zhang et al., 2012). In addition to these primers, RAPD primers that were not known to give polymorphism in pomegranate were also randomly selected from random primer sets and used in this study. The list of all the primers used in this study was given in the Supplementary File 1, 2. PCR reactions were performed in MJ Mini PTC1148 or ICycler

(Bio-Rad, USA) thermal cycler using tubes or PCR plate, respectively. Each PCR mixture contained 1X PCR buffer solution (50 mM KCl, 10 mM Tris HCl pH 9.0 at 25 °C, 1% Triton X-100), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 10 µM RAPD primer or each primer specific to SSR primers, 1.25 U Taq polymerase (Thermo, USA) and 25–75 ng of DNA. The profile of the PCR program was at 94 °C for 5 min for initial denaturation followed by 35–40 cycles of 30 s to 1 min at 94 °C for denaturation, 30 s to 1 min at 35–65 °C for primer binding, and 1–3 min at 72 °C for primer elongation followed by one cycle at 72 °C for 10 min for final primer extension. Amplified PCR products were separated on 1.5%–3% agarose gel in 1X TAE buffer [50X (2 M Tris-base, 0.1 M EDTA pH 8.0, 5.7% glacial acetic acid)] and photographed under UV light using Mini BIS-Pro (DNR, Israel) gel imaging system or Qsep 100 fragment analyzer system (BiOptic, Taiwan).

2.4. Scoring and trait association of markers

Only the intense and clear polymorphic bands produced repetitively in bulked DNA in different PCR machines and PCR reactions performed at different times were selected for further analysis. The consistency of the amplification of the selected polymorphic bands between the two DNA bulks was confirmed by PCR analyses using individual DNAs of plants forming the bulk groups. As in bulk DNA analysis, polymorphic bands that are consistently reproducible in the use of individual plants' DNA have been confirmed to be associated with sunburn trait.

2.5. Conversion of RAPD to SCAR markers

The polymorphic DNA fragment consistently associated with resistance or susceptibility to sunburn were excised from the agarose gel and purified by Qiaquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified DNA was cloned into pGEM-T easy vector using T-A cloning with pGEM-T easy PCR cloning system (Promega, USA) as suggested by the manufacturer. The clones were transformed into *Eserichia coli* JM109 cells by heat shock transformation. The bacterial colonies were screened with colony PCR using M13 forward (F) and reverse (R) primers binding site present outside of the T-A cloning site. Positive colonies were selected and grown in liquid media with ampicillin and plasmid was purified using Mini Prep Plasmid DNA isolation Kit (Qiagen, Germany) according to the manufacturer's instructions. The purified plasmids were digested with EcoR I enzyme (New England Biolab, USA) to confirm the presence of the insert DNA. Finally, plasmids containing the RAPD fragments were sequenced bidirectionally by Sanger sequencing using universal M13 F and M13 R primers. The sequences were analyzed using Vector NTI suite (InforMax, Frederick, MD) and forward (5' CCAGCCGAACCACGTAAGTCG 3') and reverse (5' CCAGCCGAACTGAAGCAACTG 3') SCAR primers were designed based on this sequence. PCR using SCAR primers was conducted in a 25-µL reaction mixture containing 1X PCR buffer solution (50 mM KCl, 10 mM Tris HCl pH 9.0 at 25 °C, 1% Triton X-100), 1.25 mM $MgCl_2$, 0.2 mM dNTP, 10 µM from each SCAR primer, 1.25 U Taq polymerase (Thermo, USA), and 50 ng of DNA. The amplification was carried out in MJ Mini PTC1148 thermocycler (Bio-Rad, USA). The thermocycler was programmed at 94 °C for 5 min initial denaturation for one cycle and 35 cycles at 94 °C for 30 s denaturation, 60 °C for 30 s primer annealing, 72 °C for 1 min primer extension followed by one cycle of final primer extension at 72 °C for 10 min. PCR products were separated in 1.5% agarose gel by electrophoresis in TAE buffer [50X (2 M Tris-base, 0.1 M EDTA pH 8.0, 5.7% glacial acetic acid)] and photographed under UV light using Mini BIS-Pro (DNR, Israel) gel imaging system.

3. Results

In order to develop molecular markers associated with sunburn resistance using BSA analysis, hybrid progeny plants obtained from different combinations of Ernar, Hicaznar, and Fellahyemez varieties were reevaluated for sunburn resistance for 2 more years in this study and each plant was grouped again as resistant, sensitive, and moderately resistant to sunburn. The results of these 2-year observations showed that the most sunburn resistant genotypes were obtained from the combinations of Hicaznar × Fellahyemez and Fellahyemez × Hicaznar. On the other hand, the most sensitive genotypes to sunburn were obtained from the crosses between Ernar × Hicaznar, Hicaznar × Ernar combinations. Therefore, in this study, hybrid individuals belonging to Fellahyemez × Ernar and Ernar × Fellahyemez combinations (17-06, 16-145, 20-138, 16-174, 16-42) as the most sunburn-resistant hybrids and individuals of Ernar × Hicaznar and Hicaznar × Ernar combinations (19-81, 59-64, 17-22, 18-20, 19-93) as most sunburn-sensitive hybrids were used for the identification of markers associated with sunburn using BSA analysis.

For BSA analysis, DNAs isolated from individual genotypes of most sunburn-resistant and most susceptible genotypes were mixed in equal amounts separately in two different tubes. The bulked DNAs were subjected to PCR analysis using primers specific to SSR and RAPD primers. Although PCR amplifications were successful with all 24 pairs of SSR primers, none of the SSR primers produced polymorphic DNA bands between bulked DNA samples. In RAPD PCR analysis, while some of the primers did not produce any amplification, the majority of 260 primers used produced multiple DNA fragments ranging from 100 bp to 5000 bp. Among these primers, thirteen of them gave polymorphic bands indicating the differences between the groups that were most susceptible to sunburn and most

resistant to sunburn. To confirm that these polymorphic bands do indeed show the differences between the groups, PCR reactions were repeated several times using the DNA of each of the individuals forming these groups. As a result of these repetitive PCR analyses, polymorphic bands showing the differences between the groups were consistently obtained from only two primers (OPAI-08 and OPP14). In addition, the PCR reactions with OPAI-08 and OPP14 primers were repeated several times using different PCR machines and DNA samples isolated at different times in order to validate the amplification of the polymorphic bands between DNA samples forming two different groups (Figures 2 and 3).

As a result of the PCR analysis using OPAI-08 primer, a band of approximately 650 bp was consistently amplified from individual progeny plants sensitive to sunburn, but this band was not amplified from individual progeny plants resistant to sunburn. On the other hand, as a result of PCRs performed with OPP14 primer, a band of approximately 900 bp was obtained from individuals susceptible to sunburn, while this band was not produced from individuals resistant to sunburn.

To further confirm that the resulting polymorphic bands which differentiate between the most resistant and/or sensitive to sunburn are indeed associated with sunburn resistance or susceptibility, PCR was performed using primers specific to OPAI-08 and OPP14 and DNAs isolated from the other individual plants that were sunburn resistant, sunburn susceptible and moderately sunburn resistant in the population (Figures 4 and 5). The results showed that the markers were able to differentiate among progenies with different degrees of resistance or susceptibility to sunburn.

One of the polymorphic bands obtained from the amplification of the OPP14 primer, enabling us to distinguish between sunburn susceptible and resistant individuals, was converted into SCAR markers in this study. For this purpose, the polymorphic DNA fragment obtained from amplification of DNA of the sunburn susceptible individual (18-20) was extracted from the gel, cloned, and sequenced. The SCAR marker was produced by designing a pair of primers based on this obtained sequence and named as PgSCARGYH1. This SCAR marker was also tested using DNA from the other individual plants identified as resistant or susceptible to sunburn based on the observations in the population (Figure 6).

4. Discussion

Sunburn reduces the marketing value of fruits. It has become a serious problem especially in grape, apple, pear, plum, citrus, and pomegranate due to increasing temperatures and drought in recent years (Yazici and Ercisli, 2017; Xue et al., 2021). Sunburn in pomegranate fruit is under the influence of many factors. Previous studies have been tried to find a correlation between the sunburn problem and the anatomical characteristics of pomegranate fruit, climatic factors, or the genetics of the cultivars. These studies revealed that climatic factors and genetics are more effective in the formation of sunburn in pomegranate fruit (Hooks et al., 2021). In addition, it has been reported by different researchers that resistance to sunburn differs between cultivars grown under the same ecological conditions; therefore, it was concluded that genetics plays an important role in sunburn resistance in pomegranate (Schrader et al., 2003; Hooks et al., 2021). Consistent with the results of previous studies, it has been observed in this study that the genetics is contributing significantly to sunburn resistance of pomegranate genotypes grown in the same ecological conditions. Therefore, considering all results obtained from this

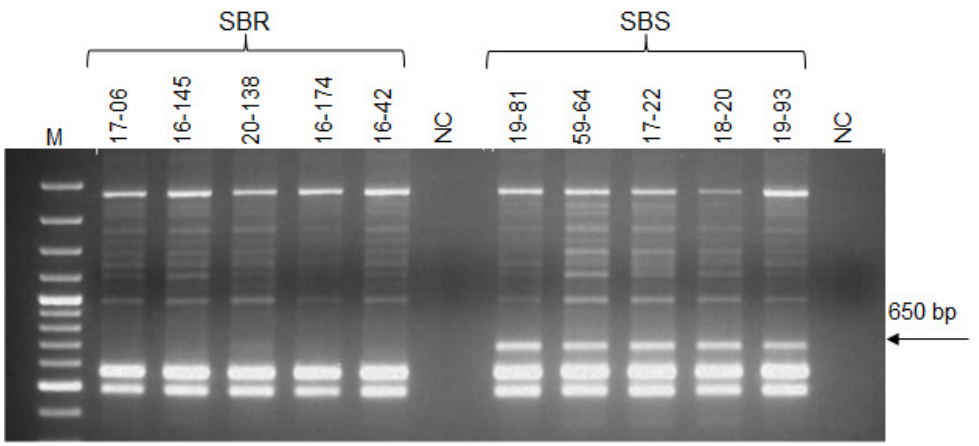

Figure 2. Amplification products of OPAI-08 primer in different pomegranate genotypes in sunburn-resistant and -sensitive groups. M: 100 bp molecular weight marker, SBR: sunburn resistant individuals, NC: negative control, SBS: sunburn sensitive individuals, and arrows indicate polymorphic bands.

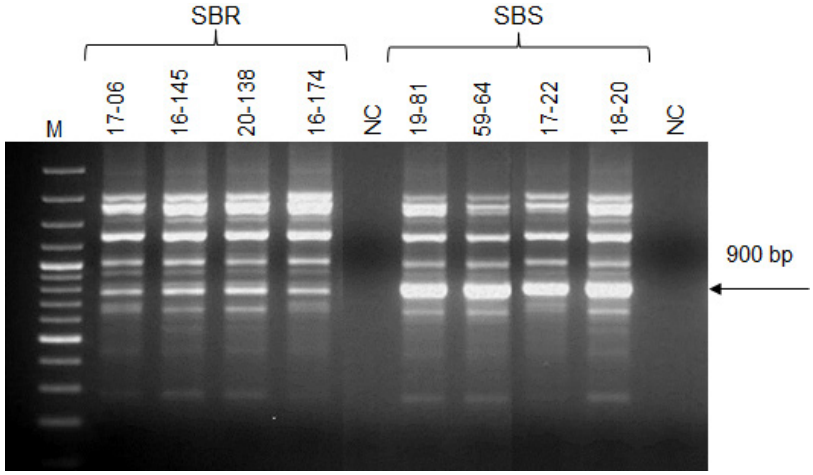

Figure 3. Amplification products of OPP-14 primer in different pomegranate geneotypes in sunburn-resistant and -sensitive groups. M: 100 bp molecular weight marker, SBR: sunburn resistant individuals, NC: negative control, SBS: sunburn-sensitive individuals, and arrows indicate polymorphic bands.

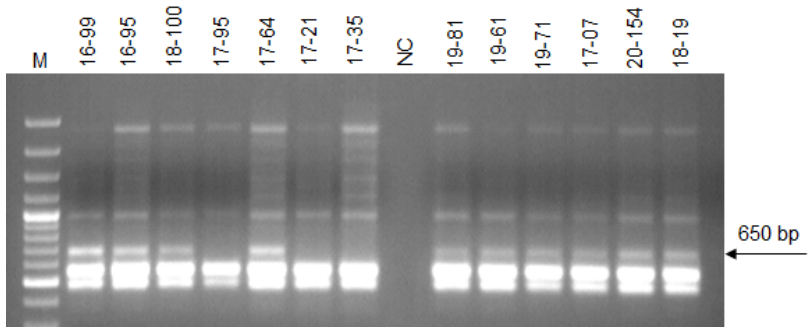

Figure 4. Testing pomegranate progenies with OPAI-08 primer. M: 100 bp molecular weight marker, NC: negative control, and arrows indicate polymorphic bands. Sunburn-resistant individuals: 17-95, 17-21, and 17-35. Sunburn-sensitive individuals: 16-99, 16-95, 18-100, 17-64, 19-81, 19-61, 19-71, 17-07, 20-154, and 18-19.

and other studies, development of a sunburn-resistant pomegranate cultivar would be the most effective method to prevent sunburn damage in pomegranate.

The development of new plant varieties is a very time-consuming process, especially in perennial plants such as fruit trees. DNA markers are used in many plants, including fruit species, to accelerate the development of new cultivars. This time-saving process also saves costs and space (Yamamoto and Terakami, 2016). Different types of DNA markers have been identified associated with tolerance to biotic stresses and flower and fruit characters including pear and plum (Fiol et al., 2021; Terakami et al., 2021).

In pomegranate, to find markers associated with different fruit characters, Zamani et al. (2007) conducted a study using 24 Iranian pomegranate genotypes and RAPD markers. However, no relationship was identified between 28 different fruit characters studied and RAPD primers analyzed. On the other hand, four SSR markers associated with fruit acidity, fruit size, and bacterial blight resistance were identified in association mapping study in pomegranate fruit (Singh et al., 2015). Marker trait associations were also studied by Harel-Beja et al. (2015) to find markers associated with some fruit characters and plant size in pomegranate using QTL analysis. Twenty-five QTLs associated with related traits were identified using a population generated from a cross between two genetically distinct cultivars. However, molecular markers associated with sunburn in pomegranate cultivars have not yet been determined in studies up to date.

In the current study, RAPD markers associated with sunburn trait in pomegranate were determined using BSA analysis. Compared to other methods for determination of markers linked with trait of interest, BSA analysis is an

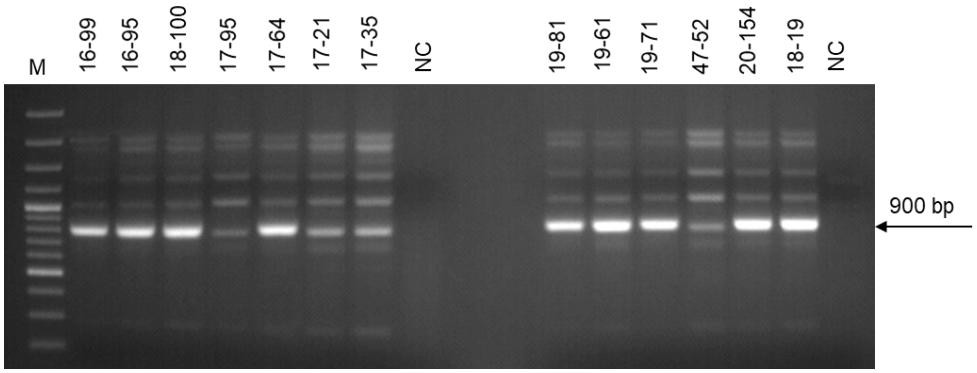

Figure 5. Testing pomegranate progenies with OPPP-14 primer. M: 100 bp molecular weight marker, NC: negative control, and arrows indicate polymorphic bands. Sunburn-resistant individuals: 17-95, 17-21, 17-35, and 47-52. Sunburn-sensitive individuals: 16-99, 16-95, 18-100, 17-64, 19-81, 19-61, 19-71, 47-52, 20-154, and 18-19 (Table 1).

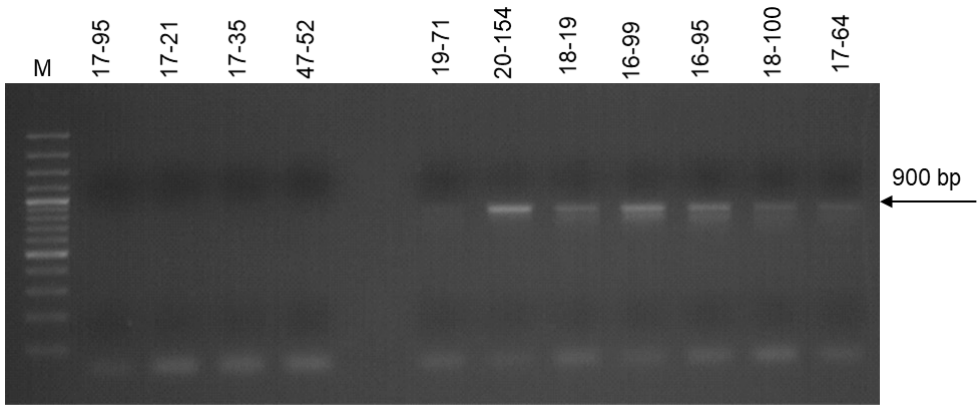

Figure 6. Testing pomegranate progenies with PgSCARGYH1 primer pair. M: 100 bp molecular weight marker. Arrows indicate polymorphic bands. Sunburn-resistant individuals: 17-95, 17-21, 17-35, and 47-52. Sunburn-sensitive individuals: 19-71, 20-154, 18-19, 16-99, 16-95, 18-100, and 17-64 (Table 1).

easier, cheaper, and rapid process (Quarrie et al., 1999; Chavez and Chaparro, 2011). For this analysis, individuals with opposite characteristics are needed in terms of the trait of interest; then, individuals with opposite traits are pooled separately and the DNAs of the two pooled samples are used to identify markers associated with different traits (Michelmore et al., 1991). In different studies to date, BSA analysis has been performed using different types of population. The population to be used in BSA analysis may be a genetically isolated population or a natural population. The plant species studied is an important factor for the determination of population type used in this analysis. Generally, F1 population type is preferred in tree species for BSA analysis (Li and Xu, 2021). The same type of population was used in peach by Tataranni et al. (2015) and poplar by Zhang et al. (2002). In the present study, the F1 population type, which is preferred for other fruits, was used.

The F1 population used in this study was a population that had been previously studied by different researchers for many characteristics, including resistance to sunburn. According to the results obtained from these studies, two groups were formed to select individuals resistant to sunburn (80%–100%) and those susceptible to sunburn (80%–100%) from the population. However, due to the absence of determination of any marker that distinguishes the groups from each other in the BSA analyses, the individuals forming the groups were reevaluated in terms of resistance to sunburn. Then, groups were reconstituted by selecting individuals that were nearly 100% resistant or 100% susceptible to sunburn for BSA analysis.

The studies carried out up to date for BSA analysis, different types of DNA markers have been used to find markers linked to the trait of interest (Avila et al., 2003; Raman et al., 2002; Lim et al., 2021). Among these DNA markers, RAPD marker has been a preferred marker for many researchers because it is easy to use, and it produces very high degree of polymorphisms. In addition to these factors that are effective in the use of the RAPD marker,

the most important factor is not requiring prior sequence information (Yang et al., 2014, Sirijan et al., 2020). Due to these advantages mentioned above, the RAPD marker was also preferred in this study.

One of the RAPD markers developed to distinguish between sunburn resistant and susceptible ones was converted into a SCAR marker in this study. SCAR marker is developed to improve the reliability and reproducibility of PCR-based assays. In addition, primers that are specific to SCARs have relatively long sequences; thus, they can produce more specific bands. All markers identified in this study were tested in other individuals in the population with varying levels of sunburn resistance or susceptibility and validity of the markers is confirmed.

In recent years, although the genomes of different cultivars of pomegranate have been completed by different researchers (Qin et al., 2017; Yuan et al., 2018; Luo et al., 2018), we do not have information about genetics of sunburn resistance, or any marker linked to sunburn trait that can be used for the breeding process of the pomegranate. In this study, molecular markers related with sunburn characteristic were developed for marker-assisted selection to identify genetically resistant and sensitive individuals in pomegranate. This is the first report of identification of markers associated with sunburn characteristics in pomegranate.

5. Conclusion

The consumption of pomegranate fruit as a strong antioxidant due to the fact that containing high flavonoids has been increasing in recent years. With the increasing consumption of pomegranate, the demand for different pomegranate varieties by the consumer has also been increasing. As in other fruit species, a long time period is necessary for obtaining a new pomegranate variety. In this study, molecular markers linked to sunburn resistance, which has become a major problem with global warming in recent years, have been developed in order to carry out pomegranate breeding faster and more effectively. These molecular markers will enable the pomegranate industry to develop new sunburn-resistant pomegranate varieties in a shorter time, with less money and space. In addition, pomegranate genetic resources will be screened for resistance to sunburn to determine candidate genotypes and varieties resistant to sunburn in a short time without waiting for fruit development.

Acknowledgment

This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK), project number; 114O380. The markers obtained as a result of this study were patented by the Turkish Patent and Trademark Office with the patent number "TR 2018 15742 B" on 21/09/2021.

References

Abu-bakar AR, Ashraf N, Ashraf M (2013). Effect of plant biostimulants on fruit cracking and quality attributes of pomegranate cv. Kandhari kabuli. Academic Journals 8 (44): 2171-2175. https://doi.org/10.5897/SRE2013.5702

Alamuti MK, Zeinalabedini M, Derazmahalleh MM, Roodbar Shojaie T, Irandoost HP et al. (2012). Extensive genetic diversity in Iranian pomegranate (*Punica granatum* L.) germplasm revealed by microsatellite markers. Scientia Horticulturae 146: 104-114. https://doi.org/10.1016/j.scienta.2012.07.029

Al-Qurainy F, Al-Ameri AA, Khan S, Nadeem M, Gaafar ARZ et al. (2018). SCAR marker for gender identification in date palm (*Phoenix dactylifera* L.) at the seedling stage. International Journal of Genomics 2018. https://doi.org/10.1155/2018/3035406

Al-Sadi AM, AL-Fahdi AR, Al-Yahyai RA, Al-Ghaithi AG, Al-Said FA et al. (2015). Genetic analysis suggests a shared origin of *Punica granatum* cultivars in Oman with cultivars from the center of origin, Iran. Genetic Resources and Crop Evolution 62: 815–821. https://doi.org/10.1007/s10722-015-0256-0

Avila CM, Sillero JC, Rubiales D, Moreno MT, Torres AM (2003). Identification of RAPD markers linked to the Uvf-1 gene conferring hypersensitive resistance against rust (Uromyces viciae-fabae) in *Vicia faba* L. Theoretical and Applied Genetics 107 (2): 353-358. https://doi.org/10.1007/s00122-003-1254-8

Chavez DJ, Chaparro JX (2011). Indentification of markers linked to seedlessness in Citrus kinokuni hort. ex Tanaka and progeny using bulked segregant analysis. HortScience 46 (5): 693-697. https://doi.org/10.21273/HORTSCI.46.5.693

Diaz-Mula HM, Tomas-Barberan FA, Garcia-Villalba R (2019). Pomegranate fruit and juice (cv. Mollar), rich in ellagitannins and anthocyanins, also provide a significant content of a wide range of proanthocyanidins. Journal of Agricultural and Food Chemistry 67 (33): 9160–9167. https://doi.org/10.1021/acs.jafc.8b07155

Dong Z, Hegarty JM, Zhang J, Zhang W, Chao S et al. (2017). Validation and characterization of a QTL for adult plant resistance to stripe rust on wheat chromosome arm 6BS (Yr78). Theoretical and Applied Genetics 130 (10): 2127-2137. https://doi.org/10.1007/s00122-017-2946-9

Doyle JJ, Doyle LL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Ercisli S, Gadze J, Agar G, Yildirim N, Hizarci Y (2011a). Genetic relationships among wild pomegranate (*Punica granatum*) genotypes from Coruh Valley in Turkey. Genetic and Molecular Research 10 (1): 459-64. https://doi.org/ 10.4238/vol10-1gmr1155

Ercisli S, Kafkas E, Orhan E, Kafkas S, Dogan Y at al. (2011b). Genetic characterization of pomegranate *(Punica granatum* L.) genotypes by AFLP markers. Biological Research 44: 345-350. https://doi.org/10.4067/S0716-97602011000400005

Farooqi MQU, Sa KJ, Hong TK, Lee JK (2016). Bulk segregant analysis (BSA) for improving cold stress resistance in maize using SSR markers. Genetics and Molecular Research 15 (4): 10-4238. https://doi.org/10.4238/gmr15049326

Fiol A, García-Gómez BE, Jurado-Ruiz F, Alexiou K, Howad W et al. (2021). Characterization of Japanese Plum (*Prunus salicina*) PsMYB10 alleles reveals structural variation and polymorphisms correlating with fruit skin color. Frontiers in Plant Science 12: 665267. https://doi.org/10.3389/fpls.2021.655267

Grover A, Sharma PC (2016). Development and use of molecular markers: past and present. Critical Reviews in Biotechnology 36 (2): 290–302. https://doi.org/10.3109/07388551.2014.959891

Harel-Beja R, Sherman A, Rubinstein M, Eshed R, Bar-Ya'akov I et al. (2015). A novel genetic map of pomegranate based on transcript markers enriched with QTLs for fruit quality traits. Tree Genetics & Genomes 11 (5): 1-18. https://doi.org/10.1007/s11295-015-0936-0

Hooks T, Niu G, Masabni J, Sun Y, Ganjegunte G (2021). Performance and Phytochemical Content of 22 Pomegranate (*Punica granatum*) Varieties. Hortscience 56 (2): 217–225. https://doi.org/10.21273/HORTSCI15551-20

Lansky EP, Newman RA (2007). Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. Journal of ethnopharmacology 109 (2): 177-206. https://doi.org/10.1016/j.jep.2006.09.006

Laurens F, Aranzana MJ, Arus P, Bassi D, Bonany J et al. (2012). The new EU project FruitBreedomics: an integrated approach for increasing breeding efficiency in fruit tree crops. In: XX. PAG Plant & Animal Genome; SanDiego, United States. pp. 8-p.

Leite DC, Pinheiro JB, Campos JB, Di Mauro AO, Unêda-Trevisoli SH (2016). QTL mapping of soybean oil content for marker-assisted selection in plant breeding program. Genetics and Molecular Research 15 (1). https://doi.org/10.4238/gmr.15017685

Li Z, Xu Y (2021). Bulk segregation analysis in NGS era: a review for its teenage years. The Plant Journal 109 (6): 1355-1374. https://doi.org/10.1111/tpj.15646

Lim SE, Sa KJ, Lee JK (2021). Bulk segregant analysis identifies SSR markers associated with leaf- and seed-related traits in Perilla crop (Perilla frutescens L.). Genes Genomics (4): 323-332. https://doi.org/10.1007/s13258-021-01056-5

Luo X, Cao S, Hao Z, Hou L, Cao D et al. (2018). Analysis of genetic structure in a large sample of pomegranate (*Punica granatum* L.) using fluorescent SSR markers. The Journal of Horticultural Science and Biotechnology 93 (6): 659-665. https://doi.org/10.1080/14620316.2018.1432994

Madadi M, Zamani Z, Fatahi R (2017). Assessment of genetic variation within commercial Iranian pomegranate (*Punica granatum* L.) cultivars, using ISSR and SSR markers. Turkish Journal of Agriculture-Food Science and Technology 5 (6): 622-628. https://doi.org/10.24925/turjaf.v5i6.622-628.1100

Mehlenbacher SA, Brown RN, Nouhra ER, Gokirmak T, Bassil NV et al. (2006). A genetic linkage map for hazelnut (*Corylus avellana* L.) based on RAPD and SSR markers. Genome 49 (2): 122-133. https://doi.org/10.1139/g05-091

Mercati F, Sunseri F (2020). Genetic diversity assessment and marker-assisted selection in crops. Genes 11 (12): 1481. https://doi.org/10.3390/genes11121481

Michelmore RW, Paran I, Kesseli RV (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proceedings of The National Academy of Sciences 88 (21): 9828-9832. https://doi.org/10.1073/pnas.88.21.9828

Nadeem MA, Nawaz MA, Shahid MQ, Doğan Y, Comertpay G et al. (2018). DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. Biotechnology & Biotechnological Equipment 32 (2): 261-285. https://doi.org/10.1080/13102818.2017.1400401

Narjesi V (2021). Effects of different shade netting treatments on some quantitative and qualitative characteristics of pomegranate fruits cv. Malas-e- Saveh. Journal of Agrıcultural Scıence And Sustaınable Productıon 31 (1): 275-293. https://doi.org/10.22034/saps.2021.12815

Nguyen CD, Verdeprado H, Zita D, Sanada-Morimura S, Matsumura M et al. (2019). The development and characterization of near-isogenic and pyramided lines carrying resistance genes to brown planthopper with the genetic background of japonica rice (*Oryza sativa* L.). Plants 8 (11): 498. https://doi.org/10.3390/plants8110498

Nantawan U, Kanchana-udomkan C, Bar I, Ford R (2019). Linkage mapping and quantitative trait loci analysis of sweetness and other fruit quality traits in papaya. BMC Plant Biology 19 (449): 1-11. https://doi.org/10.1186/s12870-019-2043-0

Onur C, Tibet H, Ataseven Isık E (1999). Pomegranate breeding through hybridization. In: Proceedings of Turkey III. National Horticulture Conference; Ankara, Turkey. pp. 58-61.

Paran I, Michelmore RW (1993). Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. Theoretical Applied Genetics 85: 985–993. https://doi.org/10.1007/BF00215038

Parveresh M, Talebi M, Sayed-Tabatabaei BE (2012). Molecular diversity and genetic relationship of pomegranate *(Punica granatum* L.) genotypes using microsatellite markers. Scientia Horticulturae 138: 244-252. https://doi.org/10.1016/j.scienta.2012.02.038

Patil PG, Singh NV, Parashuram S, Bohra A, Sowjanya R et al. (2020). Genome-wide characterization and development of simple sequence repeat markers for genetic studies in pomegranate (*Punica granatum* L.). Trees 34 (4): 987-998. https://doi.org/10.1007/s00468-020-01975-y

Qin G, Xu C, Ming R, Tang H, Guyot R et al. (2017). The pomegranate (*Punica granatum* L.) genome and the genomics of punicalagin biosynthesis. The Plant Journal: for Cell and Molecular Biology 91 (6): 1108–1128. https://doi.org/10.1111/tpj.13625

Quarrie SA, Lazi-Jani V, Kovaevi D, Steed A, Peki S (1999). Bulk segregant analysis with molecular markers and its use for improving drought resistance in maize. Journal of Experimental Botany 50 (337): 1299-1306. https://doi.org/10.1093/jexbot/50.337.1299

Raman H, Moroni J, Sato K, Read B, Scott B (2002). Identification of AFLP and microsatellite markers linked with an aluminium tolerance gene in barley (*Hordeum vulgare* L.). Theoretical and Applied Genetics 105 (2): 458-464. https://doi.org/10.1007/s00122-002-0934-0

Sarkhosh A, Zamani Z, Fatahi R, Ranjbar H (2009). Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions of fruit characteristics and RAPD markers. Scientia Horticulturae 121 (3): 313-319. https://doi.org/10.1016/j.scienta.2009.02.024

Sarkomi FH, Moradinezhad F, Khayyat M (2019). Pre-harvest bagging influences sunburn, cracking and quality of pomegranate fruits. Journal Of Hortıculture And Postharvest Research 2 (2): 131-142. https://doi.org/10.22077/JHPR.2019.2247.1044

Schrader L, Zhang J, Sun J (2003) Environmental stresses that cause sunburn of apple. Acta Horticulturae 618 (618): 379-405. https://doi.org/ 10.17660/ActaHortic.2003.618.47

Sharma Ranjana, Datta SC, Varghese Eldho (2018). Effect of Surround WP®, a kaolin-based particle film on sunburn, fruit cracking and postharvest quality of 'Kandhari' pomegranates. Crop Protection 114: 18-22. https://doi.org/10.1016/j.cropro.2018.08.009

Shen R, Messer WP (2021). Predicting the Genomic Resolution of Bulk Segregant Analysis. *G3* Genes|Genomes|Genetics 12 (3). https://doi.org/10.1093/g3journal /jkac012

Singh N, Abburi V, Ramajayam D, Kumar R, Chandra R et al. (2015). Genetic diversity and association mapping of bacterial blight and other horticulturally important traits with microsatellite markers in pomegranate from India. Molecular Genetics and Genomics 290 (4): 1393-1402. https://doi.org/10.1007/s00438-015-1003-0

Sirijan M, Drapal M, Chaiprasart P, Fraser PD (2020). Characterisation of Thai strawberry (*Fragaria* × *ananassa* Duch.) cultivars with RAPD markers and metabolite profiling techniques. Phytochemistry 180: 112522. https://doi.org/10.1016/j.phytochem.2020.112522

Sowa S, Paczos-Grzęda E (2020). Identification of molecular markers for the *Pc39 gene* conferring resistance to crown rust in oat. Theoretical and Applied Genetics 133**:** 1081-1094. https://doi.org/10.1007/s00122-020-03533-z

Terakami S, Adachi Y, Takeuchi Y, Takada N, Nishio S et al. (2021). Development of an SSR marker set for efficient selection for resistance to black spot disease in pear breeding. Breeding Science 71 (2): 240-252. https://doi.org/ 10.1270/jsbbs.20136

Tataranni G, Spada A, Pozzi C, Bassi D (2015). AFLP-based bulk segregant analysis for tagging the slow-ripening trait in peach [*Prunus persica* (L.) Batsch]. The Journal of Horticultural Science and Biotechnology 85 (1): 78-82. https://doi.org/10.1080/14620316.2010.11512634

Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18 (24): 7213-7218. https://doi.org/ 10.1093/nar/18.24.7213

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic-markers. Nucleic Acids Research 18: 6531–6535. https://doi.org/ 10.1093/nar/18.22.6531.

Xu Y, Crouch JH (2008). Marker-assisted selection in plant breeding: from publications to practice. Crop Science 48: 391-407. https://doi.org/ 10.2135/cropsci2007.04.0191

Xue Y, Cao B, Liang H, Yang J, Gao P (2021). Environmental shifts have important impacts on the functional traits and bioactive products of medicinal crop Cornus officinalis. Industrial Crops and Products 162: 113304. https://doi.org/10.1016/j.indcrop.2021.113304

Yang L, Khan M, Mei Z, Yang M, Zhang T et al. (2014). Development of RAPD-SCAR markers for Lonicera japonica (Caprifolicaceae) variety authentication by improved RAPD and DNA cloning. Revista de Biología Tropical 62 (4): 1649-1657. https://doi.org/ 10.15517/rbt.v62i4.13493

Yamamoto T, Terakami S (2016). Genomics of pear and other Rosaceae fruit trees. Breeding Science. 66 (1): 148–159. https://doi.org/ 10.1270/jsbbs.66.148

Yazıcı K, Ercisli S (2017). Characterization of hybrid pomegranate genotypes based on sunburn and cracking traits related to maturation time. Journal of Applied Botany and Food Quality 90: 132-139. https://doi.org/10.5073/JABFQ.2017.090.016

Yazıcı K, Sahin, A (2016). Characterization of pomegranate (*Punica granatum* L.) hybrids and their potential use in further breeding. Turkish Journal of Agriculture and Forestry 40 (6): 813-824. https://doi.org/10.3906/tar-1604-120

Yuan Z, Fang Y, Zhang T, Fei Z, Han F et al. (2018). The pomegranate (*Punica granatum* L.) genome provides insights into fruit quality and ovule developmental biology. Plant Biotechnology Journal 16 (7): 1363–1374. https://doi.org/10.1111/pbi.12875

Zamani Z, Sarkhosh A, Fatahi R, Ebadi A (2007). Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. The Journal of Horticultural Science and Biotechnology 82 (1): 11-18. https://doi.org/10.1080/14620316.2007.11512192

Zarei A, Sahraroo A (2018). Molecular characterization of pomegranate (*Punica granatum* L.) accessions from Fars Province of Iran using microsatellite markers. Horticulture, Environment, and Biotechnology 59 (2): 239-249. https://doi.org/10.1007/s13580-018-0019-x

Zargar SM, Farhat S, Mahajan R, Bhakhri A, Sharma A (2016). Unraveling the efficiency of RAPD and SSR markers in diversity analysis and population structure estimation in common bean. Saudi Journal of Biological Sciences 23 (1): 139-149. https://doi.org/10.1016/j.sjbs.2014.11.011

Zhang B, Huang MR, Zhuge Q, Han ZM, Yin TM (2002). Identification of markers linked to resistance locus of Marssonina leaf spot in poplars by bulked segregant analysis (BSA). Yi Chuan 24 (5): 543-547.

Zhang YP, Tan HH, Cao SY, Wang XC, Yang G (2012). A novel strategy for identification of 47 pomegranate (*Punica granatum*) cultivars using RAPD markers. Genetics and Molecular Research 11 (3): 3032-3041. https://doi.org/10.4238/2012.May.30.1

Supplementary File 1. Names and sequences of RAPD primers used.

Primer name	Sequence	Primer name	Sequence	Primer name	Sequence	Primer name	Sequence
OPAY-01	GTCCACCTCT	OPS-01	CTACTGCGCT	OPR-01	TGCGGGTCCT	OPQ-01	GGGACGATGG
OPAY-02	TGCGAAGGCT	OPS-02	CCTCTGACTG	OPR-02	CACAGCTGCC	OPQ-02	TCTGTCGGTC
OPAY-03	TTTCCGGGAG	OPS-03	CAGAGGTCCC	OPR-03	ACACAGAGGG	OPQ-03	GGTCACCTCA
OPAY-04	AAGGCTCGAC	OPS-04	CACCCCCTTG	OPR-04	CCCGTAGCAC	OPQ-04	AGTGCGCTGA
OPAY-05	TCGCTGCGTT	OPS-05	TTTGGGGCCT	OPR-05	GACCTAGTGG	OPQ-05	CCGCGTCTTG
OPAY-06	GGCTTCGCAA	OPS-06	GATACCTCGG	OPR-06	GTCTACGGCA	OPQ-06	GAGCGCCTTG
OPAY-07	GACCGTCTGT	OPS-07	TCCGATGCTG	OPR-07	ACTGGCCTGA	OPQ-07	CCCCGATGGT
OPAY-08	AGGCTTCCCT	OPS-08	TTCAGGGTGG	OPR-09	TGAGCACGAG	OPQ-08	CTCCAGCGGA
OPAY-09	CCGATCCAAC	OPS-09	TCCTGGTCCC	OPR-10	CCATTCCCCA	OPQ-09	GGCTAACCGA
OPAY-10	CAAGGCCCCT	OPS-10	ACCGTTCCAG	OPR-11	GTAGCCGTCT	OPQ-10	TGTGCCCGAA
OPAY-11	ACGCGCCTTC	OPS-11	AGTCGGGTGG	OPR-12	ACAGGTGCGT	OPQ-11	TCTCCGCAAC
OPAY-12	CTGTCGGCGT	OPS-12	CTGGGTGAGT	OPR-13	GGACGACAAG	OPQ-12	AGTAGGGCAC
OPAY-13	CCGCTCGTAA	OPS-13	GTCGTTCCTG	OPR-14	CAGGATTCCC	OPQ-13	GGAGTGGACA
OPAY-14	GGTGGGTAGA	OPS-14	AAAGGGGTCC	OPR-15	GGACAACGAG	OPQ-14	GGACGCTTCA
OPAY-15	CCAAGAGGCA	OPS-15	CAGTTCACGG	OPR-16	CTCTGCGCGT	OPQ-15	GGGTAACGTG
OPAY-16	GGTGTGGTTC	OPS-16	AGGGGGTTCC	OPR-17	CCGTACGTAG	OPQ-16	AGTGCAGCCA
OPAY-17	GGTGATTCGG	OPS-17	TGGGGACCAC	OPR-18	GGCTTTGCCA	OPQ-17	GAAGCCCTTG
OPAY-18	ACCCCAACCA	OPS-18	CTGGCGAACT	OPR-19	CCTCCTCATC	OPQ-18	AGGCTGGGTG
OPAY-19	AACTTGGCCC	OPS-19	GAGTCAGCAG	OPR-20	ACGGCAAGGA	OPQ-19	CCCCCTATCA
OPAY-20	TCATTCGCCC	OPS-20	TCTGGACGGA	OPM-01	GTTGGTGGCT	OPQ-20	TCGCCCAGTC
OPK-01	CATTCGAGCC	OPL-01	GGCATGACCT	OPM-02	ACAACGCCTC	OPN-01	CTCACGTTGG
OPK-02	GTCTCCGCAA	OPL-02	TGGGCGTCAA	OPM-03	GGGGGATGAG	OPN-02	ACCAGGGGCA
OPK-03	CCAGCTTAGG	OPL-03	CCAGCAGCTT	OPM-04	GGCGGTTGTC	OPN-03	GGTACTCCCC
OPK-04	CCGCCCAAAC	OPL-04	GACTGCACAC	OPM-05	GGGAACGTGT	OPN-04	GACCGACCCA
OPK-05	TCTGTCGAGG	OPL-05	ACGCAGGCAC	OPM-06	CTGGGCAACT	OPN-05	ACTGAACGCC
OPK-06	CACCTTTCCC	OPL-06	GAGGGAAGAG	OPM-07	CCGTGACTCA	OPN-06	GAGACGCACA
OPK-07	AGCGAGCAAG	OPL-07	AGGCGGGAAC	OPM-08	TCTGTTCCCC	OPN-07	CAGCCCAGAG
OPK-08	GAACACTGGG	OPL-08	AGCAGGTGGA	OPM-09	GTCTTGCGGA	OPN-08	ACCTCAGCTC
OPK-09	CCCTACCGAC	OPL-09	TGCGAGAGTC	OPM-10	TCTGGCGCAC	OPN-09	TGCCGGCTTG
OPK-10	GTGCAACGTG	OPL-10	TGGGAGATGG	OPM-11	GTCCACTGTG	OPN-10	ACAACTGGGG
OPK-11	AATGCCCCAG	OPL-11	ACGATGAGCC	OPM-12	GGGACGTTGG	OPN-11	TCGCCGCAAA
OPK-12	TGGCCCTCAC	OPL-12	GGGCGGTACT	OPM-13	GGTGGTCAAG	OPN-12	CACAGACACC
OPK-13	GGTTGTACCC	OPL-13	ACCGCCTGCT	OPM-14	AGGGTCGTTC	OPN-13	AGCGTCACTC
OPK-14	CCCGCTACAC	OPL-14	GTGACAGGCT	OPM-15	GACCTACCAC	OPN-14	TCGTGCGGGT
OPK-15	CTCCTGCCAA	OPL-15	AAGAGAGGGG	OPM-16	GTAACCAGCC	OPN-15	CAGCGACTGT
OPK-16	GAGCGTCGAA	OPL-16	AGGTTGCAGG	OPM-17	TCAGTCCGGG	OPN-16	AAGCGACCTG
OPK-17	CCCAGCTGTG	OPL-17	AGCCTGAGCC	OPM-18	CACCATCCGT	OPN-17	CATTGGGGAG
OPK-18	CCTAGTCGAG	OPL-18	ACCACCCACC	OPM-19	CCTTCAGGCA	OPN-18	GGTGAGGTCA
OPK-19	CACAGGCGGA	OPL-19	GAGTGGTGAC	OPM-20	AGGTCTTGGG	OPN-19	GTCCGTACTG
OPK-20	GTGTCGCGAG	OPL-20	TGGTGGACCA	Y-4	GTTTCGCTCCT	OPN-20	GGTGCTCCGT
OPA-04	AATCGGGCTG	OPBD-17	GTTCGCTCCC	Y-6	GTTTCGCTCCC	OPE-01	CCCAAGGTCC

Supplementary File 1. (Continued).

Primer name	Sequence	Primer name	Sequence	Primer name	Sequence	Primer name	Sequence
OPA-19	CAAACGTCGG	OPBC-08	GGTCTTCCCT	Y-9	CTGCTGGGACA	OPE-02	GGTGCGGGAA
OPH-07	CAAACGTCGG	OPH-18	GAATCGGCCA	Y-22	GGACCCAACCT	OPE-03	CCAGATGCAC
OPH-08	GAAACACCCC	OPY-06	AAGGCTCACC	Y-30	GTGTGCCCCAC	OPE-04	GTGACATGCC
OPAD-10	AAGAGGCCAG	OPY-07	AGAGCCGTCA	Y-34	AAGCCTCGTCT	OPE-05	TCAGGGAGGT
OPAG-08	AAGAGCCCTC	OPY-11	AGACGATGGG	Y-48	ACGACCGACAC	OPE-06	AAGACCCCTC
OPAG-12	AAGAGCCCTC	OPY-13	GGGTCTCGGT	Y-51	TGGTGGCGTTA	OPE-07	AGATGCAGCC
OPAG-20	CTCCCAGGGT	OPBA-03	GTGCGAGAAC	Y-54	TGGTGGCGTTC	OPE-08	TCACCACGGT
OPAH-16	TGCGCTCCTC	OPBA-06	GGACGACCGT	Y-57	ACCCCCGACTA	OPE-09	CTTCACCCGA
OPAH-19	CAAGGTGGGT	OPBB-09	AGGCCGGTCA	D-5	GTCAGAGTCCT	OPE-10	CACCAGGTGA
OPAH-02	GGCAGTTCTC	OPBB-13	CTTCGGTGTG	OPAD18	ACGAGAGGCA	OPE-11	GAGTCTCAGG
OPAI-08	GGAAGGTGAG	OPAC-11	CCTGGGTCAG	OPAE14	GAGAGGCTCC	OPE-12	TTATCGCCCC
OPAI-18	AAGCCCCCCA	OPAD-02	CTGAACGCTG	OPAH20	CACTTCCGCT	OPE-13	CCCGATTCGG
OPB-01	TCGCAGCGAG	OPAD-04	GTAGGCCTCA	OPAJ08	TCGCGGAACC	OPE-14	TGCGGCTGAG
OPB-12	TGATGGCGTC	OPAD-13	GGTTCCTCTG	OPAJ14	GTGCTCCCTC	OPE-15	ACGCACAACC
OPB-20	CCTTGACGCA	OPAD-16	AACGGGCGTC	OPAK19	ACCGATGCTG	OPE-16	GGTGACTGTG
OPD-17	GGACCCTTAC	OPAE-10	CTGAAGCGCA	OPA02	TGCCGAGCTG	OPE-17	CTACTGCCGT
OPX-19	TTTCCCACGG	OPB-10	CTGCTGGGAC	OPA05	AGGGGTCTTG	OPE-18	GGACTGCAGA
OPBA-06	GGACGACCGT	OPAD-15	TTTGCCCCGT	OPA10	GTGATCGCAG	OPE-19	ACGGCGTATG
OPBB-03	TCACGTGGCT	OPP-02	TCGGCACGCA	OPA13	CAGCACCCAC	OPE-20	AACGGTGACC
OPBB-04	ACCAGGTCAC	OPP-14	CCAGCCGAAC	OPB05	TGCGCCCTTC	OPC08	TGGACCGGTG
OPBB-07	GAAGGCTGGG	MG-01	AGCGCCGACG	OPC05	GATGACCGCC	OPC10	TGTCTGGGTG
OPBB-08	TCGTCGAAGG	MG-11	AGGAGCTGCC	OPC06	GAACGGACTC	OPC12	TGTCATCCCC
OPBB-10	ACTTGCCTGG	MG-16	GAAGAACCGC			OPI18	TGCCCAGCCT
OPBD-07	GAGCTGGTCC					OPR08	CCCGTTGCCT

Supplementary File 2. Names and sequences of SSR primers used.

Primer name	Nucleotide sequence	Primer name	Nucleotide sequence
ABRII-MP26-F	5-TTTCTCGAAGAATTGGGTAA-3	FM994087-F	5-GCAAAGGAAACAAAAACAAA-3
ABRII-MP26-R	5-CTGAGTAAGCTGAGGCTGAT-3	FM994087-R	5-TGATTTGATCCTCAGCTTCT-3
ABRII-MP28-F	5-ATCCTCTGTCTTTGTGTTCG-3	FM994097-F	5-ATGAATGAGGAAGACGAAAA-3
ABRII-MP28-R	5-TGAGTAATTCCGGTCAGAAG-3	FM994097-R	5-GTGCTCCATCCATACAAAAT-3
ABRII-MP42-F	5-GAGCAGAGCAATTCAATCTC-3	FM994094-F	5-GCCTATCTCGTGATCACATC-3
ABRII-MP42-R	5-AACAATTTCCCATGTTTGAC-3	FM994094-R	5-AATGGGAGCGGACTAACTAT-3
EPS01-F	5-TCTATTCCACATAGAAAGAGGGG-3	FM994095-F	5-GGACTAGCACAACTCGTAGC-3
EPS01-R	5-ATGATGTCTATGCAATTGGCTG-3	FM994095-R	5-CAACAAAATGAGAAGGTGGT-3
EPS03-F	5-CGCTGGTCACACTACTTACTCG-3	pg4-F	F: CTGATGTAATGGCTGAGCAAA
EPS03-R	5-TTGTAGTGGAAGACACAGCAGC-3	pg4-R	R: GCACTTGAACAAAGAGAATGC
EPS04-F	5-AAAGGGGAAAAAGACGAAGAAG-3	pg6-F	F: GGTTGCTCATCCCTTGACTC
EPS04-R	5-CCCTGTCCTTAAGTCTGAGTGG-3	pg6-R	R: GCGTCTGTCAGTGTCTTAGGC
EPS05-F	5-TTGTTGGGTATTCCTCTTCTC-3	pg8-F	F:CACCATAGACTTAAACGAGCACAA
EPS05-R	5-ACATCATACACCTTGCCCTC-3	pg8-R	R: GAAGCTCCATTGCCTCGTCC
EPS06-F	5-AAATCGCATCCCTCCGTCT-3	pg17-F	F: CATCAGACTACGATGGCACT
EPS06-R	5-CTGTTCGCCAGGGTAAAGA-3	pg17-R	R: GCATAATAGCCTTCAATTTACA
EPS08-F	5-TTCCCGAGAAAGTTGCATATCT-3	pg18-F	F: TCTAAGGGCAGAATGGCACT
EPS08-R	5-TAGTCCGTGAGGATTTTGTCCT-3	pg18-R	R: TGGCACTAGATCCGTAAATCT C
EPS09-F	5-TAATCCCATTCCAAACAAGTCC-3	PGCT006-F	F:TTGAATTGATGTAACGCTTG
EPS09-R	5-ATATTGACGGAGGCTTCACTGT-3	PGCT006-R	R:GAGGAAAGTCGTTTGAAGTG
EPS10-F	5-TAGCACAGGGGAAATCTGAAAT-3	PGCT015-F	F:GACGCCTTTAGTTTGCTCCA
EPS10-R	5-GGAAGAGTTTGGTTCAGGATTG-3	PGCT015-R	R:CTCGGGACAGGACTTGGAAT
EPS19-F	5-TGGGGATTATCGTTGTCTTCA-3	PGCT033-F	F:TAATAAGCTGCCCCGAAGTC
EPS19-R	5-TCCAAGCTGAACTCGTTCCT-3	PGCT033-R	R:CGGTGATGTCCCTATTGGAG