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An optimized PCR protocol with newly designed primers for reliable molecular selection of high oleic type sunflower

Yüksek oleik tip ayçiçeğinin güvenilir moleküler seçiminde yeni tasarlanmış primerler ile optimize PCR protokolü

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ABSTRACT

High oleic sunflower is one of the most significant oilseed crops due to the stability of its oil in processing and desirable characteristics for health. Determination of high oleic sunflower by standard methods such as gas chromatography is time consuming and expensive. On the other hand, marker-assisted selection analysis with molecular markers associated with high oleic acid trait is a useful and powerful tool in order to facilitate sunflower breeding programs. In this study, we compared three molecular markers which have been used for selection of the high oleic sunflower varieties. We also describe an optimized PCR protocol with newly designed two primer pairs targeting normal sequence of FAD2 gene as internal control and direct analysis of the inserted DNA sequences which is known to be closely linked to the Pervenets mutation. According to results of our study, showing the insertion site which is linked to the Pervenets mutation by the insertion specific PCR protocols is more reliable than the SSR marker for selection of the high oleic sunflower varieties. Because we have not been able to get successful results with the available PCR protocols described for the insertion site, we report here a novel multiplex PCR protocol with newly designed primers enabling reliable discrimination of the high oleic and low oleic sunflower genotypes in a single PCR tube, which offers some advantages to the breeders by means of saving money and time.

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ÖZ

Yüksek oleik tip ayçiçeği, işlenme sürecindeki kararlı yapısı ve sağlık açısından istenen özelliklere sahip olması nedeniyle en önemli yağ bitkilerinden birisidir. Yüksek oleik içeriğine sahip ayçiçeğinin gaz kromatografisi gibi standart metotlarla tayini zaman alıcı ve pahalıdır. Diğer yandan, yüksek oleik asit özelliği ile ilişkili moleküler belirteçlerle yapılan seleksiyon ayçiçeği yetiştiriciliğini kolaylaştırmak açısından kullanışlı ve güçlü bir araçtır. Bu çalışmada, yüksek oleik tip ayçiçeği çeşitlerinin seçiminde kullanılan üç moleküler belirteç karşılaştırılmıştır. Bu çalışma ile ayrıca, kontrol olarak FAD2 geninin normal dizisini ve Pervenets mutasyonuyla yakından ilişkili olduğu bilinen ilave DNA dizilerinin doğrudan analizini hedefleyen yeni tasarladığımız primerler ile optimize ettiğimiz yeni bir PCR protokolü tanımlanmıştır. Çalışmamızın sonuçlarına göre, yüksek oleik tip ayçiçeği çeşitlerinin seçiminde Pervenets mutasyonuyla yakın ilişkili olan ilave bölgeyi özgün PCR protokolleri ile göstermek SSR belirtecinden daha güvenilirdir. İlave bölgeye yönelik mevcut PCR protokolleri ile başarılı sonuçlar elde edemediğimiz için, bu çalışmada yetiştiricilere/ıslahçılara para ve zamandan tasarruf etmek gibi avantajlar sunan, yüksek ve düşük oleik tip ayçiçeği genotiplerinin tek bir PCR tüpünde güvenilir şekilde ayrımını sağlayan, yeni primer çiftlerinin kullanıldığı yeni bir multipleks PCR protokolü bildirilmektedir.

1. Introduction

Sunflower is one of the most significant oil crops in the world. It is grown in the world generally for its edible oil which contributes approximately 12% of the global edible oil production (Rauf et al. 2017). The sunflower oil contains high level of unsaturated fatty acids (88%); linoleic acid (48-74%), oleic acid (14-40%), as well as saturated fatty acids such as palmitic acid (4-9%) and stearic acid (1-7%) (Nagarathna et al. 2011; Singchai et al. 2013). It is beneficial for human

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consumption because of its favorable fatty acid composition (Baydar and Erbas 2005) and also unsaturated fatty acids is advantageous for lowering the cholesterol content in a human body (Nagarathna et al. 2011). Classic sunflower varieties are low oleic type (LO) whereas new varieties that are qualified as high oleic (HO) have been developed. High oleic acid containing diets have been reported to be most effective for preventing cardiovascular diseases (Delplanque et al. 1997; Broun et al. 1999; Lacombe et al. 2009). Recently, oleic type sunflower production and consumption started rapidly both for healthy frying oil, and also non-food purposes. High (>80%) or mid oleic type (60-70%) sunflower oil is significant in the world due to high oleic acid sunflower oil is more appropriate for frying and beneficial for health (Evci et al. 2016). High oleic oils are also significant in food processing due to being more stable in exposure to high temperatures (Dimitrijevic et al. 2017). Non-food applications in particular require oleic acid content that is stable and higher than 90% (Vannozzi 2006).

Increase of oleic acid content has become one of the major goals to improve vegetable oil quality (Lacombe et al. 2004). In order to reach this aim, sunflower lines and hybrids which have high oleic acid content in their seeds have been obtained by selection programs from HO Pervenets mutant by chemical mutagenesis (Soldatov 1976). Pervenets population was obtained by 0.5% DMS solution application to the seed of VNIIMK8931 variety (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenets population is higher than 65% whereas this content in normal LO variety is about 20% (Berville et al. 2009). HO sunflower varieties are widely used in the world because of the interest in oleic acid and also the agronomic performance of HO varieties carrying the Pervenets mutation compared with the LO varieties (about 1.2 million ha, CETIOM 2002). Afterwards, new cultivars with modified fatty acid content were developed but still Pervenets variety is the most preferable source of high oleic acid content (Skoric et al. 2007; Alberio et al. 2016; Cvejic et al. 2016; Dimitrijevic et al. 2017). There are many reports indicating that genetic and environmental factors affects the oleic acid contents in sunflower (Schuppert et al. 2006; Izquierdo and Aguirrezabal 2008; Fernandez-Martinez et al. 2009; Demurin and Borisenko 2011; Evci et al. 2016; Dimitrijevic et al. 2017). It is indicated that high temperatures are necessary for high oleic acid yield in sunflower (van der Merwe et al. 2015; Neto et al. 2016).

The determination of high oleic type sunflower hybrids, genotypes or varieties is very significant in plant breeding studies. The phenotypic determination (fatty acid analysis) does not allow rapid and early determination of HO genotypes and also cannot provide differentiation of homozygotes from heterozygotes for the mutation. The use of molecular markers has become popular tool for the genetic and breeding studies/programs and it is rapid, cheaper and simple when suitable markers were developed (Varshney et al. 2005; Lacombe et al. 2009). Therefore, marker assisted selection (MAS) analysis is necessary at genomic level allowing rapid and earlier determination of homozygous HO genotypes for sunflower breeding studies. Oleoyl-phosphatidyl choline desaturase (FAD2) has three genes (FAD2-1, FAD2-2 and FAD2-3) in sunflower which has role in the synthesis of linoleic acid from oleic acid (Harwood 1996; Hongtrakul et al. 1998; Schuppert et al. 2006; Berville et al. 2009; Dimitrijevic et al. 2017). FAD2-1 has main role in synthesis of linoleic acid, and the mutation in this gene cause increase in oleic acid content in sunflower seeds (Martinez-Rivas et al. 2000, 2001; Garcia-Diaz et al. 2002). There are dominant and codominant molecular markers such as RFLP, SSR, HO PCR specific fragment and FAD2-1 gene specific primers in order to identify the mutation at FAD2-1 gene region and to detect HO genotypes in the literature (Hongtrakul et al. 1998; Schuppert et al. 2006; Berville et al. 2009; Lacombe et al. 2009; Dimitrijevic et al. 2017). These developed markers are not always able to effectively identify genotypes that have high oleic content. Consequently, the developed markers and methods need to be evaluated in different sunflower populations, genotypes, varieties and also genetic backgrounds. The aims of this study were to design appropriate and easy-to-use new primer sets and PCR protocols for detecting FAD2-1 mutation in order to select high oleic type sunflower, and to evaluate the effectivity of the other marker types developed by different researchers.

2. Materials and Methods

2.1. Plant materials

For the purpose of molecular screening on high oleic genotypes, commercial sunflower varieties with high oleic (HO) acid and low oleic (LO) acid content (four HO and three LO) and high oleic hybrid genotype (Sample number 8) were used (Table 1). Fresh leaf samples of genotypes were collected, labeled with individual number and kept at -80 ºC until further use.

2.2. DNA isolation

Leave samples were homogenized using Retsch® Model MM300 Mixer Mill just before DNA isolation. Vivantis GF-1 Plant DNA Extraction Kit was used for DNA isolation. DNA concentration was measured with Qubit® 2.0 Fluorometer and the quality of DNA was checked by 1% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum ST5. Each of DNA sample was diluted as 50 ng per µl and was stored at -20 ºC for later uses.

Table 1. Characteristics of studied commercial varieties based on oleic acid content and molecular marker analysis.

Sample number	Characteristics of genotypes	Oleic Acid (%)	SSR (N1-1F/N1-1R)	HO PCR specific fragment (N1-3F/N2-1R)	INDEL marker (FAD2-F4/FAD2-R1)	FAD2-NF/FAD2-NR / FAD2-IS-F/FAD2-IS-R
1	Low Oleic	-	246	-	-	- / +
2	High Oleic	>80	246	+	+	+ / +
3	High Oleic	>80	246	+	+	+ / +
4	Low Oleic	-	246 / 249	-	-	- / +
5	High Oleic	>80	246	+	+	+ / +
6	High Oleic	>80	246 / 249	+	+	+ / +
7	Low Oleic	-	246 / 249	-	-	- / +
8	High Oleic	88.1	246	+	+	+ / +

'+' : Presence of specific band, '-' : Absence of specific band

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2.3. PCR analysis

Firstly, genotyping of high oleic (HO) and low oleic (LO) commercial sunflower varieties and hybrid individual was performed with three primer pairs; SSR (N1-1F/N1-1R) (Berville et al. 2009), HO PCR specific fragment (N1-3F/N2-1R) (Berville et al. 2009), and INDEL marker (FAD2-F4/FAD2-R1) (Schuppert et al. 2006). PCR with these primers was performed as described by Berville et al. (2009) and Schuppert et al. (2006). PCR amplification products were controlled by 2% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum ST5. SSR (N1-1F/N1-1R) fragments were scored in a *Beckman Coulter* GenomeLab™ GeXP Genetic Analysis System and fragment sizes were calculated by its Software.

Secondly, we designed novel primer pairs FAD2-N-F/FAD2-N-R for normal sequence of FAD2 gene and FAD2-IS-F/FAD2-IS-R for the insertion specific sequences linked to the Pervenets mutation, and optimized the PCR protocol for genotyping of same sunflower individuals mentioned above. Multiplex PCR amplification was carried out using 10 µl final volume containing 100 ng of template DNA, 2 mM $MgCl_2$, 1X reaction buffer, four dNTPs (each 0.2 mM), 10 pmol of each primer and 1.5 U of Taq-polymerase. The PCR profile for FAD2-N-F/FAD2-N-R and FAD2-IS-F/FAD2-IS-R primer sets consisted of 5 min denaturing at 94 ºC, followed by 35 cycles of 1 min denaturing at 95 ºC, 1 min annealing at 60 ºC and 1 min extension at 72 ºC, with a final extension of 5 min at 72 ºC. PCR amplification products were controlled by 2% agarose gel electrophoresis, stained with RedSafe Nucleic Acid Staining Solution and visualized by Gel Imaging System Vilber Lourmat Quantum ST5. The size of PCR products on gels for each primer was determined by using Vision Capt Software (Vilber Lourmat).

3. Results and Discussion

Various sunflower lines and hybrids have been studied to distinguish HO genotypes from LO genotypes by different researchers and molecular marker types such as RAPD or SSR (Dehmer and Friedt 1998; Nagarathna et al. 2011; Grandon et al. 2012; Singchai et al. 2013; Bilgen 2016; Dimitrijevic et al. 2017). Nagarathna et al. (2011) studied around 350 sunflower genotypes including RHA-lines, cms lines, inbreds and germplasm lines to screen for high oleic acid. In Nagarathna et al. (2011), to genotype the sunflower lines for high oleic content, HO PCR specific fragment (N1-3F/N2-1R) were chosen and also the seeds were used for the determination of fatty acids (linoleic acid, oleic acid, palmitic acid and stearic acid) using gas chromatography. They reported that the genotypes having a specific band (at 800 to 900 bp) showed high oleic content. Singchai et al. (2013) studied the developed lines that used as the representative of low and high oleic acid sunflowers for genotyping. They screened 37 SSR primers including 34 primers of ORS set, 2 primers of ha set and N1-3F/N2-1R primer to identify DNA samples from two lines (high and low oleic acid contents). Out of the 37 SSR primers screened for polymorphism, 10 SSR primers including N1-3F/N2-1R generated differentiating bands between the high and low oleic content lines. With the 10 SSR markers they studied, Singchai et al. (2013) reported that it is possible to identify the genetic markers linked to high oleic acid trait which may be useful for further sunflower breeding program.

It is widely accepted that the Pervenets mutation is closely associated with the polymorphic region near to the Δ12-desaturase gene. It has also been shown that the 246 bp of PCR fragment indicating 16 repeats of TTA triplets in this polymorphic SSR locus is strongly associated with Pervenets mutation (Berville et al. 2009). Therefore, determination of the repeat number on this trinucleotides repeat region has been used for molecular marker assisted selection of the HO sunflower varieties. In this study, four different primer pairs (one of them was newly designed) were used in order to determine HO genotypes (Table 1). Genotypes, based on repeat number, of studied commercial sunflower varieties were determined for the SSR locus by the N1-1F/N1-1R fluorescent labeled primer pair. According to the DNA fragment analysis; homozygous (246/246 bp) and heterozygous (246/249 bp) genotypes were identified (Figure 1). In order to confirm HO sunflower genotypes, all studied individuals were screened with HO PCR specific fragment (N1-3F/N2-1R). The insertion mutation as the second molecular marker was detected by the 870 bp PCR fragment across the 5' insertion point by N1-3F/N2-1R primers (Berville et al. 2009). The results showed that HO genotypes showed a specific band at about 870 bp length which was absent in LO genotypes (Figure 2). In addition, discrimination of HO and LO genotypes were done by using INDEL marker FAD2-F4/FAD2-R1 primer set for all studied individuals. According to FAD2-F4/FAD2-R1 primer results, HO genotypes has specific band at about 653 bp (Figure 3).

The HO and LO varieties were also determined by multiplex PCR with two primer pairs FAD2-N-F/FAD2-N-R and FAD2-IS-F/FAD2-IS-R described in this study (Figure 4). Our results showed that FAD2-N-F/FAD2-N-R primer pair gave a band at around 530 bp length which was present in all studied genotypes and used for internal control. The second primer FAD2-IS-F/FAD2-IS-R which is insert specific primer has specific band at around 695 bp which was present in HO varieties and absent in LO varieties (Figure 4).

Our recent study has shown that 16 TTA triplet repeats can be found in some LO sunflower varieties. So, the SSR marker is able to indicate the Pervenets mutation but not in all the sunflower varieties. Pervenets mutation results from an insertion of a DNA sequence into a region at the downstream of the Δ12-desaturase gene. Current research in addition to previously reported recent studies have shown that the PCR protocols aiming to show the presence of the inserted sequence are able to detect HO varieties more confidently than the SSR marker. Finally, we decided to develop a reliable PCR protocol targeting to show the inserted DNA sequence which is known as the Pervenets mutation. Our protocol consists of conventional multiplex PCR and agarose gel electrophoresis combination. As one primer pair is specific to insertion region that yields a PCR band if the Pervenets mutation present, the other primer pair is specific to Δ12-desaturase gene sequences as positive control in order to show that the PCR works properly. The second primer pair is necessary to prevent false negative results in the samples that have no Pervenets mutation. Using the PCR protocol described here, we were able to reliably differentiate the HO and LO sunflower varieties. In conclusion, molecular markers are advantageous for breeders by means of saving money and time. Plants breeders, who want to develop new HO genotypes, are able to discard LO genotypes by using available molecular markers.

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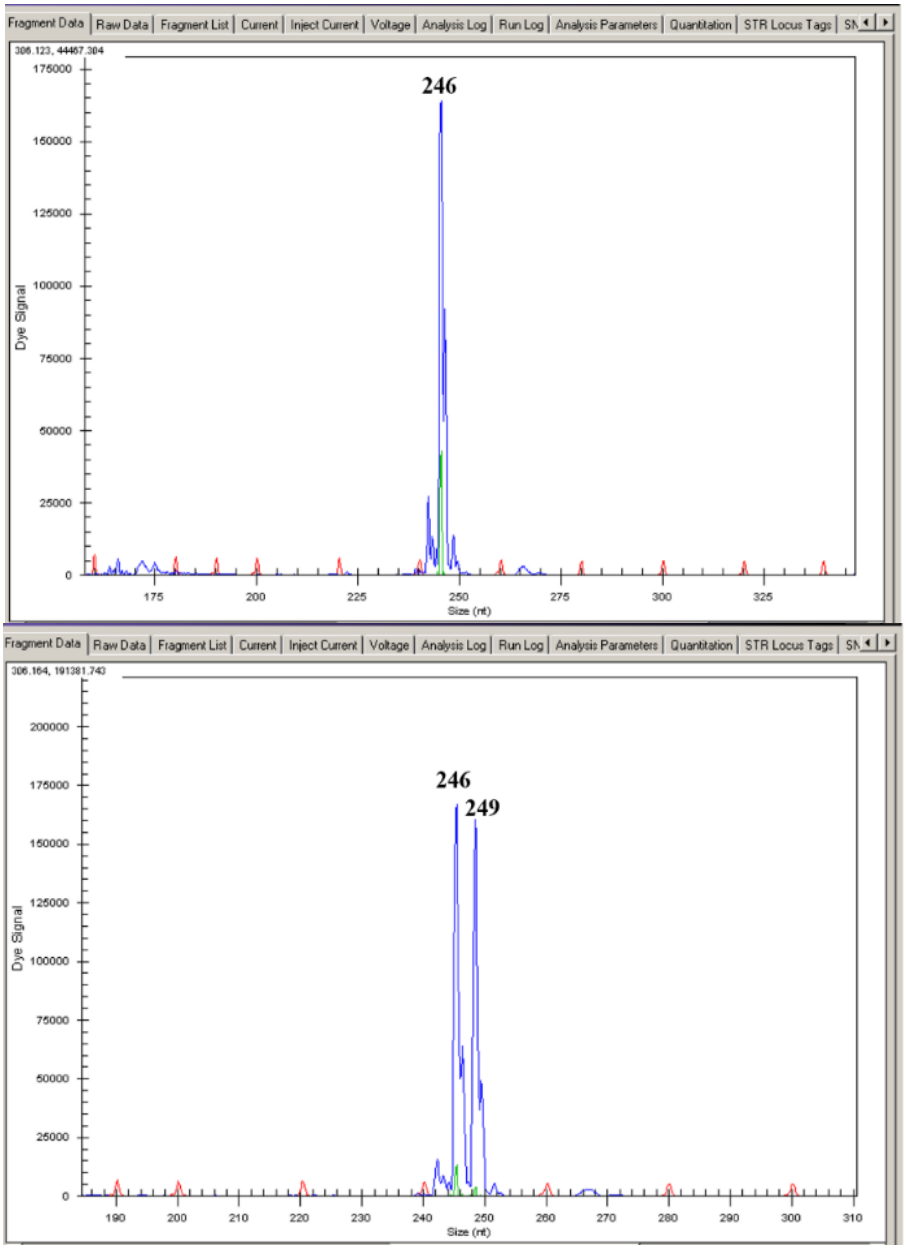

Figure 1. DNA fragment analyses results for studied sunflower genotypes.

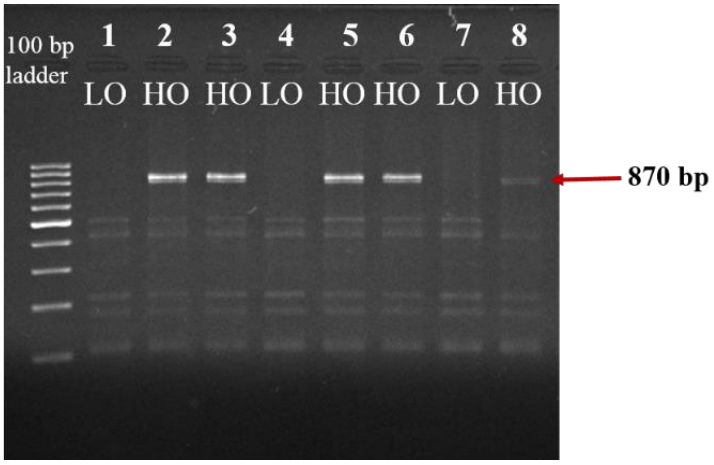

Figure 2. PCR amplification of HO and LO genotypes with HO PCR specific fragment (N1-3F/N2-1R).

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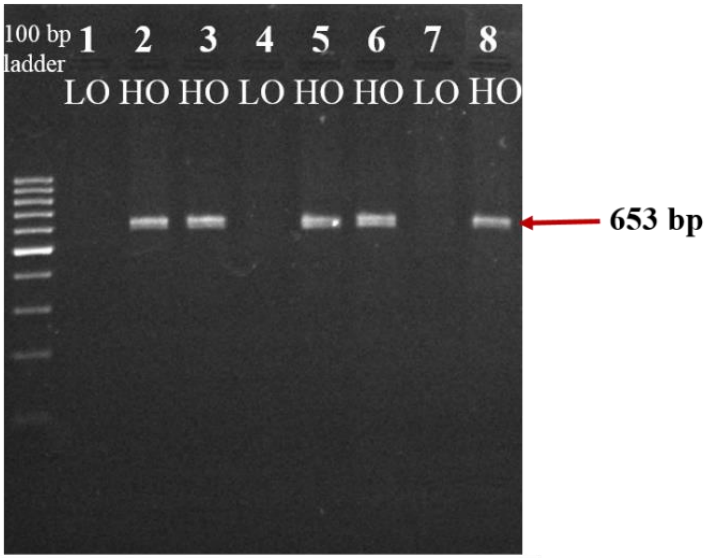

Figure 3. PCR amplification of HO and LO genotypes with INDEL marker (FAD2-F4/FAD2-R1).

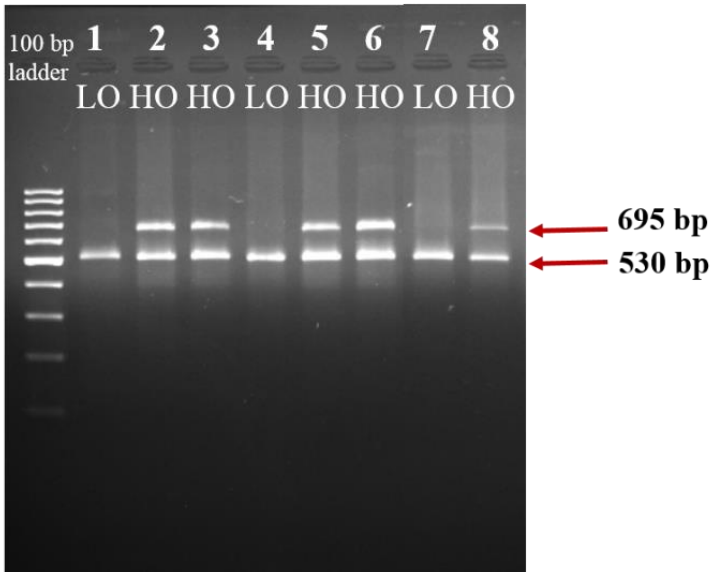

Figure 4. PCR amplification of HO and LO genotypes with FAD2-N-F/FAD2-N-R and FAD2-IS-F/FAD2-IS-R.

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