CHARACTERIZATION OF SUNFLOWER INBRED LINES WITH HIGH OLEIC ACID CONTENT BY DNA MARKERS

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ABSTRACT

Sunflower is one of the most important oilseed crops due to high oxidative stability of its oil with high oleic acid content. Screening high oleic sunflower genotypes by standard methods such as Gas Chromatography (GC) is time consuming and expensive. Using molecular markers associated with high oleic acid trait is a useful tool in order to facilitate sunflower breeding program. For the purpose of genotyping the sunflower lines for high oleic content, two markers were chosen; SSR marker and HO PCR specific fragment. The results showed that high oleic containing hybrids expressed a specific SSR band at 246 bp length, and also HO PCR specific fragment at 870 bp length. The results were confirmed by determining the fatty acid composition. The results of this work allowed to validation of two DNA markers in sunflower inbred lines for high oleic acid traits.

Key words: *Helianthus annuus* L.*,* Marker-assisted selection, SSR, Oleic acid composition

INTRODUCTION

Sunflower is one of the most important oil crops in the world. It is produced in the world generally for human and non-food purposes (cosmetics, paints, etc.) due to the oil and fatty acid composition of the seed being adapted to these uses. Sunflower oil contains high level of unsaturated fatty acids (88%); linoleic acid (48-74%), oleic acid (14-40%) and also saturated fatty acids; palmitic acid (4-9%) and stearic acid (1-7%) (Singchai et al., 2013; Nagarathna et al., 2011). It is desirable for human consumption because of its favorable fatty acid composition (Baydar and Erbas 2005). Oleic sunflower production and consumption started rapidly both for healthy frying oil, and also non-food purposes in recent years. Non-food applications in particular require oleic acid content that is stable and higher than 90% (Vannozzi 2006). Diets containing vegetable oil with high oleic acid content have been reported to be most effective for preventing cardiovascular diseases (Delplanque et al., 1997; Broun et al., 1999). 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 (High oleic) Pervenet mutant by chemical mutagenesis (Soldatov 1976). The mean content of oleic acid of the seeds from Pervenet population is higher than 65% whereas this content in normal LO varieties is about 20% (Berville et al., 2009). Because of the interest in oleic acid and also the agronomic performance of HO varieties carrying the Pervenets mutation compared with the LO varieties, these varieties are widely used in the world (about 1.2 million ha, CETIOM 2002). 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 and it is rapid, cheaper and simple when suitable markers were developed (Varshney et al., 2005). Therefore, marker assisted selection (MAS) analysis is necessary at genomic level allowing rapid and earlier determination of homozygous HO genotypes for sunflower breeding studies.

The aims of this study are characterization of sunflower inbred lines with high oleic acid content by DNA markers and evaluate the effectivity of two marker types developed by Berville et al. (2009).

MATERIALS AND METHODS

Plant materials

For the purpose of screening on high oleic acid genotypes, around 300 sunflower F_3 (K2-R-SN-9/13) individuals obtained from a cross between high oleic acid and low oleic acid lines were used. Leaves were collected from the field, labeled with individual number and stored kept at -80ºC until further use.

DNA isolation

Before DNA isolation leave samples were homogenized with Retsch® Model MM300 Mixer Mill. Different manual DNA isolation methods (Dellaporta et al., 1983; Doyle and Doyle 1987; Li et al., 2007; Azmat et al., 2012; Souza et al., 2012; Healey et al., 2014) and also DNA isolation kits (NANObiz Plant Genomic DNA Isolation Kit, Vivantis GF-1 Plant DNA Extraction Kit and i-genomic Plant DNA Extraction Mini Kit) were tested in order to obtain high quality and quantity DNA for PCR analysis. Finally i-genomic Plant DNA Extraction Mini Kit was selected and used for DNA isolation from all samples. Concentration of each DNA 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 the extracted DNA was diluted as 50 ng per µl and was stored at -20 ºC for later uses.

PCR analysis

Genotyping of high oleic (HO) and low oleic (LO) sunflower individuals was performed with two primer pairs; SSR (N1-1F/N1-1R) and HO PCR specific fragment (N1-3F/N2-1R) that were chosen from the patent obtained by Berville et al. (2009) (Table 1). PCR amplification was carried out using 20 µl 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 (forward primer WellRed D4 fluorescent dye labeled) and 1.5 U of Taq-polymerase. The PCR profile for SSR (N1-1F/N1-1R) consisted of 5 min denaturing at 94 ºC, followed by 35 cycles of 1 min denaturing at 94 ºC, 1 min annealing at 50 ºC and 1 min extension at 72 ºC, with a final extension of 10 min at 72 ºC. The PCR profile for HO PCR specific fragment (N1-3F/N2-1R) consisted of 5 min denaturing at 94 ºC, followed by 35 cycles of 1 min denaturing at 94 ºC, 1 min annealing at 58 ºC and 1 min extension at 72 ºC, with a final extension of 10 min at 72 ºC. Amplified PCR 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 (Figure 1 and

Figure 2). 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.

Table 1. Characteristics of markers used to analyze HO and LO sunflower genotypes

No	Primer type	Primer name	Primer sequences (5′-3′)
1	SSR	N1-1F	TTGGAGTTCGGTTTATTTAT
		N1-1R	TTAGTAAACGAGCCTGAAC
2	HO PCR specific fragment	N1-3F	GAGAAGAGGGAGGTGTGAAG
		N2-1R	AGCGGTTATGGTGAGGTCAG

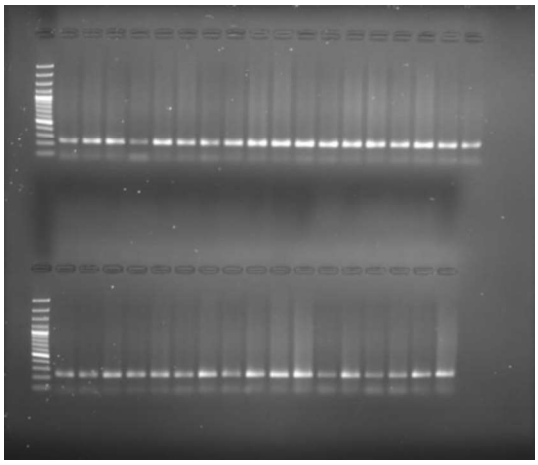

Figure 1. Amplified fragments with SSR (N1-1F/N1-1R) primer for sunflower individuals (First lane is 100 bp DNA Ladder)

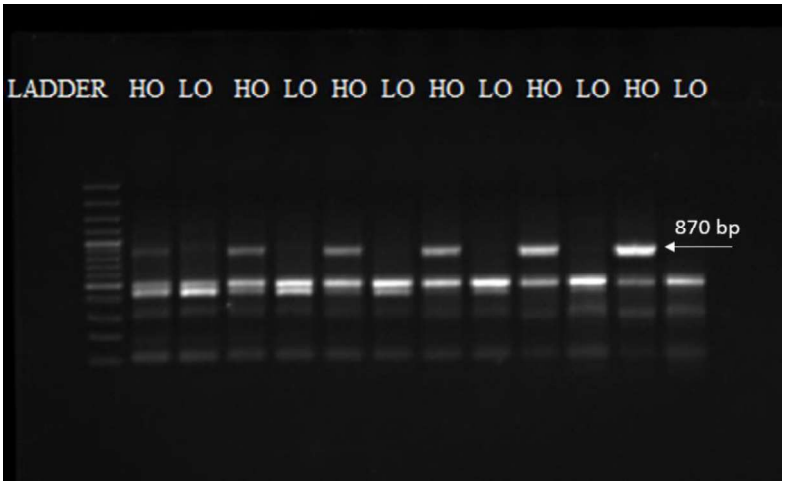

Figure 2. PCR amplification of HO and LO genotypes with HO PCR specific fragment (N1-3F/N2-1R) (First lane is 100 bp DNA Ladder)

PCR amplification of SSR (N1-1F/N1-1R) primer leads to 243/246/249 bp fragments corresponding to 15/16/17 TTA repeats, respectively. The HO genotypes has 16 TTA repeats whereas LO genotypes has 17 TTA repeats for the studied sunflower individuals. DNA sequence analysis with reverse primer was carried out to confirm repeat motifs corresponding to HO genotypes (Figure 3 and 4).

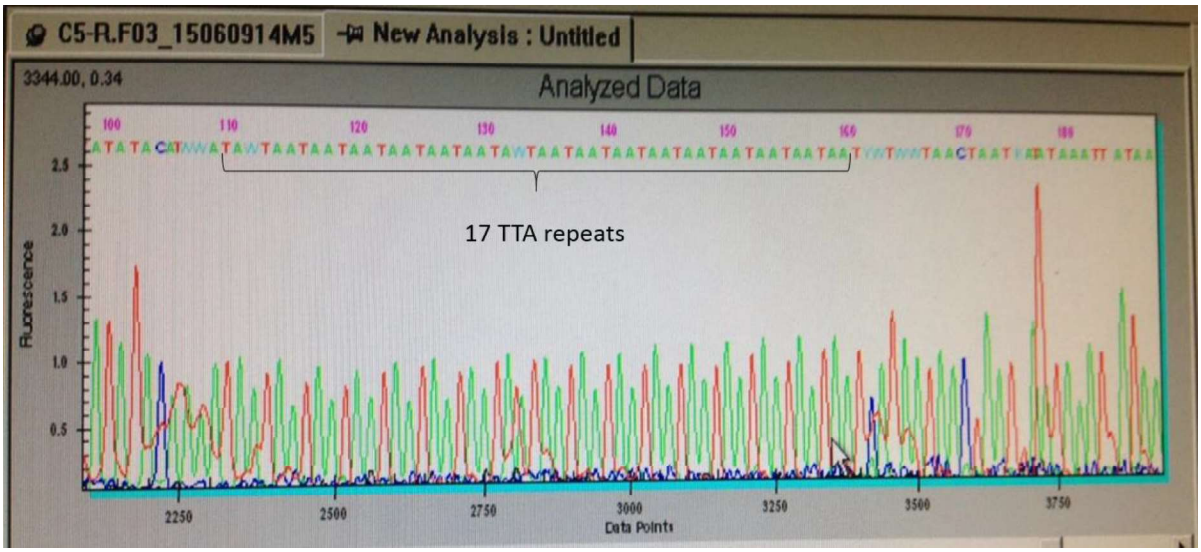

Figure 3. DNA sequence analysis of LO genotypes that have 17 TTA repeats (249 bp)

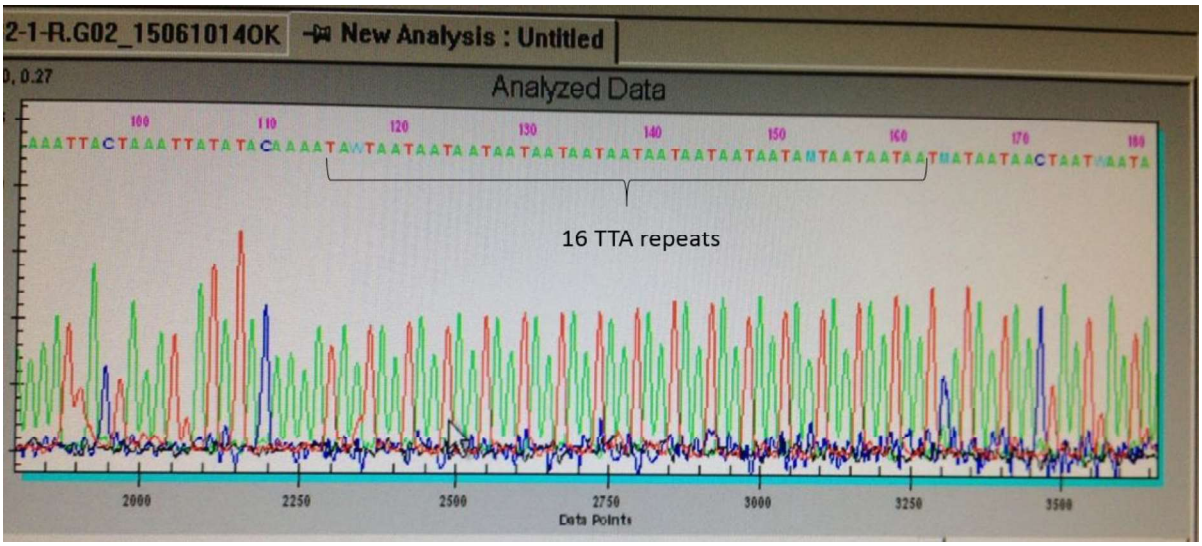

Figure 4. DNA sequence analysis of HO genotypes that have 16 TTA repeats (246 bp)

RESULTS AND DISCUSSION

The Pervenet mutation was labelled by the polymorphism of the SSR locus located on the Δ12-desaturase gene intron (Berville et al., 2009). Alleles and genotypes of studied sunflower individuals were determined for analyzed SSR (N1-1F/N1-1R) locus. According to DNA fragment analysis for SSR locus 246/246 Homozygous, 249/249 Homozygous and 246/249

Heterozygous genotypes were identified (Figure 5). In order to confirm HO sunflower genotypes, all studied individuals were screened with HO PCR specific fragment (N1-3F/N2-1R). The Pervenet mutation was labelled by the 870 bp PCR fragment across the 5' insertion point by HO PCR specific fragment (N1-3F/N2-1R) (Berville et al., 2009). The results showed that high oleic containing sunflower individuals (HO genotypes) showed a specific band at about 870 bp length which was absent in low oleic (LO) genotypes (Figure 2).

After evaluation of all studied sunflower individuals (Totally 300 F_3), 183 of them were HO genotypes, the others were LO genotypes. The results were confirmed by determination of fatty acid composition using gas chromatography in all the studied individuals. According to fatty acid analysis, the oleic acid content was obtained from 60-92% for HO genotypes and below 60% (minimum 22.8%) for LO genotypes.

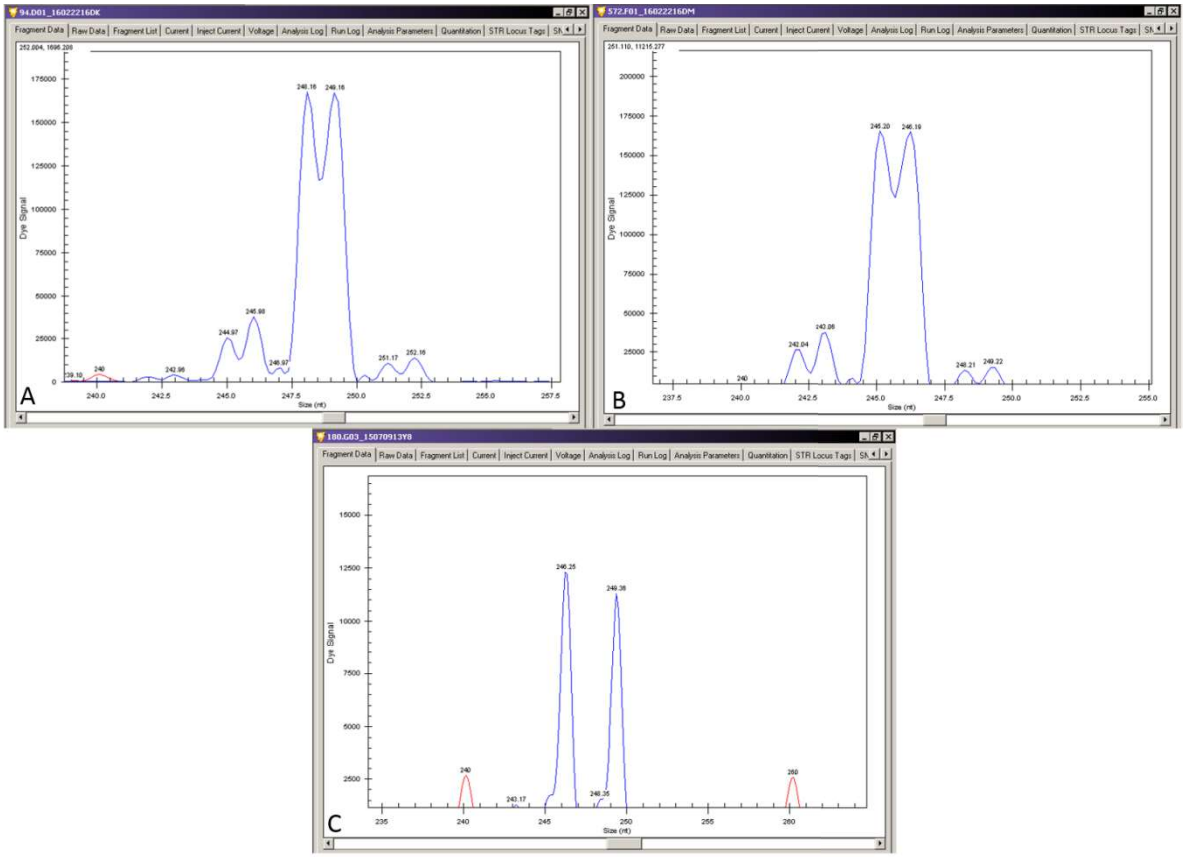

Figure 5. DNA fragment analyses results for (N1-1F/N1-1R) primer A) 249 bp (Homozygous LO genotype), B) 246 bp (Homozygous HO genotype), C) 246 bp/249 bp (Heterozygous HO genotype)

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). Nagarathna et al., (2011) studied around 350 sunflower genotypes including RHA-lines, cms lines, inbreds and germplasm lines to screening on high oleic acid. In Nagarathna et al., (2011) For the purpose of genotyping 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 thirty seven 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.

As a conclusion PCR analysis with selected primers enabling to amplify either the Δ12HOS allele and thus the Pervenet mutations (N1-3F/N2-1R) or the SSR locus (N1-1F/N1-1R), lead to discriminate HO and LO genotypes. Consequently, these primers may be used in selection programs to identify genotypes carrying the Pervenet mutation. However, these markers especially SSR (N1-1F/N1-1R) need to be further validation in different sunflower populations in order to confirm their capability to identify high and low oleic acid contents.

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