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USE OF SSR MARKERS IN IDENTIFICATION OF SUNFLOWER ISOGENIC LINES IN LATE GENERATIONS OF BACKCROSSING

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SUMMARY

In the present study, thirteen SSRs (Simple Sequence Repeats) were employed for screening the level of similarity between sunflower parental lines and progeny obtained in final stages of selection. Another aim of the study was to compare the success of making isogenic lines through selection. Line P (donor line) was used as a donor of downy mildew gene Pl_6 and backcrossed with four commercial inbred lines (A, B, C and D) susceptible to downy mildew. HAP3, a DNA-specific primer, was used to test the success of transfer of Pl_6 gene from the donor line to the progeny lines in the F_6 and F_7 generations. It was found that all progeny lines were resistant to downy mildew. Results obtained by using thirteen primers showed that progeny lines were similar to their respective recipient parent, but further studies have to be conducted using a larger number of primers and a larger number of genotypes.

Key words: **sunflower, *Helianthus annuus* L., isogenic line, backcross, SSR, downy mildew**

INTRODUCTION

Molecular markers offer specific advantages in assessment of genetic diversity and in trait-specific crop improvement, but are also used for cultivar identification, as well as for the study of polymorphism and genetic similarity in major crops such as maize (Carvalho *et al.*, 2004), soybean (Narvel *et al.*, 2000), potato (Solano Solis *et al.*, 2007), common bean (Duarte *et al.*, 1999), sorghum (Smith *et al.*, 2000), coconut (Perera *et al.*, 1998) and others.

Classical backcrossing is a time-consuming process, due to the number of backcrosses needed to achieve desired results. Selection based on phenotypic char-

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acteristics can be unreliable because of the environmental effects and measurement errors. By using molecular markers, required number of backcrosses can be significantly decreased, which makes their use a cost-efficient method (Knapp, 1998). Molecular markers provide an insight in the variability of the progeny at the molecular level in each generation (Moose and Mumm, 2008). The efficiency of the marker-assisted backcrossing depends on experimental design, especially on the marker density and position, population size and selection strategy (Frisch and Melchinger, 2005).

The level of polymorphism and genetic relationship have been studied by means of different molecular markers such as RAPDs (Random Amplified Polymorphic DNA), RFLPs (Restriction Fragment Length Polymorphisms), AFLPs (Amplified Fragment Length Polymorphisms) and, more recently, SSRs (Wünsch and Hormaza, 2002). Because of their multiallelic and highly polymorphic nature, microsatellite markers or SSRs are currently becoming the preferred technique for the molecular breeding in plant species. In sunflower (*Helianthus annuus* L.), microsatellites have been particularly useful in the studies of phylogenic relationships, genotype identification and calculation of genetic relationship between inbred lines (Smith *et al.*, 2000; Paniego *et al.*, 2002; Solodenko *et al.*, 2005; Antonova *et al.*, 2006; Hvarleva *et al.*, 2007).

In the present study, thirteen SSRs were employed for screening the level of similarity between parental lines and their progeny obtained by selection. Line P (donor line) was used as a donor of Pl_6 gene (downy mildew resistance gene) and crossed with four commercial lines (A, B, C and D), which are susceptible to downy mildew. The aim of this study was to check the success of backcrossing and to determine the degree of similarity of backcross progenies to both parents using SSR markers, as well as to determine if chosen SSR primers could be used for this purpose. HAP3, a DNA-specific marker, was used to determine whether Pl_6 gene has been successfully transferred from the donor line to the progeny plants.

MATERIALS AND METHODS

Leaf samples were taken from original inbred lines: donor line P and four recipient lines: A, B, C, D, and their backcross progeny $A_{1\text{-}10}$, $B_{1\text{-}10}$, $C_{1\text{-}10}$ and $D_{1\text{-}10}$, respectively. Modified CTAB protocol (Permingeat *et al.*, 1998) was used for DNA extraction.

PCR mix of 15 μl contained 1 × buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μl mixed primer, 1 U enzyme, 25 μg BSA, and 40 ng DNA. The primers used for PCR amplification were: ORS 7, ORS 78, ORS 307, ORS 316, ORS 349, ORS 509, ORS 561, ORS 610, ORS 613, ORS 1079, ORS 1114, ORS 1144, ORS 1209 and HAP3. In PCR reaction with ORS primers DNA was amplified at 95°C for 2 min, 1 cycle of 94°C of 30 sec, 64°C for 30 sec, 65°C for 45 sec, followed by 6 cycles in which temperature was decreased by 1°C per cycle, followed by 32 cycles of 94°C for 30 sec, 58°C for 30 sec, and 65°C for 45 sec. Final extension was at 65°C for 20 min. For HAP3, PCR reaction described by Panković *et al.* (2007) was used. All PCR products were separated on 2% MetaPhor agarose gel.

Genetic relationship between the parental and their progeny lines was determined by UPGMA analysis (STATISTICA 7.0; StatSoft, *Inc.*, Tulsa, OK 74104, USA).

RESULTS AND DISCUSSION

ORS primers, being codominant, are generally used for sunflower genotyping in order to determine the genetic diversity between wild and cultivated sunflower, or sunflower lines and hybrids (Solodenko and Sivolap, 2005; Hvarleva *et al.*, 2007). In this study we used thirteen SSR primers, created by Tang *et al.* (2002) for testing sunflower isogenic lines in final stages of selection, in order to determine the degree of similarity between the parental lines and their F_6 and F_7 progeny lines. Eight primers showed presence of polymorphism between parental lines. ORS 509, ORS 610 and ORS 1209 primers amplified multiple bands and thus proved to be the most informative of all primers used in genotyping of the F_6 and F_7 generations of progeny lines and their parental lines (Table 1). ORS 509 amplified the largest number of bands of all primers used (five in total). In the work of Solodenko and Sivolap (2005), ORS 509 amplified only three bands in all of the investigated inbred lines. Two out of the five bands on PCR profiles of inbred lines in our experiment were of the same size as the bands obtained by Solodenko and Sivolap (2005). The DNA fragment length in the SSR profiles of the tested inbreds varied from 184-202 bp.

Table 1: Genetic profile of parental and progeny lines obtained by amplification with 13 ORS primers in the F_6 and F_7 generation of backcrossing.

	P	A	B	C	D	$A_{1\text{-}10}$	$B_{1\text{-}10}$	$C_{1\text{-}10}$	$D_{1\text{-}10}$
ORS 7	272	272	272	272	272	272	272	272	272
ORS 78	165	165	165	165	165	165	165	165	165
ORS 307	118	118	150	193	118	118	150	193	118
ORS 316	202	202	195	202	202	202	195	202	202
ORS 349	254	254	254	254	254	254	254	254	254
ORS 509	81	81	81	81	81	81	81	81	81
	120	120	120	120	120	120	120	120	120
	202	202	183	183	183	202	183	183	183
			194				194		
ORS 561	373	373	373	400	400	373	373	400	400
			400				400		
ORS 610	168	178	178	178	178	178	178	178	178
	232	232	232	232	232	232	232	232	232
	313	313	313	313	313	313	313	313	313
ORS 613	239	239	239	239	239	239	239	239	239
ORS 1079	402	402	360	402	374	402	360	402	374
ORS 1114	252	280	269	252	269	280	269	252	269
ORS 1144	181	181	178	202	178	181	178	202	178
ORS 1209	328	328	328	328	328	328	328	328	328
	512	512	512	512	512	512	512	512	512
	643	643	643	643	643	643	643	643	643

PCR profiles obtained from ORS 509 and ORS 1209 showed that progeny lines $B_{1\text{-}10}$ and $D_{1\text{-}10}$ in the F_6 generation were not completely identical with their respective recipient parent. ORS 509 and ORS 1209 amplified some non-specific bands, in most of the progeny plants of back crosses with the line B ($B_{1\text{-}10}$) and the line D ($D_{1\text{-}10}$). In the F_7 generation in all progeny lines of line D, ORS 509 amplified the same bands as in recipient line (D). In the progenies of line B the same primer amplified some, bands originating from donor line (P). Non-specific bands were not amplified. ORS 1209 amplified four bands of the same size in all parental and progeny lines. Although ORS 610 amplified three bands in the work of Hvarleva *et al.* (2007), in our study this primer amplified four bands in all parental lines and F_6 progeny lines, so there was no need to use this primer in the analysis of the next generation.

Primers ORS 307, ORS 1079, ORS 1114, and ORS 1144 were also highly informative in our study, amplifying three bands. In the work of Tang and Knapp (2003) and Zhang *et al.* (2005) the same number of bands in elite inbred lines was amplified by using ORS 307, while in the work of Hvarleva *et al.* (2007) this primer amplified two bands. ORS 1144 amplified seven bands in wild and domesticated sunflower species and two in inbred lines (Solodenko and Sivolap, 2005).

In progeny lines $B_{1\text{-}10}$ and $D_{1\text{-}10}$, in the F_6 generation, bands that belonged to both parental lines were amplified by ORS 1079 (Figure 1), therefore, this primer was used to test the degree of similarity of progeny lines with their maternal line in the next generation of selection.

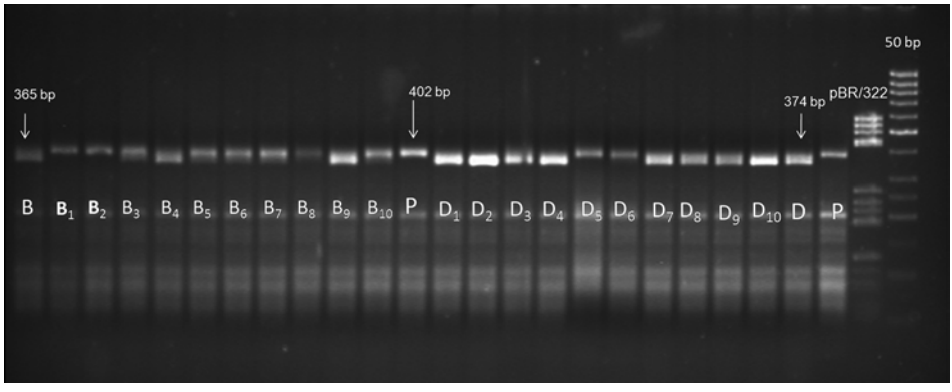

Figure 1: PCR amplification profile of parental lines (B and D) and their respective progeny lines from crosses with line P ($B_{1\text{-}10}$ and $D_{1\text{-}10}$) (the F_6 generation) obtained with ORS 1079 primer.

ORS 1079 showed that progeny of line D ($D_{1\text{-}10}$, the F_7 generation) was genetically identical to this line, while only one progeny plant, B_5, was genetically identical with its recipient line B, two progeny plants, B_2 and B_7, were identical with the donor line (P) and the rest of the progeny plants had bands from both parents (Figure 2).

In our experiment, ORS 316 and ORS 561 amplified two allele combinations. These primers amplified three and five alleles in sunflower elite inbred lines, respectively (Tang and Knapp, 2003). ORS 561 amplified some non-specific bands or bands that originated from both parental lines, B and P, in F_6 and F_7 progeny genotypes of $B_{1\text{-}10}$.

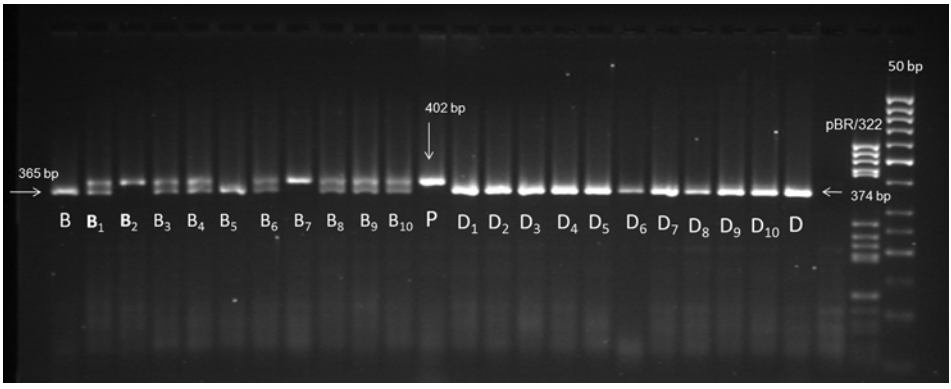

Figure 2: PCR amplification profile of parental lines (B and D) and their respective progeny lines from crosses with line P ($B_{1\text{-}10}$ and $D_{1\text{-}10}$) (the F_7 generation) obtained with ORS 1079 primer.

ORS 7 and ORS 78 amplified only one band in the tested inbred lines and their progenies. This is not in agreement with the results of Haverleva *et al*. (2007) and Solodenko and Sivolap (2005) who found these markers to be polymorphic, amplifying two and three bands, respectively. ORS 78 amplified a band of the same size as in the work of Solodenko and Sivolap (2005). Beside ORS 7 and ORS 78, ORS 349 and ORS 613 also did not appear to be reliable markers for further research because of their monomorphic nature. Tang and Knapp (2003) found ORS 349 to be monomorphic for elite inbred lines, but polymorphic for exotic domesticated and wild sunflower populations.

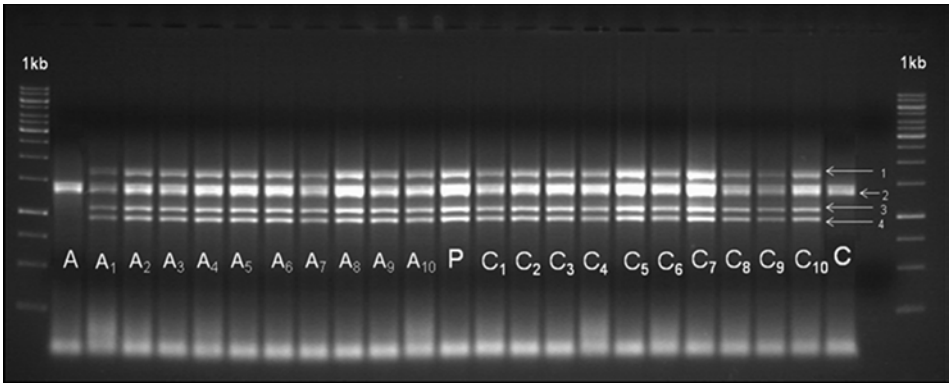

Figure 3: HAP3 primer amplified 4 bands (1. 1720 bp, 2. 1330 bp, 3. 1060 bp, 4. 940 bp) in resistant P and all progeny plants $A_{1\text{-}10}$ and $C_{1\text{-}10}$ (the F_7 generation), and one band in susceptible recipient lines A and C.

HAP3 was chosen for its specific use in detecting the presence of Pl_6, a downy mildew resistance gene (Panković *et al.*, 2007). HAP3 primer amplified 4 bands (1720 bp, 1330 bp, 1060 bp and 940 bp) in donor line P and all progeny lines in the F_6 and F_7 generations, and one band (1330 bp) in recipient lines A, B, C and D, confirming that downy mildew resistance gene has been successfully transferred from line P to all progeny lines (Figure 3).

Bands generated in F_7 progenies and parental lines using all thirteen ORS primers and HAP3 primer were analyzed by assigning 1 or 0 depending on the presence or absence of each allele form. The dendrogram showed that there was a great difference between parental line P (donor line) and recipient lines A, B, C and D (Figure 4).

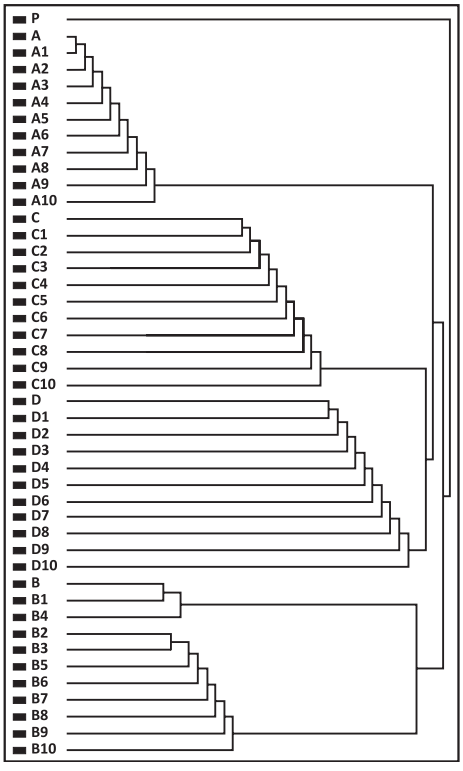

Figure 4. Dendrogram based on SSR data of parental and progeny lines

All progeny lines showed the highest similarity to their recipient parental line (*e.g.*, A_1 to A_{10} belong to the same sub-cluster as their recipient line A, *etc.*). However, due to the presence of bands that originated from both parental lines in F_7 progeny lines $B_{1\text{-}10}$, there was an additional sub-cluster formed in B sub-cluster. Consequently, an additional backcross should be carried out in order to create a population that will be genetically close (preferably identical) to its maternal line, and still be resistant to downy mildew. An improvement in the level of similarity between progeny lines $D_{1\text{-}10}$ and line D was shown, as all progeny lines showed the same profile as their recipient line, after electrophoresis.

CONCLUSIONS

Progeny lines formed clusters with their respective recipient line, while line P formed a separate cluster. This shows that the combination of primers used in this study is sufficiently informative. ORS 509, ORS 610 and ORS 1209 proved to be the most informative primers, because they amplified bands that differed between recipient lines and donor line P, thus clearly distinguishing these lines. Besides ORS 509 and 1209, ORS 1079 amplified bands that belonged to both parental lines in some individual plants of $B_{1\text{-}10}$ and $D_{1\text{-}10}$ in F_6 generation. All F_7 progeny lines $D_{1\text{-}10}$ had the same profile as their recipient line D, but there were still some plants

in F_7 generation of $B_{1\text{-}10}$ that did not show the same profile as their recipient line B. These results showed that an additional backcross should be carried out. Downy mildew resistance gene Pl_6 was successfully transferred to all progeny plants. The results obtained by using thirteen ORS primers showed that progeny lines are similar to their respective recipient line, but further studies are needed which would include new primers on greater number of genotypes.

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REFERENCES

Antonova, T.S., Guchetl, S.Z., Tchelustnikova, T.A. and Ramasanova, S.A., 2006. Development of marker system for identification and certification of sunflower lines and hybrids on the basis of SSR-analysis. Helia 29(45): 63-72.

Carvalho, V.P., Ruas, C.F., Ferreira, J.M., Moreira, R.M.P. and Ruas, P.M., 2004. Genetic diversity among maize (*Zea mays* L.) landraces assessed by RAPD markers. Genetics and Molecular Biology 27(2): 228-236.

Duarte, J.M., Bosco dos Santos, J. and Cunha Melo, L., 1999. Comparison of similarity coefficients based on RAPD markers in the common bean. Genetics and Molecular Biology 22(3): 427-432.

Frisch, M. and Melchinger, A.E., 2005. Selection theory for marker-assisted backcrossing genetics. Genetics 170: 909-917.

Hvarleva, Tz., Bakalova, A., Chepinski, I., Hristova-Cherbadji, M. and Hristov, M. and Atanasov, A., 2007. Characterization of Bulgarian sunflower cultivars and inbred lines with microsatellite markers. Biotechnology & Biotechnological Equipment 21(4): 408-412.

Knapp, S.J., 1998. Marker-assisted selection as a strategy for increasing the probability of selecting superior genotypes. Crop Sci. 38:1164-1174.

Moose, S.P. and Mumm, R.H., 2008. Molecular plant breeding as the foundation for 21st century crop improvement. Plant Physiology 147: 969-977.

Narvel, J.M., Fehr, W.R., Chu, W., Grant, D. and Shoemaker, R.C., 2000. Simple sequence repeat diversity among soybean plant introductions and elite Genotypes. Crop Sci. 40: 1452–1458.

Paniego, N., Eschaide, M., Munoz, M., Fernandez, L., Torales, S., Faccoi, P., Fuxan, I., Carrera, M., Zandomeny, R., Suarez, E. and Hopp, H., 2002. Microsatellite isolation and characterization in sunflower (*Helianthus annuus* L.). Genome 45: 34-43.

Panković, D., Radovanović, N., Jocić, S., Satović, Z. and Škorić, D., 2007. Development of codominant amplified polymorphic sequence markers for resistance of sunflower to downy mildew race 730. Plant Breeding 126(4): 440-444.

Perera, L., Russell, J.R., Provan, J., McNicol, J.W. and Powell, W., 1998. Evaluating genetic relationships between indigenous coconut (*Cocos nucifera* L.) accessions from Sri Lanka by means of AFLP profiling. Theor. Appl. Genet. 96: 545-550.

Permingeat, H.R., Romagnoli, M.V. and Vallejos, R.H., 1998. A simple method for isolating high yield and quality DNA from cotton (*Gossypium hirsutum* L.) leaves. Plant Mol. Biol. Rep. 16: 1-6.

Smith, J.S.C., Kresovich, S., Hopkins, M.S., Mitchell, S.E., Dean, R.E., Woodman, W.L., Lee, M. and Porter, K., 2000. Genetic diversity among elite sorghum inbred lines assessed with simple sequence repeats. Crop Sci. 40: 226-232.

Solano Solis, J., Morales Ulloa, D. and Anabalón Rodríguez, L., 2007. Molecular description and similarity relationships among native germplasm potatoes (*Solanum tuberosum* ssp. *tuberosum* L.) using morphological data and AFLP markers. Electron. J. Biotechnol. [online]. 10(3): 436-443. ISSN 0717-3458.

Solodenko, A. and Sivolap, Y., 2005. Genotyping of *Helianthus* based on microsatellite sequences. Helia 28(42): 19-26.

Tang, S. and Knapp, S.J., 2003**.** Microsatellites uncover extraordinary diversity in native American land races and wild populations of cultivated sunflower. Theor. Appl. Genet. 106(6): 990-1003.

Tang, S., Kishore, V.K. and Knapp, S.J., 2003. PCR-multiplex for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theor. Appl. Genet. 107(1): 6-19.

Tang, S., Yu, J.-K., Slabaugh, M., Shintani, D., and Knapp, S., 2002. Simple Sequence Repeat Map of the Sunflower Genome. Theor. Appl. Genet. 105(8): 1124-1136.

Wünsch, A. and Hormaza, J.I., 2002. Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. Euphytica 125: 59-67.

Zhang, L.S., Le Clerc, V., Li, S. and Zhang, D., 2005. Establishment of an effective set of simple sequence repeat markers for sunflower variety identification and diversity assessment. Can. J. Bot. 83(1): 66-72.