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THE USE OF PCR-BASED MARKERS IN THE EVALUATION OF RESISTANCE TO DOWNY MILDEW IN NS-BREEDING MATERIAL

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SUMMARY

The aim of this work was the application of previously developed and development of new PCR markers for the evaluation of sunflower resistance to downy mildew. Twenty sunflower inbred lines were investigated. Plant resistance to downy mildew was determined by the whole seedling immersion method. Genomic DNA was extracted from the first pair of leaves, and its polymorphism was investigated by RAPD, SSR and several published markers for disease resistance.

The presence of the markers Ha-NBS 7/R, Ha-NBS 8/R, Ha-NBS 9/R, in *Pl6* donor lines (Ha-335, JM-8) and in resistant progeny (Ha-26, G12, G10, G11) confirm that HAP3 could be useful for the detection of the *Pl6* gene.

DNA polymorphism, which coincided with disease resistance, was revealed with one RAPD (UBC 119 fragment 900-1000 bp) and one SSR primer (ORS37 fragment 600-700 bp). Amplified fragments segregated in the same way, i.e., they appeared in 50% of the resistant genotypes. The non-expecting SSR fragment was purified, cloned and sequenced. The results indicated that this fragment is not a part of a coding sequence. Specific primers for the amplification of this fragment have been designed and the investigation of the inheritance of this SCAR marker is under way. None of the applied markers appeared in all resistant genotypes.

In order to select lines for making crosses for use in further investigation, the obtained results were also used for the calculation of genetic distances between genotypes (simple matching coefficient) and the construction of a dendrogram (UPGMA method).

Key words: **sunflower, downy mildew, RAPD, SSR, DNA polymorphism, cluster analysis**

INTRODUCTION

Downy mildew severely affects sunflower yield. The facts that new races of *Plasmopara halstedii* (Farl.) Berl and de Toni emerge continuously and that the resist-

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ance to chemical control has appeared recently (Gulya *et al*., 1999) impose the need for developing tools for marker-assisted selection in order to develop disease resistant lines more efficiently.

Several genes are supposed to confer resistance to one or more races of *P. halstedii*. Most of these were reviewed by Slabaugh *et al*. (2003). Among them, *Pl1* and *Pl2* confer resistance to race 1 (virulence phenotype 100), and races 1 and 2 (virulence phenotype 300), respectively. *Pl6*, which was introgressed from *H. annuus*, confers resistance to races 1, 2, 3 and 4 (virulence phenotype 730). Bouzidi *et al*. (2002) suggest that *Pl6* locus contains at least 11 tightly linked genes. They have designed specific primers, which amplify 13 markers covering a genetic distance of about 3 cM centered on the *Pl6* locus. Sequence analysis indicated that *Pl6* contains conserved genes of the TIR-NBS-LRR class of plant resistance genes.

It was stated earlier that race 4 is predominant in Yugoslavia (Maširević, 1998). As in most commercial sunflower breeding programs, the main goal of the NS breeding program is the development of disease resistant lines, particularly to downy mildew. The aim of this investigation was to introduce the resistance to downy mildew in inbred lines with good combining abilities. Here we have focused on two lines: Ha-26 and Ha-19, parental components of mid-early and early hybrids, respectively (Škorić *et al*., 2000). Both lines were crossed with line JM-8, a source of *Pl6* gene. The progeny was subjected to several backcrosses with recurrent parental lines. Ha-26 was also improved by introducing resistance to *Plasmopara halstedii* from Ha-335, another source of *Pl6* (Miller and Gulya, 1988, 1991), noted as Ha-26(R). Screening and selection of resistant individuals after crossings were performed by the whole seedling immersion method. Beside the described material, several inbred lines from the NS breeding program and seven differential lines for downy mildew were also examined.

The same material was screened with published markers for plant resistance genes *Pl6* (Bouzidi *et al*., 2002), *Pl2* (Brahm *et al*., 2000), *Or5* (Lu *et al*., 2000) as well as with several RAPD and SSR markers.

MATERIALS AND METHODS

Twenty sunflower inbred lines were investigated; (S=sensitive; R=resistant to downy mildew race 730):

Two nearly isogenic lines: Ha-26(S) and Ha-26(R), lines with high general combining ability.

Sunflower lines from the NS breeding program: *cms*-1-90 (S); Ha-98N (S); OCMS-26 B (S); Ha-74 (S); Ha-19 (S); G16 (R)**; JM-8 (R); *cms*-3-8 (R); G12 (R)**; G10 (R)*; G11 (R)*

*inbred lines descending from the cross (Ha-26 × JM-8A) × Ha-26.

** inbred lines descending from the cross (Ha-19A × JM-8A) × Ha-19A.

Differential set of inbred lines for downy mildew: Rha-265 (USDA)(S); Rha-274 (USDA) (S); PMI3 (INRA) (S); 803-1 (IFVC)(S); PM-17 (USDA) (R); QHP1 (INRA) (R); Ha-335 (USDA) (R).

The analysis of resistance to *Plasmopara halstedii*, race 4, most abundant in Yugoslavia, was performed by the whole seedling immersion method (Miller and Gulya, 1991). For each genotype DNA was isolated from frozen leaves (Dellaportha *et al.*, 1983) sampled from 10 plants. RAPD analysis was performed as in Williams *et al.* (1990). SSR's were analyzed with primers synthesized according to the sequences obtained from Prof. Knapp, Oregon State University, USA. The primer for a SCAR marker connected to *Pl2* locus published by Brahm *et al.* (2000) was also used in this investigation. PCR conditions for SSR's and SCAR were as in Tang and Knapp (2001). RAPD markers were separated on 2% agarose gels. SSR's and SCAR markers were separated on 3% Metaphore agarose and 6% sequencing gels.

The data obtained with the applied primers was also used for the investigation of DNA polymorphism of the examined genotypes. Screening for presence/absence of polymorphic fragments was used for the calculation of simple matching coefficient (SM) between all pairs of genotypes. For each comparison SM was calculated as SM=(a+d)/n; where a=the number of bands in common to both accessions ("+ +" matches), d=the number of missing bands in common to both accessions ("– –" matches) and n=total number of bands (includes "+ +", "– –", "– +" and "+ –") (Staub *et al.*, 2000). The similarity measurements were converted to Genetic distance (GD) measurements as (1-SM) × 100 (Spooner *et al.*, 1996). The pairwise distance matrix was used for cluster analysis by UPGMA (Statistica for Windows, v.5.0, StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

PCR markers for resistance to downy mildew

It was stated earlier that in Serbia and Montenegro (Yugoslavia) the predominant *Plasmopara race* is race 4 (730) (Maširević, 1998), but the response of differential lines to the infection indicated that the isolate that we used in this investigation has changed (race 756; Table 1).

Table 1: Resistance of the examined differential lines to *Plasmopara halstedii* infection. Beside the original name of the differential line is the code used for the new nomenclature of *Plasmopara* races (Limpert and Muller, 1994). The differential line D7 was not available in this investigation

Differential line	Ha-26	Rha-265	Rha-274	PMI 3	PM-17	803-1	HAR-4	QHP1	Ha-335
	D1	D2	D3	D4	D5	D6	D7	D8	D9
resistance	-	-	-	-	+	-	/	-	-
race	1	2	4	1	0	4-	/	0	0
race		7			5			0	

As the amplification pattern obtained with primer HaP1 was different from the one obtained by Bouzidi *et al*. (2002), the marker Ha-NBS 2/R could not be identified. Conversely, the amplification patterns obtained with the primers HaP2 and HaP3 mainly resembled the published ones. The markers Ha-NBS 7/R, Ha-NBS 8/R, Ha-NBS 9/R were present in genotypes Ha-26(R), Ha-335, JM-8, G12, G10, G11. The DNA of inbred line Rha-274 contained only the marker Ha-NBS 7/R (results not shown). Fragments obtained in the reaction with the primer HaP3 and DNA extracts of the examined sunflower genotypes are presented in Figure 1. DNAs of the genotypes which were sensitive/resistant to downy mildew infection were applied on lanes 1-10, and 11-19 respectively. The markers Ha-NBS 11/R, Ha-NBS 13/R and Ha-NBS 14/R were present in the genotypes Ha-26(R), Ha-335, JM-8, G12, G10 and G11.

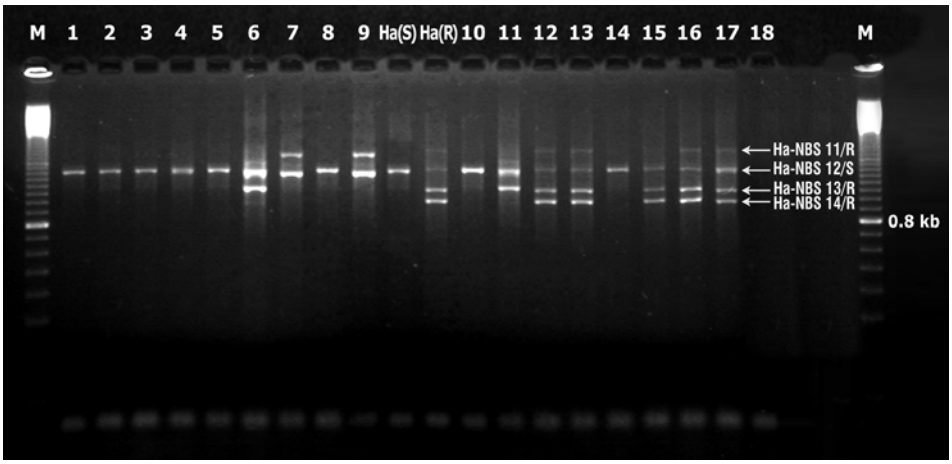

*Figure 1: PCR fragments obtained with primer HaP3, which covers about 3cM of Pl6 locus, and DNAs of the examined genotypes. Gel positions resemble the profiles obtained with DNAs from the following genotypes: 1 cms-1-90; 2 Ha-98N; 3 OCMS-26; 4 Ha-74; 5 Ha-19; 6 Rha-265; 7 Rha-274; 8 PMI 3; 9 803-1; Ha-26(S); Ha-26(R); 10 PM-17; 11 QHP1; 12 Ha-335; 13 JM-8; 14 cms-3-8; 15 *G12; 16 *G10; 17 *G11; 18 negative control, M=100bp ladder. The arrows indicate the position of markers.*

A SCAR marker for the *Pl2* gene, published by Brahm *et al*. (2000) was also tested. We observed the difference in the intensity of the band ~ 1000bp long, which was strongly amplified in the differential lines Rha-274 (D3), the source of the *Pl2* gene, and in 803-1 (D6) (results not presented).

Ha-26(S) and Ha-26(R) were screened by 50 RAPD primers (UBC, Amersham Pharmacia Biotech). The primer UBC 119 revealed reproducible polymorphism, a fragment 900-1000 bp long, which was detected in Ha-26(R), JM-8, G-10 and G11 (Figure 2).

Fifteen SSR primers were also screened. PCR with SSR primer ORS 37 resulted with an unexpected polymorphic fragment of 600-700 bp (Figure 3). No

further polymorphism could be detected after separation on 6% sequencing gel. The polymorphic SSR segregated in the same way as the RAPD marker, i.e., it was detected in Ha-26(R), JM-8, G-10 and G11 (Figure 3).

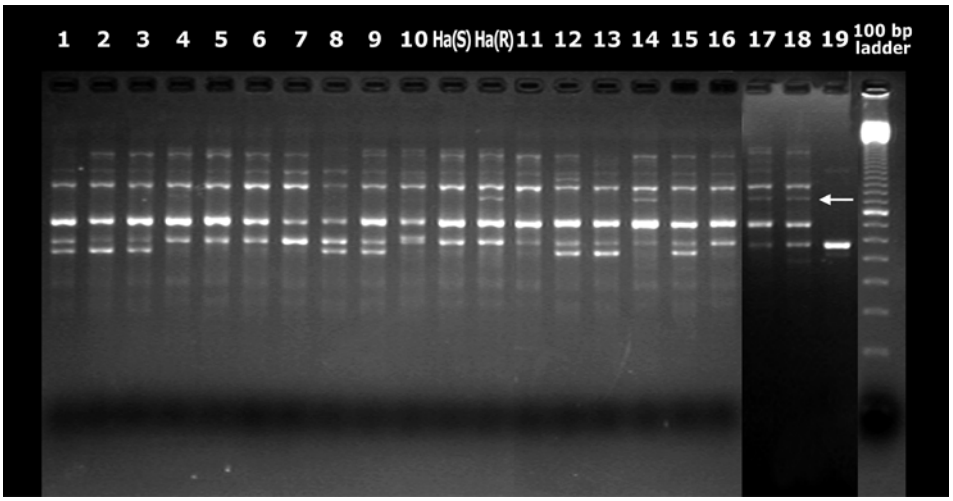

*Figure 2: The amplification of RAPD fragments obtained with primer UBC 119 and DNAs from the following genotypes: 1 cms-1-90; 2 Ha-98N; 3 OCMS-26 ; 4 Ha-74; 5 Ha-19; 6 G16; 7 Rha-265; 8 Rha-274; 9 PMI 3; 10 803-1; Ha-26(S); Ha-26(R); 11 PM-17; 12 QHP1; 13 Ha-335; 14 JM-8; 15 cms-3-8; 16 *G12; 17 *G10; 18 *G11; 19 negative control, M=100bp ladder. Fragment from 900-1000 bp, which was detected in resistant genotypes Ha-26(R), JM-8A, G10 and G11 is marked with an arrow.*

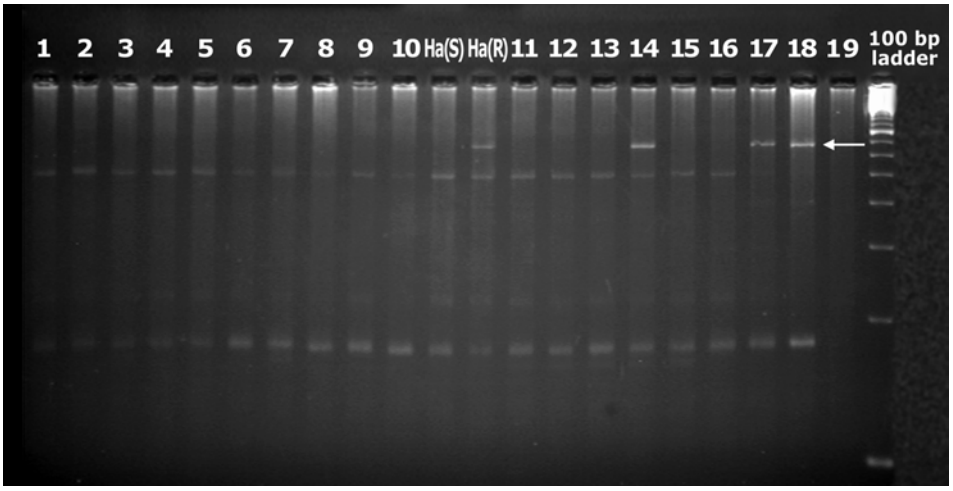

Figure 3: The amplification of SSR fragments with primer ORS 37 and DNA of the examined genotypes (the disposition is the same as in Figure 1). Marker (600-700 bp) is marked with an arrow. Fragments are separated on 2% agarose.

Both RAPD and SSR markers appeared in the same four out of the six *Pl6*-positive genotypes. In order to characterize this SSR marker, the fragment was cut

from the four lanes of the gel, purified and cloned by the use of the vector pCR®2.1-TOPO® and chemically competent *E. coli* cells. After DNA isolation from white colonies and digestion with EcoR I, the presence of the cloned fragment was confirmed in most samples (Figure 4).

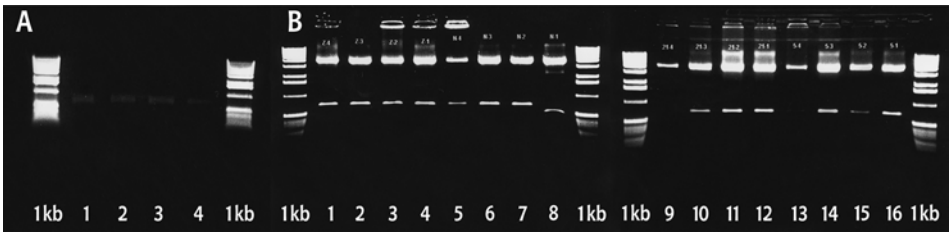

Figure 4: ***A*** *SSR marker, cut and purified from four samples on the gel 1. Ha-26(R) infected, 2. Ha-26(R) uninfected, 3. G10 and 4. JM-8.*
B *Fragment was cloned (pCR®2.1-TOPO®), DNA was isolated from white colonies and digested with EcoRI, and the presence of the cloned fragment was confirmed in most of the samples from different sources: Ha-26(R) infected (positions 1-4), Ha-26(R) uninfected (positions 5-8), G10 (positions 9-12) i JM-8 (positions 13-16).*

The sequencing of the cloned fragments indicates that it was not a part of the coding sequence. A specific primer designed to amplify this fragment will be used in further investigation.

Our results indicate first that the applied isolate of *Plasmopara halstedii* might have changed, and that an investigation of race composition should be performed in more detail. Second, the presence of the markers Ha-NBS 7/R, Ha-NBS 8/R and Ha-NBS 9/R in *Pl6* donor lines (Ha-335, JM-8) and in resistant progeny [Ha-26(R), G12, G10, G11] confirms that HAP3 could be useful for the detection of the *Pl6* gene.

Genetic distance among the examined sunflower genotypes

The results of the screening of all investigated primers were used for the investigation of DNA polymorphism among the 18 sunflower inbred lines. Based on the presence/absence of 48 polymorphic markers, SM coefficient and genetic distance between 18 pairs of inbreds were determined, as explained previously in section Material and Method. Genetic distance ranged from 7 to 75%. The lowest GD was between Ha-26(S) and Ha-26(R), the highest between JM-8 and Rha-274 (Table 2). The observed range of GDs was similar to that in Berry *et al.* (1994), who used 57 RFLP probes to determine the interrelationships between a diverse set of 24 sunflower inbred lines. Genetic distances were used for cluster analysis by UPGMA method (Statistica) and the dendrogram was constructed (Figure 5). Inbred lines were separated in two major groups, named A and B. Group A consisted of three differential lines for downy mildew which are restorer lines. The separation of restorers and maintainers was shown earlier by the use of different marker systems such as RFLP (Berry *et al.*, 1994, Gentzbittel *et al.*, 1994) and AFLP (Hongtrakul *et al.*, 1997). Group B consisted of two subgroups: subgroup B1 with lines from a *Pl6*

Table 2: Genetic distances between all possible pairs of lines calculated as dissimilarities

Genotyp	Dissimilarity (1-SM)																	
	cms-1-90-A	Ha-98 NA	Ha-26 B	Ha-74 A	Ha-19 A	Rha- 265 (*Pl1*)	Rha-274 (*Pl2*)	Pml 3	803-1	Ha-26(S)	Ha-26(R)	PMI-17	QH P1	Ha 335 (*Pl6*)	JM-8A	*cms* 3-8 A	G12	G10
cms-1-90-A	0	37	22	30	33	40	54	37	41	41	51	37	33	50	50	37	50	46
Ha-98 NA	37	0	30	26	28	52	50	37	50	41	49	24	44	54	46	28	50	60
Ha-26 B	22	30	0	26	22	40	54	26	52	41	49	33	30	46	46	15	37	43
Ha-74 A	30	26	26	0	20	45	52	28	41	20	28	33	42	41	46	33	41	49
Ha-19 A	33	28	22	20	0	45	54	38	54	37	42	44	42	46	46	29	21	46
Rha- 265(*Pl1*)	40	52	40	45	45	0	34	45	39	45	50	57	31	59	59	39	59	38
Rha-274 (*Pl2*)	54	50	54	52	54	34	0	46	17	52	56	63	56	67	75	46	58	43
Pml 3	37	37	26	28	38	45	46	0	46	33	33	29	30	50	58	29	46	43
803-1	41	50	52	41	54	39	17	46	0	48	51	56	51	67	67	42	54	34
Ha-26(S)	41	41	41	20	37	45	52	33	48	0	7	37	35	39	43	48	39	37
Ha-26(R)	51	49	49	28	42	50	56	33	51	7	0	47	42	33	33	56	35	43
PMI-17	37	24	33	33	44	57	63	29	56	37	47	0	40	52	42	33	54	49
QH P1	33	44	30	42	42	31	56	30	51	35	42	40	0	40	47	33	49	43
Ha 335 (*Pl6*)	50	54	46	41	46	59	67	50	67	39	33	52	40	0	17	56	38	40
JM-8A	50	46	46	46	46	59	75	58	67	43	33	42	47	17	0	56	33	40
cms 3-8 A	37	28	15	33	29	39	46	29	42	48	56	33	33	56	56	0	42	49
G12	50	50	37	41	21	59	58	46	54	39	35	54	49	38	33	42	0	26
G10	46	60	43	49	46	38	43	43	34	37	43	49	43	40	40	49	26	0

introduction program and the rest of NS and differential lines separated in sub-group B2.

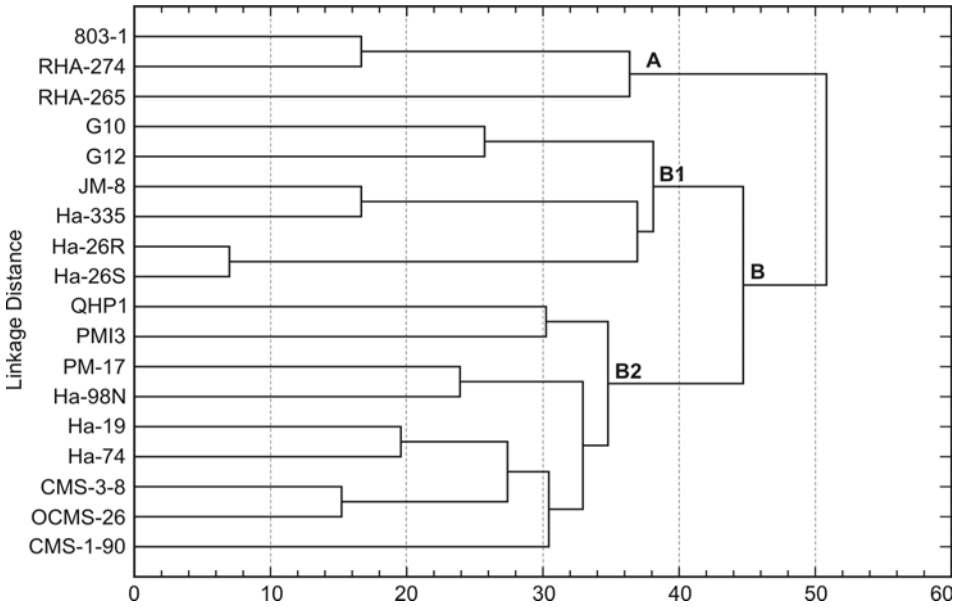

Figure 5: Dendrogram showing the similarities among the 18 examined sunflower inbred lines. Clusters were identified by UPGMA analysis (Statistica for Windows, v.5.0, StatSoft, Inc., Tulsa, OK, USA).

Recent studies on the inheritance and allelic relationships of resistance genes suggest digenic inheritance of resistance to races 1 (100) and 2 (200) (Rahim *et al.*, 2002) and two dominant resistance genes to race 330, with other types of regulation such as complementary and epistatic relationships which also occur (Molinero-Ruiz *et al.*, 2002). The low GD between the resistant Ha-26 and the sensitive Ha-26, found in our paper, indicates that these lines might provide a good material for the molecular study of allelic test for the *Pl6* gene. Namely, although eleven resistance genes to downy mildew have been identified, the allelic relationships among these genes are not clear.

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USO DE LOS MARCADORES PCR EN LA INVESTIGACIÓN DE RESISTENCIA A TIZÓN DE GENOTIPOS DEL PROGRAMA DE MEJORAMIENTO DE NS

RESUMEN

El objetivo de este trabajo ha sido la aplicación de los marcadores PCR publicados, tanto como el desarrollo de nuevos marcadores, en la investigación de la resistencia de girasol a tizón. Se investigaron veinte líneas consanguíneas de girasol. La resistencia de las plantas a tizón fue determinada por el método de hundimiento de la plántula entera. Tras el aislamiento del ADN genómico del primer par de hojas, el polimorfismo fue investigado con la aplicación de RAPD, SSR y varios marcadores para la resistencia a enfermedades.

La presencia de los marcadores Ha-NBS 7/R, Ha-NBS 8/R y Ha-NBS 9/R, en donadores del gen *Pl6* (Ha-335, JM-8) y en las líneas resistentes (Ha-26, G12, G10, G11), originadas por cruzamiento de las líneas de donador y las líneas irresistentes, confirma que aplicando el primer HAP3, se puede detectar con éxito la presencia del gen *Pl6*.

El polimorfismo de ADN, que está en armonía con la característica de ser resistente a tizón, fue detectado aplicando un primer RAPD (fragmento UBC 119; 900-1000 bp) y un primer SSR (fragmento ORS37; 600-700 bp). Ambos fragmentos multiplicados se presentaron en 50% de genotipos resistentes. El fragmento SSR, que no era esperado, es depurado, clonado y secuencionado. Los resultados indican que este fragmento no es parte de la secuencia codificadora. Fue disenado un primer específico para la multiplicación de este fragmento, y la investigación de la herencia de este marcador SCAR está en curso. Ninguno de los marcadores empleados se ha presentado en todos los genotipos resistibles.

Los resultados obtenidos también fueron empleados para calcular las distancias genéticas entre genotipos (coeficiente "simple matching") y para la construcción de dendrograma (método UPGMA), para seleccionar líneas para el cruzamiento, las cuales se van a utilizar en las futuras investigaciones.

USAGE DES MARQUEURS PCR DANS L'ÉVALUATION DE RÉSISTANCE DE GÉNOTYPES À LA ROUILLE D'APRÈS LE PROGRAMME DE PLANTES CULTIVÉES DE NOVI SAD

RÉSUMÉ

Le but de cette étude est l'application des marqueurs PCR déjà publiés et le développement des nouveaux dans l'évaluation de résistance du tournesol à la rouille. Vingt lignes cultivées de tournesol ont été examinées. La résistance de plantes à la rouille est déterminée par la méthode d'immersion totale de semailles. Après l'isolation de A.D.N. génomique de première paire de feuilles, le polymorphisme a été examiné par application des marqueurs RAPD, SSR, et quelques marqueurs déjà publiés pour la résistance aux maladies.

La présence des marqueurs Ha-NBS 7/R, Ha-NBS 8/R et Ha-NBS 9/R, dans les donneurs de gène P16 (Ha-335, JM-8) et les lignes résistantes (Ha-26, G12, G10, G11), obtenues par croisement de lignes donneurs et lignes irrésistibles, confirme que l'application de "primer" HAP3 peut être efficace pour détecter le gène *P16*.

Le polymorphisme A.D.N qui est en corrélation avec le trait de résistance à la rouille, est dépisté par l'application d'un RAPD "primer" (fragment UBC 119; 900-1000 bp) et d'un SSR "primer" (fragment ORS37; 900-700 bp). Les deux fragments multipliés se sont avérés chez 50% de génotypes résistants. Le fragment SSR qui n'a pas été attendu est purifié, cloné et disposé en séquence. Les résultats indiquent que ce segment ne fait pas partie d'une séquence codée. Pour multiplier ce fragment un "primer" spécifique a été créé, et les recherches d'hérédité du marqueur SCAR sont en cours. Aucun marqueur appliqué ne s'est manifesté dans tous les génotypes résistants.

Les résultats obtenus sont aussi utilisés pour calculer la distance génétique entre les génotypes ("simple matching" coefficient) et construire de "dendrogramme" (la méthode UPGMA) afin de sélectionner les lignes de croisement qui seront mises en œuvre dans les recherches futures.