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AFLP/SSR mapping of resistance genes to *Alectra vogelii* in cowpea (*Vigna unguiculata* L. WALP)

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Abstract

The parasite weed *Alectra vogelii* (Benth) causes significant yield reduction of cowpea in Africa. To find and map the resistance gene to *A. vogelii* in cowpea, a F_2 population from a cross involving a resistant parent IT81D-994 and a susceptible TVX3236 was screened. Amplified fragment length polymorphism (AFLP) in combination with Single Sequence Repeat (SSR) analysis was used to identify markers that may be linked to the gene Rav_3 conferring resistance to *A. vogelii* in the cowpea cultivar IT81D-994. The analysis of F_2 individuals scored for resistance showed that a single dominant gene conditioned *A. vogelii* resistance in IT81D-994 with a probability of 30 to 50%. Sixty-six AFLP/SSR markers were identified. Using MAPMAKER, four linkage groups were found. The first group showed 33 markers linked to the susceptible gene. The closest identified marker was 21.4 centimorgans away from the susceptible gene. The primer combination that showed the closest linkage was 809ACG8 (E-ACG 8 + UBC-809). The second group indicated 4 markers linked together while the third and the fourth groups showed 2 markers linked together respectively. No markers were found linked to the resistance gene Rav_3 conferring the resistance in the cultivated cowpea cultivar IT81D-994.

Key words: Parasite, weeds, legumes, molecular markers

Résumé

Cartographie AFLP/SSR de genes de résistance à Alectra vogelii *chez le niébé (*Vigna unguiculata *L. Walp)*

La plante parasite Alectra vogelii *(Benth) provoque une réduction significative du rendement de niébé en Afrique. Pour repérer et cartographier le gène de résistance à* A. vogelii*, une population F2 issue d'un croisement impliquant le cultivar résistant IT81D-994 et le cultivar sensible TVX3236 a été criblée. La technique Amplified fragment length polymorphism (AFLP) en combinaison avec des Séquences Simples Répétés (SSR) a été utilisée pour identifier les marqueurs qui seraient liés au gène* Rav_3 *qui confère la résistance à* A. vogelii *chez le cultivar de niébé IT81D-994. L'analyse des individus F2 montrant une résistance indique qu'un seul gène dominant conditionne la résistance à* A. vogelii *chez IT81D-994 avec une probabilité de 30 à 50 %. Soixante six marqueurs AFLP/SSR ont été identifiés. A l'aide de MAPMAKER, 4 groupes de liaison ont été trouvés. Le premier groupe montre 33 marqueurs liés au gène sensible. Le marqueur le plus proche identifié est à 21.4 centimorgans du gène sensible. La combinaison d'amorces ayant des liaisons les plus proches était 809ACG8 (E-ACG 8 + UBC-809). Le second groupe montre 4 marqueurs liés ensemble tandis que le troisième et le quatrième groupe montrent 2 marqueurs liés entre eux respectivement. Aucun marqueur n'a été trouvé lié au gène* Rav_3 *qui confère la résistance au cultivar de niébé cultivé IT81D-994.*

***Mots clés** : Parasite, herbes, légumineuses, marqueurs moléculaires*

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is not only an important pulse crop in many areas of the world, but also the most important food legume in West and Central Africa (Myers *et al.*, 1996). This region represents over 78% of the 12.5 million ha of cowpea grown worldwide (Cissé & Hall, 2003). One of its most serious production constraints is *Alectra vogelii*. However, information on cowpea yield losses resulting from *A. vogelii* infestation is scarce (Rambakudzibga *et al.*, 2002). Yield loss resulting from this parasite ranges from 41% (Lagoke *et al.*, 1997) to total crop loss of the highly susceptible cultivar Black-eye (Riches, 1990).

A. vogelii can produce as many as 600,000 seeds per plant (SP-IPM, 2003). The large numbers of seed produced makes difficult the mechanical control of the parasite. Furthermore, up to 75% of the crop damage is done before seeds emerge from the ground (Singh & Emechebe, 1991). Thus, the identification of *Alectra*-resistant genes and their incorporation into breeding programs could be the most successful strategy to combat the parasite. Identification of resistant genotypes is essential in the process of developing resistant varieties. The uses of molecular tools can serve to identify desirable genotypes with high precision. Molecular markers for different traits have been identified (Myers *et al.*, 1996; Ouédraogo *et al.*, 2001). However, there are no markers for *Alectra* resistance in cowpea. Tagging *Alectra*-resistance gene in cowpea by using molecular markers is the first step towards cloning these genes. Up to now, such markers have been used to detect resistant genes in cowpea. For instance, One RFLP marker, bgD9b, was found to be tightly linked to the aphid resistance gene *Rac1* (Myers *et al.*, 1996). Ouédraogo *et al.*, (2001) identified AFLP markers which are tightly linked to different race-specific *Striga gesneroides* resistance genes in cowpea. The AFLP technique is able to detect a large number of polymorphic bands in a single lane rather than high levels of polymorphism at each locus such as the case for Single Sequence Repeat (SSR) methods (Garcia *et al.*, 2004). The AFLP technique has shown to be more efficient in detecting polymorphism than either RFLP or RAPD (Garcia-Mas *et al.*, 2000). The SSR technique is also characterized by its higher levels of detection of polymorphism (Akkaya *et al.*, 1995; Grivet & Noyer, 1999). Thus, the combination of both AFLP and SSR techniques offers a good opportunity to increase the levels of polymorphism that can be revealed.

This study aims at selecting SSR primers that fit with the AFLP technique for the screening of a cowpea population and to identify markers linked to *A. vogelii* resistance genes in cowpea.

2. Materials and methods

2.1 Plant material

A cross was carried out between IT81D-994 (*A. vogelii* resistant) and TVX3236 (*A. vogelii* susceptible). The DNA isolated following the CTAB method described by Van Gysel *et al.* (1998) for 52 F_2 individuals resulting from this cross, including the parents were provided for AFLP/SSR analysis by the Department of Biology of the University of Virginia (USA) following screening and phenotyping. The phenotypic score data for the F_2 was subjected to a Chi-square analysis and the hypothesis of 3:1 resistant to susceptible was tested.

2.2 AFLP/SSR primers

Fifteen *Eco*R1 primers were used in combination with 26 SSR primers. The *Eco*R1 primers were designated by E and were represented by three selective nucleotides. The E primers were either labeled with IRDye™ 700 or IRDye™ 800 at the 5'end. The dye-labeled E-primer and gel matrix were purchased from LI-COR® Biosciences (Lincoln, Nebraska). E-primers used were E-ATG 7, E-AGG 7, E-ACT 7, E-ACA 7, E-AAG 7, E-AGA 7, E-AAC 7, E-ACC 7, E-ATC 7, E-GGC 7, E-ACG 8, E-GCG 8, E-AGC 8, E-AAG 8, and E-GCC 8**.**

The SSR primers (Table1) were synthesized by the Biotechnology Laboratory of British Columbia University (Vancouver, Canada). The RETRO 1 primer was developed by the Department of Biology of the University of Virginia.

Table 1: SSR primers and RETRO 1 primers, sequences and base patterns used for the analysis

Primer names	Sequences (3' > 5')	Base patterns (3' > 5')
UBC-801	ATA TAT ATA TAT ATA TT	$(AT)_8$ T
UBC-802	ATA TAT ATA TAT ATA TG	$(AT)_8$ G
UBC-803	ATA TAT ATA TAT ATA TC	$(AT)_8$ C
UBC-804	TAT ATA TAT ATA TAT AA	$(TA)_8$ A
UBC-805	TAT ATA TAT ATA TAT AC	$(TA)_8$ C
UBC-806	TAT ATA TAT ATA TAT AG	$(TA)_8$ G
UBC-807	AGA GAG AGA GAGAGA GT	$(AG)_8$ T
UBC-808	AGA GAG AGA GAGAGA GC	$(AG)_8$ C
UBC-809	AGA GAG AGA GAGAGA GG	$(AG)_8$ G
UBC-810	GAG AGA GAG AGA GAG AT	$(GA)_8$ T
UBC-811	GAG AGA GAG AGA GAG AC	$(AG)_8$ C
UBC-812	GAG AGA GAG AGA GAG AA	$(AG)_8$ A
UBC-813	CTC TCT CTC TCT CTC TT	$(CT)_8$ T
UBC-814	CTC TCT CTC TCT CTC TA	$(CT)_8$ A
UBC-815	CTC TCT CTC TCT CTC TG	$(CT)_8$ G
UBC-816	CAC ACA CAC ACA CAC AT	$(CA)_8$ T
UBC-817	CAC ACA CAC ACA CAC AA	$(CA)_8$ A
UBC-822	TCT CTC TCT CTC TCT CA	$(TC)_8$ A
UBC-832	ATA TAT ATA TAT ATA TYC**	$(AT)_8$ YC**
UBC-833	ATA TAT ATA TAT ATA TYG**	$(AT)_8$ YG**
UBC-835	AGA GAG AGA GAG AGA GYC**	$(AG)_8$ YC**
UBC-837	TAT ATA TAT ATA TAT ART**	$(TA)_8$ RT**
UBC-840	GAG AGA GAG AGA GAG YT**	$(GA)_8$ YT**
UBC-844	CTC TCT CTC TCT CTC TRC**	$(CT)_8$ RC**
UBC-850	GTG TGT GTG TGT GTG TYC**	$(GT)_8$ YC**
RETRO 1*	TCC AAC GAC GTC GCA AAA A	-

** RETRO 1 is not a SSR primer but is a primer derived from cowpea.*
***Type of degenerate nucleotide: Y = pYrimidine (C, T); R = puRine (A, G).*

2.3. AFLP/SSR analysis

AFLP analysis was conducted as described by Vos *et al.* (1995) with the following modifications. The pre-amplification reaction was performed with 2 µl of template DNA using 10 µl of a mix of a diluted 1:100, *Eco*R1 + 1 primer and the *Mse*1 + 1 primer and, 1 µl of dNTPs (10 mM) and 0.05µl of *Taq* polymerase in a total volume of 25 µl . The cycle profile was 24 cycles of 30 s at 94 ˚C, 60 s at 56 ˚C and 60 s at 72 ˚C. Amplification products were either diluted 1: 8 or 1:10 in autoclaved water.

Minor modification was done in the selective amplification. The SSR primers were used instead of using *Mse*1 + 3. The amplification mixture contained 2 µl of template DNA from the diluted first pre-amplification product, the same amount of *Taq* polymerase was used as in the first reaction, 1 µl of dNTPs (1 mM), *Eco*R1 + 3, 1 µl of diluted 1 : 6 SSR primer in a 11.5 µl total reaction volume. A "touch-down" PCR was performed in (Eppendorf) Mastercycler. After 1 cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C, the temperature was lowered 0.7 °C in each cycle for 11 cycles and was followed by 22 cycles of 30 s at 94 °C, 60 s at 56 °C and 60 s at 72 °C. The reaction products were analyzed on KB^{plus} 6.5% denaturing polyacrylamide gels (Ready-to-use Acrylamide Gel Matrix containing urea and TBE). LI-COR DNA Analyzer was used for the gel electrophoresis. LI-COR 25 cm plates and 0.25 mm thick spacers and rectangular combs (63 wells) were used. After a 30 min pre-run, the wells were flushed to remove urea precipitate and 1.0 µl of each denatured sample was loaded using the 8-channel Hamilton syringe.

Binary scoring with 1 for a band presence or 0 for a band absence was performed. Each band was considered as a locus. Linkage analysis of the set of markers was conducted by using MAPMAKER 3.0b (Lander *et al.*, 1987; Lincoln & Lander, 1993).

3. Results and discussion

3.1 Inheritance of Alectra resistance

The results of F_2 individuals scored for Alectra resistance is shown in (Table 2). Thirty- six F_2 individuals were resistant and sixteen individuals were susceptible. The probability for the expected 3 resistant: 1 susceptible segregation in F_2 was 30 – 50% (Table 2). The Chi-squared analysis confirmed that the 52 F_2 populations were segregating for *Alectra* resistance as expected (3 resistant: 1 susceptible). Indeed, Atokple *et al.* (1995) found that a single dominant gene conditioned *Alectra* resistance in IT81D-994.

3.2 Selection of SSR markers

The result of the selected primers is shown in (Table 3). Fifty-two AFLP/SSR primers combinations allowed the selection of 8 SSR primers and the RETRO 1 primer. The 9 selected primers showed good bands with both *EcoR1* 700 and *EcoR1* 800 primers.

Table 2: Segregation for monogenic *Alectra vogelii* resistance (3:1) in F_2 population

	F_2 population reaction	
	Resistant	Susceptible
Observed	36	16
Expected	39	13
Chi-square	0.92	-
$P_{1\ df}$	0.30 - 0.50	-

Table 3: Sequences of SSR primers selected with AFLP technique

Primer names	Sequences (3' > 5')	Base patterns (3' > 5')
UBC-814	CTC TCT CTC TCT CTC TA	$(CT)_8$ A
UBC-815	CTC TCT CTC TCT CTC TG	$(CT)_8$ G
UBC-816	CAC ACA CAC ACA CAC AT	$(CA)_8$ T
UBC-817	CAC ACA CAC ACA CAC AA	$(CA)_8$ A
UBC-835	AGA GAG AGA GAG AGA GYC**	$(AG)_8$ YC**
UBC-840	GAG AGA GAG AGA GAG YT**	$(GA)_8$ YT**
UBC-844	CTC TCT CTC TCT CTC TRC**	$(CT)_8$ RC**
UBC-850	GTG TGT GTG TGT GTG TYC**	$(GT)_8$ YC**

***type of degenerate nucleotide: Y = pYrimidine (C, T); R = puRine (A, G).*

3.3 AFLP/SSR primers for screening F_2 population

With the nine suitable primers, 36 reactions were run, corresponding to 72 AFLP/SSR primer combinations (Table 4). The best *Eco*R1 primers, which can be used in combination with SSR markers, were E-ACA7, E-ACC7, E-AGA7, E-ACG8 and E-GCC8. These primers showed more polymorphic bands than the others. The total number of polymorphic profiles was 52 while 26 monomorphic gels were found (Table 5). The monomorphism observed in some gels and revealed by a number of primer combinations (Table 5) is not the unique fact of using AFLP/SSR primers. Indeed, Barbosa *et al.* (2003) using the classic AFLP technique, with radioactivity, have found 774 polymorphic bands and 326 monomorphic bands.

Table 4: AFLP/SSR primer combinations used for screening of the 52 F_2 populations for *Alectra* resistance gene

SSR primers	AFLP primers														
	***Eco*R1 700**									**_Eco_R1 800**					
	ATG	AGG	ACT	ACA	AAG	AGA	AAC	ACC	ATC	GGC	ACG	GCG	AGC	AAG	GCC
814	b**	c	d					e	a		b**	c**	a	d	e
815			b	a		c		d		e	b	c***	d	a	e
816			e				ac	d		b	e	d	a	c	b
817				e		a	b	c	d		c	d**	e	b	a
835			d	b			a	c	e		e	c	b	d	a
840		a	e	d		c**		b			c**	e	a	d	b
844			b	c**		a		d	e**		a	b**	d	c**	e
850			a	e		f	b	c	d		d	e	ac	b	f
RETRO 1*			a	e		b	d	c			e	d	c	b	a

The letters indicated the duplex between an SSR primer and both EcoR1 700 and EcoR1 800.
** RETRO 1 is not a SSR primer but is a primer derived from cowpea.*
***The combination doesn't work.*
**** The combination doesn't work: No band was observed.*

Table 5: *Eco*R1 primers amplified either polymorphic or monomorphic in fragments with AFLP/SSR primer combinations

***Eco*R1 primers**	**Number of polymorphic profiles**	**Number of monomorphic profiles**
E-ACT7	4	2
E-ACA7	4	1
E-AAG7	0	2
E-AGA7	4	0
E-AAC7	4	2
E-ACC7	8	3
E-ATC7	2	2
E-GGC7	2	0
E-ACG8	7	2
E-GCG8	1	2
E-AGC8	6	3
E-AAG8	2	4
E-GCC8	8	3
Total	52	26

This AFLP modified technique gave promising results. It showed polymorphism within the *Alectra* F_2 individuals (Fig. 1a) like the classic AFLPs using no radioactivity (Fig. 1b). The tested AFLP/SSR technique has two advantages compared to the classic AFLP technique. Firstly, it is less laborious and time consuming than the former technique. Secondly, it avoids the used of a radioactive material. Therefore, it can be used in small income laboratories. However, it has a lower sensitivity in detecting polymorphism and in generating more bands.

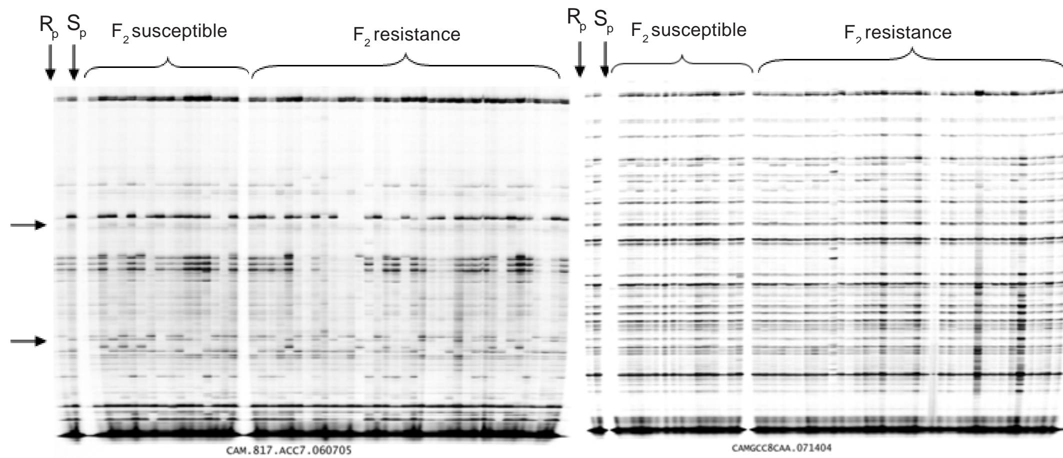

Figure 1a: AFLP/SSR profile generated from primers UBC-817 + E-ACC_{700} in *Alectra vogelii* F_2 populations. Rp and Sp indicate resistance and susceptible parents respectively. Horizontal arrows show polymorphic bands between parents and individuals.

Figure 1b: AFLP profile generated from primers E-ACC_{700} + M-CAA in *Alectra vogelii* F_2 populations (Skizim *et al.* 2004, unpublished) using a classic AFLP technique without radioactivity. Rp and Sp indicate resistance and susceptible parents respectively. Horizontal arrows show polymorphic bands between parents and individuals.

3.4 Identification of AFLP/SSR markers

Sixty-six AFLP/SSR markers were identified. Using MAPMAKER 3.0b, four linkage groups were found (Table 6). The first group showed 33 markers linked to the susceptible gene. The closest identified marker was 21.4 centimorgans away from the susceptible gene (Table 7). The primer combination that showed the closest linkage was 809ACG8 (E-ACG 8 + UBC-809). The second group indicated 4 markers linked together while the third and the fourth groups showed 2 markers linked together respectively.

No marker was found linked to the resistance gene Rav_3 conferring the resistance in the cultivated cowpea cultivar (IT81D-994), which was the resistant parent.

Table 6: Linkage Groups at min LOD 3.00, max Distance 50.0, using MAPMAKER 3.0b

Linkage Groups	AFLP/SSR markers linked to the susceptible gene
Group 1	5 6 7 8 9 10 11 12 13 16 17 18 19 20 21 22 25 33 34 35 36 37 39 41 42 43 44 49 51 55 57 61 66
Group 2	23 38 40 48
Group 3	27 64
Group 4	56 60

LOD: Log of the odds ratio. Each number corresponds to a locus (i.e. 13 corresponds to locus 13).

Table 7: Map of 6 markers showing the closest linkage to the susceptible gene, log-likelihood = - 110.55

AFLP/SSR markers linked to the susceptible gene	Distance (cM)
10. locus10	31.7
2. S_GENE	21.4
13. locus13	30.3
11. locus11	31.1
16. locus16	27.6
12. locus12	------
Total	142.1

cM : centimorgans.
The number 2 correspond to the susceptible gene (S_GENE)
Locus 13 is 21.4 cM away from the S_GENE. Locus 13 corresponds to the primer combination 809ACG8 (E-ACG 8 + UBC-809).

4. Conclusion

The modification of AFLP method by using SSR primers in the selective amplification reaction instead of using *Mse* 1 worked well. The *Alectra* resistance gene, Rav_3 was not linked with the AFLP/SSR markers used in this study. However, further studies are needed in order to find markers for *Alectra* resistance gene in cowpea with the AFLP/SSR markers. This technique is reproducible and can therefore be used in routine. It saves time and shows polymorphism between analyzed individuals.

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