Agricultural Science Research Journal Vol. 7(6): 190 – 197, June 2017
Available online at http://resjournals.com/journals/agricultural-science-research-journal.html
ISSN: 2026 –6073 ©2017 International Research Journals

Full Length Research Paper

Identification and Characterization of Potential SSR Markers Linked to *Striga gesneroides* (Willd.) Vatke Resistance Gene Race 2 in Cowpea in Mali

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Abstract

Cowpea [*Vigna unguiculata* (L.) Walp.], a major food legume grown in Mali, is an important dietary protein source. However, one of the major biotic constraints strongly limiting its production happens to be the SG2 strain of *Striga gesnerioides* (Willd.) Vatke. In order to identify SSR markers linked to resistance gene of the race 2 of *S. gesnerioides*, F2 populations resulting from the crossing between the variety IT97K-499-35 (Resistant parent) and the variety Amary Sho elites (Susceptible parent) and seeds of *S, gesnerioides* strain from Koporo were used. Thus, 100 individuals were characterized using four markers CP333/334, CP743/744, CP115/116 and MA 62 which were polymorphic out of the 159 markers used on the parents. This study revealed that only the dominant microsatellite marker MA62 (41.9 cM) slightly linked to the *Striga* resistance gene present in the cowpea variety IT97K-499-35. Linked to the gene by the 700 bp allele, the dominant marker MA62 consistently segregated with the *S. gesnerioides* Rsg2. The identification of the MA62 marker related to *Striga* resistance Race 2 in Mali could be opportunity for the national breeding programs to develop a marker-assisted selection (MAS) strategy for Race 2 of *S. gesnerioides.*

Key Words: Marker-assisted selection, microsatellites markers, Striga*, Vigna unguiculata,* Mali

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the most economically important indigenous African grain legumes with enriched proteins as source of food for both human and animal nourishment and a major crop in regional trade within West and Central Africa (Langyintuo *et al.,* 2003). The relatively high protein content of cowpea makes it an essential supplement to the diet of many Africans (Bressani, 1985) consuming high carbohydrates but low in protein cereals, root and tuber crops. Cowpea is being cultivated over an area of about 12.5 million hectares with an annual production of over 3 million tons world over (SINGH *et al.,* 1997). However, a major biological constraint to increase production in smallholder farms is the infection by the parasitic weed, *S. gesnerioides* (Ehlers and Hall, 1997). Cowpea yield losses associated with *S. gesnerioides* range from 83 to 100% (Cardwell and Lane, 1995).

Indeed, no single method is adequate to control the parasite. However, host plant resistance appears to have the potential to effectively and economically control the parasite since it is affordable to resource-poor farmers (Omoigui *et al.,* 2007). Over the past years, significant effort has been put into the identification of natural sources of genetic resistance within cowpea cultivars and to the selection and breeding of improved lines with resistance to *Striga* (Singh and Emechebe, 1997). Molecular markers for identification and selection of *Striga*-resistant genotypes have been developed for most

of the races of the parasite existing in West Africa. However, the differential virulence of races of *S. gesnerioides* on cowpea genotypes (Lane *et al.,* 1994) has serious implication to breeding and selection procedures. The overall efficiency and effectiveness of cowpea improvement programs can be enhanced by the application of advanced selection and breeding tools that employ molecular markers linked to traits of interest. The use of DNA marker systems, such as simple sequence repeat (SSRs) (Akkaya *et al.,* 1992) has contributed greatly to the development of genetic linkage maps for many important crop species, including cowpea (Fatokun *et al.,* 1997; Menendez *et al.,* 1997). Therefore, the focus of the current work was to identify and validate some SSR markers linked to *S. gesnerioides* race 2 in Mali.

Material and Methods

Plant Material

The plant material is constituted of two (2) parents IT97K-499-35 (resistant parent) developed by IITA and Amary Sho (susceptible parent), local variety and 100 F2 descendants resulting from their crossing. The seeds of *S. gesnerioides* from Koporo strain of Mali were used for the infestation.

Methods

Phenotyping of the F2 population

Phenotyping of population of this study has been done in the *Striga* room in the Laboratory of the University of Virginia (UVA), U.S.A in 2014.

Pot culture test for screening cowpea against *S. gesnerioides* infection

The experiment was set up at the *Striga* room in the biotechnology laboratory of the University of Virginia (UVA), U.S.A in 2014. Each pot (17 x 11 cm) was perforated, partially filled with sandy loam soil to 7.0 cm deep and inoculated with about 2 g seeds of *S. gesnerioides* from Koporo strain of Mali. The infested soil was watered for one week before planting. In June 5 2014, the pots were topped up with soil and two seeds of each cowpea F2 population derived from IT97K-499-35 (Resistant) × Amary Sho (Susceptible) were sown per pot. In all, 100 F2 population and their reactions to *S. gesnerioides* infection were phenotyped. The seedlings were thinned to one per pot. The soil was kept moist by watering regularly every two days or as and when necessary (Figure 1).

Figure 1: Cowpea F2 populations screening in the *Striga* room for phenotyping

Data collection:

The annotation scale used was as follows:

1. Resistant = 100% plants resistant; no *Striga* emergence on plot and no *Striga* symptom observed on plant.
2. Susceptible = *Striga* germinated with but no emergence
3. *Striga* emergence and plants show severe *Striga* symptoms,

Genotyping of F2 population

Molecular markers selection

Molecular characterization was done in Professor Timko's Laboratory of University of Virginia, U.S.A. in 2014. One hundred (100) plants of F2 segregating population developed from a cross between IT97-499-35 (Resistant parent) and Amary Sho (Susceptible parent) were used to characterize the association between markers and *Striga* resistance genes. The two parents are genetically and phenotypically contrasting. The data of area under *Striga* infestation were used to identify significant phenotype-marker associations for *Striga* in IT97K-499-35 and Amary Sho, resistant and susceptible parents respectively.

Table 1: Polymorphic marker used for F2 population amplification

N°	Markers	Sequence of forward_primer	Sequence of reverse_primer
1	MA 62	GTGGCCACATACATAGATCATAC	GCATTCTCTTGTCGATCTGAACC
2	CP 115/116	GGGAGTGCTCCGGAAAGT	TTCCCTATGAACTGGGAGATCTAT
3	CP 333/334	CAAAGGGTCATCAGGATTGG	TTTAAGCAGCCAAGCAGTTGT
4	CP 743/744	GAGATGCCTCCTCAGCACTC	TCTCACTCTCTCTAACCGACACA

DNA Isolation

Individual leaf samples were collected and the genomic DNA samples extracted from 14 days old cowpea leaves following the protocol described by Mace *et al.,* (2004) with minor modification. Harvested 100 to 200 mg (enough amounts) young leaf and put it in properly labeled 2 ml Eppendorf tube. To which added polyvinyl-polypyroid powder PVP-5% w/v (small amount) just before adding liquid nitrogen to protect the leaf from browning. Ground the leaf sample using tissue lyser until to get fine powder and put in properly labeled 2 ml tube. Put the tubs in liquid nitrogen until added CTAB buffer and also Pre-warmed CTAB (2% CTAB w/v, 100 mM Tris HCL pH 8.0, 20 mM EDTA pH 8.0 and 1.4 M NaCl) at 60 C in water bath. Added 1 ml CTAB buffer + Proteinase K, optional (10 mg/ml, 30 µl/1ml CTAB buffer) + 0.2% β-mercaptoethanol v/v (1.6 µl/1 ml of CTAB buffer), put it in water bath at 60 C for 1 hour and mixed it gently by inverting tubes 6 to 7 times.

DNA purification:

The sample isolated using the above methods were purified as detailed. To each tube added 200 µl 5M potassium acetate and put it in ice for 20 min and added 700 µl chloroform : 2-propanol (24:1) and inverted it gently, put it undisturbed for 3-5 min. Centrifuged for 15 min at 10000 rpm and transferred the middle (interface) aqueous layer to properly labeled new 2 ml tube using 1000 µl tips.
Added 24:1 chloroform: isoamyl alcohol the same volume left in tube, mixed it gently and left undisturbed for 3-5 min and then centrifuged for 10 min at 10000 rpm and removed the supernatant to properly labeled new 1.5 ml tubs. Added 500 µl ice cold isopropanol to get white flocculent (precipitate) and stored in -20°C for 30 min. Centrifuged for 5 min at 1000 rpm and removed the supernatant (done it carefully so as not to lose the pellet). Washed the pellet with 75% ice cold ethanol twice (the amount depends on the pellet size). Centrifuged for 5 min at 15000 rpm and removed the supernatant and left the pellet to dry at room temperature. Add 500 µl water and leave it overnight at 4^0C.

RNase Treatment:

A large amount of RNA in the sample can chelate Mg^{-2} and reduce the yield of the PCR. During this step the RNA is removed from the genomic DNA to which added 2 µl RNase and put it in incubator (oven) at 37°C for 1 h.

DNA qualification and quantification

The genomic DNA concentration was determined with Nanodrop using bio-spectrophotometer by measurement of optical density. The optical density was measured at 260 nm in a bio-spectrometer. The DNA concentration was then calculated according to the known method. The optical density (OD) was also taken at 280 nm (Correspondent to protein), 230 nm (correspondent to RNA), and 320 nm (Correspondent to the contamination).

PCR Amplifications:

Polymerase chain reaction (PCR) were carried out in a final volume of 10 µl. 0.4 D'NTP containing 100 mM of each deoxynycleotide triphosphate, 0.1 taq polymerase, 1 µl of primers (0.5 µl of forward primer, 0.5 µl reverse primer) and 1 µl genomic DNA containing 20 ng of sample, 6.5 µl of water were added to make up 10 µl total volume. The PCR amplifications were performed in an Eppendorf Mastercycler (Techne TC-512) comprising an initial denaturation at 94^oC for 4 min followed by 35 cycles of denaturation for 00:30 min, annealing at 56 °C for 00:30 min, extension at 72 °C for 00:30 min and end with final extension at same temperature for 10 min. The PCR products were resolved for 1 h 47 min at 120 V on 2% (w/v) Agarose gel in 1 × TAE buffer using a gel electrophoresis apparatus (Model V16.2 or V16; Gibco BRL, Gaithersburg, MD, USA). The gels were stained with ethidium bromide and visualized on a UV transilluminator (M-15 UVP Upland, CA 91786 USA) and photo-documented with a digital camera. The size of DNA bands in base pairs was determined using the 1 kb DNA standard ladder (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

The polymorphic scores of SSR markers were fed to the Join Map (version 4.0) software to analyze linkage map distances. A correlation matrix between the characters was generated using the Xlstat Version 2013 software. Chi-squared ($\chi 2$) tests were performed to examine the goodness of-fit between the expected Mendelian ratio for the F2 populations (3:1; resistant/susceptible), and (1:2:1 resistant/heterozygote/susceptible) and the segregation data for the SSR markers.

Results

SSR markers screening

A total of 159 SSR markers were screened to amplify IT97K-499-35 (Resistant parent) and Amary Sho (Susceptible parent) genomic DNA (Figure 2). Four SSR markers, CP333/334, CP115/116, CP7443/744 and MA62 were polymorphic between the two parents, representing 6% of the tested SSR markers (Table 1). They were selected to screen the genomic DNA of F2 segregating population.

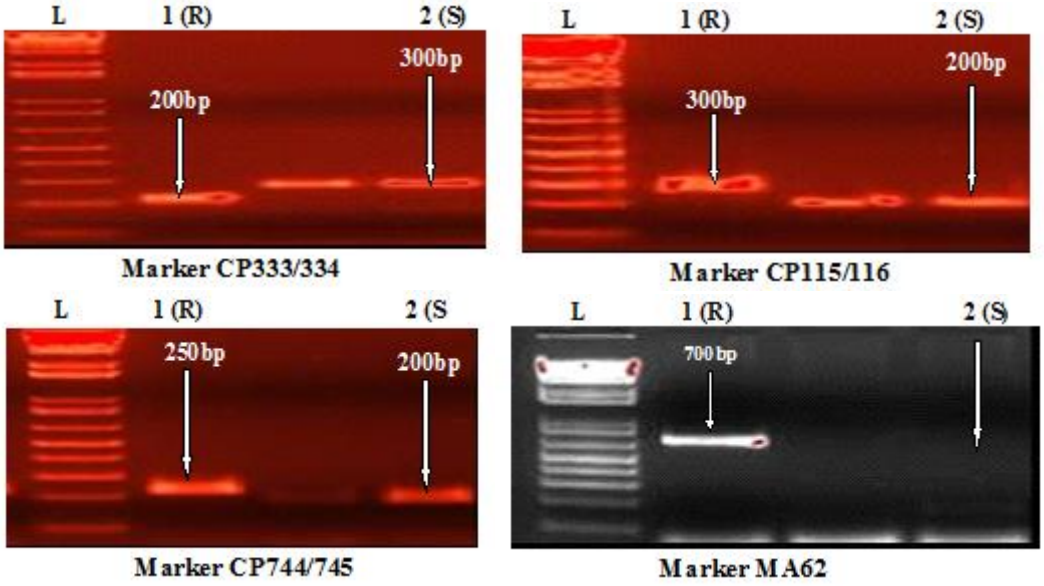

Figure 2: Simple sequence repeat profiles of four polymorphic markers CP333/334, CP115/116, CP744/745, and MA62 between IT97K-499-35 and Amary Sho. L: standard 1kb ladder. P1: IT97K-499-35, P2: Amary Sho.

Segregation of SSR markers in F2 segregating population

The distribution of different genotypes according to the marker and the frequency of alleles are consigned in Table 2. Chi square tests were performed to examine the goodness of fit between the expected Mendelian ratio for the segregation data to 1:2:1 (resistant/heterozygote/ susceptible) ratio among the genotypic data of the three SSR markers CP743/744 (Figure 4), CP333/334 (Figure 3) and CP115/116 (Figure 5) in F2 segregating progeny, and 3:1 (resistant/ susceptible) ratio among the genotypic data of MA62 the dominant SSR markers in F2 segregating progeny. The genotypic data for the SSR marker CP333/334, CP115/116, CP743/744 showed that the genotypic data does fit the 1:2:1 ratio, $\chi 2$ is significant. For the genotypic data for the SSR marker MA62, $\chi 2$ value is significant suggesting that the distribution of the genotypic data does not fit Mendel's segregation of ratio 3:1.

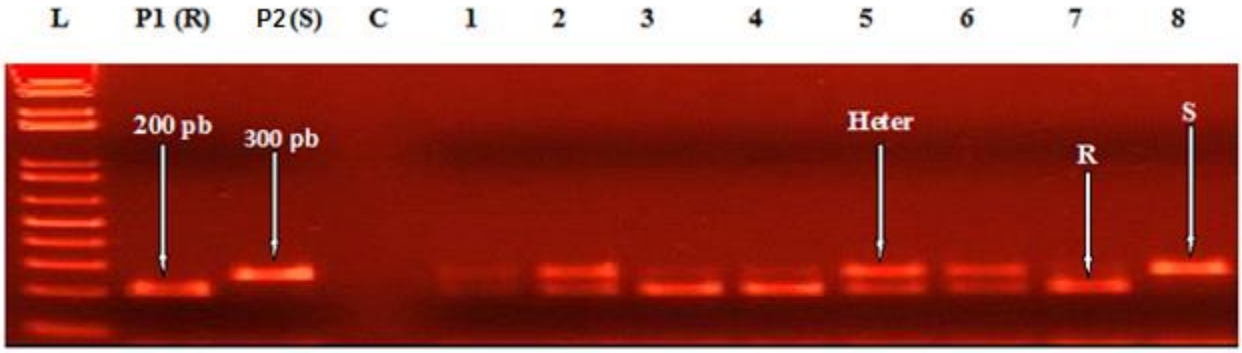

Figure 3: Electrophoretic profile of PCR products of ADN extract of the F2 with CP333/334 marker.
L: 100 bp DNA of the molecular marker, P1: allele of It 97k-499-35, P2: allele of Amary Sho, 1-7: individuals of the F2 populations,. Agarose gel concentration 2 %

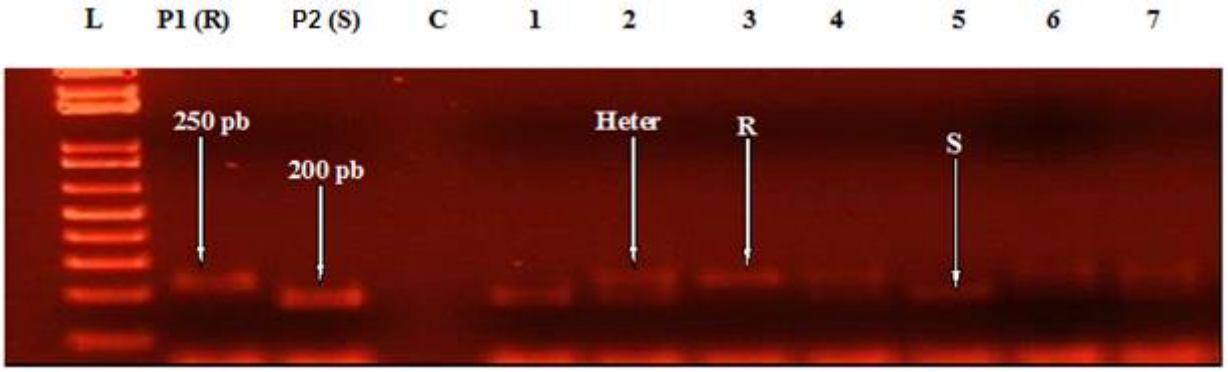

Figure 4: Electrophoretic profile of PCR products of ADN extract of the F2 with CP743/744.marker.
L: 100 bp DNA of the molecular marker, P1: allele of It97k-499-35, P2: allele of Amary Sho, 1-7: individuals of the F2 populations.
Agarose gel concentration 2 %

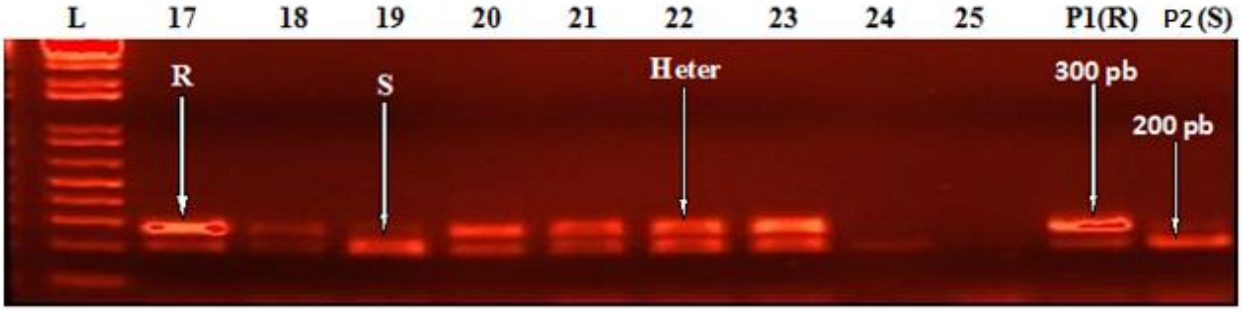

Figure 5: Electrophoretic profile of PCR products of ADN extract of the F2 with CP115/116.marker,
L: 100 bp DNA of the molecular marker, P1: allele of IT97K-499-35, P2: allele of Amary Sho, 17-25: individuals of the F2 populations,
Agarose gel concentration 2 %

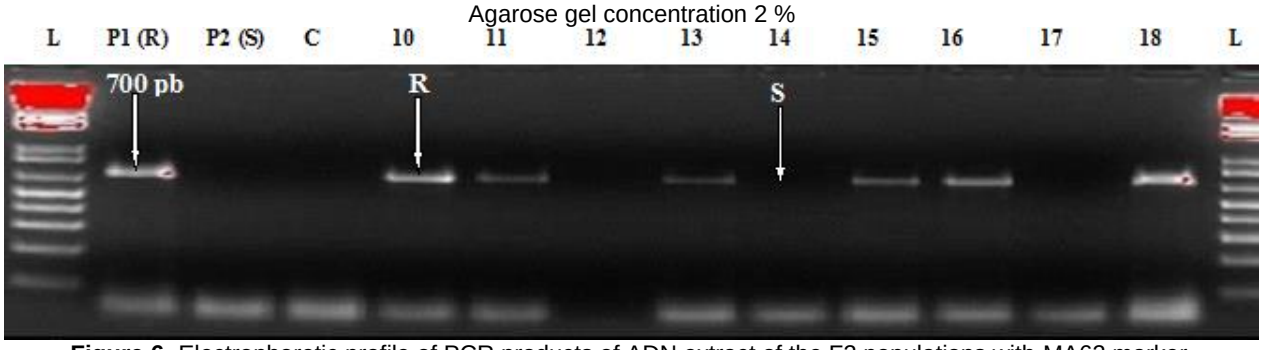

Figure 6: Electrophoretic profile of PCR products of ADN extract of the F2 populations with MA62.marker.
L: 100 bp DNA of the molecular marker, P1: allele of IT97K-499-35, P2: allele of Amary Sho, 10-18: individuals of the F2 populations,
Agarose gel concentration 2 %

Table 2: Segregation pattern, allele frequency of the four polymorphic SSR markers among F2 progeny

Markers	[a]Progeny Segregation [b]Res/Heter/Susc	Allele frequency Resistant/Susceptible	χ2
CP333/334	36/35/24	0.747/0.252	9.61*
CP115/116	14/60/17	0.813/0.186	9.44*
CP743/744	13/49/32	0.659/0.340	7.85*
MA62	73/24	0.752/0.247	146.51**

A: F2 segregating population, b: Resistant/ Heterozygote /Susceptible, c: Calculated Chi-square value (STEEL et al., 1997) according to the expected Mendelian genotypic segregation ratio 1:2:1 and 3:1, *: Significant at 5%; **: Highly significant at 1%

Map Marker Analysis:

Analysis of the 100 F2 individuals using the four polymorphic primers identified to determine the degree of linkage between the four SSR markers and *Striga*

resistance gene race–reveals that markers CP333/334, CP115/116, and CP743/744 are co-dominant whereas MA62 marker is dominant. The linkage analysis shows that all markers co-dominant and dominant are ordered as shown in Figure 7. Based on recombination frequency, the map distances between the SSR markers and Rsg2 were determined to be 41.9 cM for MA 62, 96.9 cM for CP115/116, 120.1 cM for CP333/334 and finally 245.5 cM for CP743/744. Based on The map showed in figure 8, primer MA62 with 41.9 cM seems to be tightly linked to the *S. gesnerioides* race 2 resistance gene Rsg2 while the CP743/744, CP115/116 and CP333/334 markers with distance flunked between 96.9 and 245.5 are not closely linked to the *S. gesnerioides* race 2 resistance gene *Rsg2*.

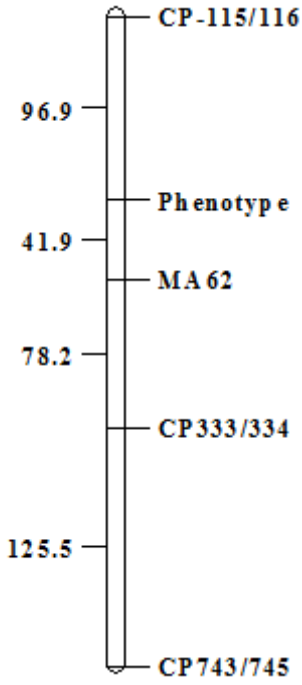

Figure 7: Map showing the linkage of the four SSR markers to S. gesnerioides race 2 resistance gene Rsg2–1 obtained by analysis of F2 progeny.

Phenotypic and Genotypic correlation:

The matrix correlation (Table 3) revealed a very significant correlation between marker genotypes and phenotypes. Thus, a positive and significant correlations were observed between MA62 marker and the phenotype (r = 0.86), marker MA62 and the marker CP115/116 (r = 0.16) and marker MA62 and the marker CP333/334 (r = 0.10).

Table 3: Correlation matrix of Pearson

Variables	Phenotypes	CP333/334	CP115/116	CP7443/744	MA62
Phenotypes	1				
CP333-334	0.359*	1			
CP115/116	0.279*	-0.021	1		
CP7443/744	0.118	0.132	0.087	1	
MA62	0.927*	0.411*	0.324*	0.166	1

** : significant at 0.05 probability level*

Discussion

The validation of the SSR markers result showed four SSR markers of which three co-dominant (CP115/116, CP333/334, CP473/744) and one dominant (MA 62) can differentiate the two parents IT97K-499-35, resistant and Amary Sho, susceptible and showed consistent polymorphic bands. At the end of the test, four markers among the 159 showed an interesting polymorphism. These results are similar to those obtained by Ouédraogo *et al.,* (2001) in their work of identifying AFLP markers related to the gene for resistance to *S. gesnerioides* in cowpea. Validation of co-dominant and dominant polymorphic markers showed, out of the four polymorphic

markers, three have a polymorphic profile characteristic of co-dominant markers because they can distinguish the heterozygotes parents from the homozygotes parents, and one showed a polymorphic profile characteristic of dominant markers because they make possible to distinguish resistant and sensitive homozygotes parents. Of the four informative SSRs, the one mapping closest to the *S. gesnerioides* Rsg2 resistance gene present in the cultivar IT97K-499-35 was MA62. Microsatellites are specific markers for revealing polymorphism individually, that is, within a particular sequence or in its vicinity (De Vienne 1998). While the technic of the AFLPs markers used by Ouédraogo *et al.,* 2001 is non-specific and reveals mass polymorphism, that is to say simultaneously from ten to one hundred polymorphic loci.

Indeed, the co-dominant markers CP115/116, CP333/334, and CP473/744 are very far from the *Striga* resistance gene race *SG2* of Mali. On the other hand, the marker MA62 with a distance of 41.9 cM is probably the marker closest to the gene and therefore consistently associated with the *SG2* resistance gene of *S. gesnerioides* present in the parent IT97K-499-35 (Li and Timko, 2009). According to Paterson *et al.,* (1996), the lower of the number of recombinant, the closer of the marker is to the gene, which is the case of marker MA62, on the other hand the higher the number of recombinant and the more the marker is removed from the gene. The MA62 marker can be used as resistance marker in the national breeding program.

The local variety preferred (Amary Sho) used in this study showed great sensitivity to *Striga* throughout the process of introgression, which was proved by the high number of *Striga* emerged or attached. The same result has been showed with SSR-1 and C-42B marker. The SSR-1 and C-42B like MA62 primers distinguished between resistant and susceptible cowpea genotypes with different discriminating power. MA62 co-segregate with *S. generoides* race 2 or *SG2* resistance gene. According to Li and Timko (2009), the SSR-1 and C-42B markers were also found to co-segregate with *S. gesnerioides* race 3 or *SG3* resistance gene. MA62 marker produced single band of 700 bp PCR products with amplification only in resistant genotypes which were absent in susceptible genotypes. According to Omoigui *et al.,* (2009) SSR-1 and C-42B identified resistant lines with a single band while the susceptible lines had no band. The implication is that MA62 might be closer to the *S. gesnerioides* race specific-resistant gene (SG2). However, the identification of susceptible and resistant conformed to the selection procedure by Singh and Emebeche (1990) and confirmed with the presence or absence of distinct markers associated with S. *gesnerioides* resistance.

Conclusion

The study revealed four polymorphic markers linked to resistance gene of race 2 of *S. gesnerioides*. One of these four markers, MA 62 is closest to this gene. Marker data were consistent with the phenotypic data facilitating the selection process. Markers are useful tools in detecting the gene of interest. The use of these markers makes easier the selection of plant traits and reduces the time needed to develop new varieties and provide a foundation upon which to begin working towards a molecular marker based breeding program for germplasm improvement in Mali. Since the resistance in this accession can be followed using the MA62 marker, it could be readily used in molecular breeding programs to improve local accessions with favorable agronomic traits.

Acknowledgements

We wish to thank the members of the Timko laboratory and Ouagadougou genetic laboratory for their assistance in various aspects of this work. This research was supported by a grant from Kirkhouse Trust 'PhD. Scholarship'. The design, execution and interpretation of the research remain wholly the responsibility of the authors.

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