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RESEARCH ARTICLE

IDENTIFICATION AND VALIDATION OF SSR MARKERS LINKED TO *STRIGA GESNEROIDES* (WILLD.) VATKE GENE RACE 2 IN COWPEA |*VIGNA UNGUICULATA* (L.) WALP] IN MALI

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp.) Is one of the most important grain legumes grown in Mali. But its production is strongly limited by the SG2 strain of *Striga gesnerioides* (Willd.) Vatke which constitutes one of the major biotic constraints. The F2 populations resulting from the crossing between the variety IT97K-499-35 (Resistant parent) and the variety M'Barawa elites (susceptible parent) and seeds of *Striga gesnerioides* strain from Koporo were used. Thus, 100 individuals were characterized using five markers CP333/334, CP743/744, CP115/ 116, SSR-1 and MA 62 which were polymorphic out of the 159 markers used on the parents. This study shows that the dominant microsatellite marker SSR1 (6.5 cM) strongly linked to the *Striga* resistance gene present in the cowpea variety IT97K-499-35 (Djiguiya). Linked to the gene by the 150 bp allele, the dominant SSR-1 marker consistently segregated with the *Striga gesnerioides* SG2. The identification of the SSR-1 marker related to *Striga* resistance Race 2 in Mali is an excellent opportunity for the national breeding programs to develop a marker-assisted breeding (MAS) strategy for *Striga gesnerioides* resistance to the SG2.

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) who consume high carbohydrates but low in protein cereals, root and tuber crops (Omoigui, 2007). It is not only an important pulse crop but also used as an excellent fodder, green manure and soil improving cover crop because of its high protein content (23-29%) and its ability to fix atmospheric nitrogen (Steele, 1972). 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, *Striga gesnerioides* (Willd) Vatke (Ehlers and Hall, 1997).

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Cowpea yield losses associated with *S. gesnerioides* range from 83 to 100% (Cardwell and Lane, 1995; Emechebe *et al.,* 1991). 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). Indeed, molecular markers for identification and selection of *Striga*-resistant genotypes have been developed for most of the races of the parasite prevalent in West Africa. However, the differential virulence of races of *Striga gesnerioides* on cowpea genotypes (Lane *et al.,* 1994; Singh, 2002) 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*. 1992, 1993, 1997; Young *et al*. 1992; Myers *et al*. 1996; Menendez *et al.,* 1997). Therefore, the focus of the current work was to identify and validate some SSR markers linked to *Striga gesneroides* race 2 in Mali.

MATERIALS AND METHODS

Plant material

The study related to 2 parents IT97K-499-35 (resistant) and M'Barawa (susceptible) and 100 F2 descendants resulting from their crossing. The infestation was made with seeds of *Striga gesnerioides* from Koporo strain of Mali.

Methodology

Phenotyping of the F2 population

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 Striga 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 11cm) was perforated, partially filled with sandy loam soil to 7.0 cm deep and inoculated with about 2g seeds of *Striga 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) × M'Barawa (susceptible) were sown per pot. In all, 100 F2 population and their reactions to *Striga 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.

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 but not emergence
3. *Striga* emergence and plants show severe *Striga* symptom

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

Table 1 Polyporphic marker used for Fé 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
5	SSR1	CAAGAAGGAGGCGAAGACTG	CCTAAGCTTTTCTCCAACTCC

Genotyping of F2 population

Molecular markers selection

Molecular characterization was done in Professor Timko's Laboratory, Department of Biology, University of Virginia, U.S.A. in 2014. A population developed from a cross between IT97-499-35 (Resistant parent) and M'Barawai (Susceptible parent) was used in this study. One hundred (100) plants of F2 segregating population 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-499635 and M'Barawa, resistant and susceptible parents respectively.

DNA Isolation

Individual leaf samples were collected and the genomic DNA samples were extracted from 14 days old cowpea leaves following the protocol described by Mace *et al*. (2004) with minor modification. Harvest 100 to 200 mg (enough amounts) young leaf and put it in properly labeled 2ml Eppendorf tube. Add polyvinyl-polypyroid powder PVP-5% w/v (small amount) just before adding liquid nitrogen to protect the leaf from browning. Grind the leaf sample using tissuelyser until to get fine powder and put in properly labeled 2ml tube (Note: Put the tubs in liquid nitrogen until you add CTAB buffer). Pre-warm CTAB (2% CTAB w/v, 100 mM Tris HCL pH 8.0, 20 mM EDTA pH 8.0 and 1.4M NaCl) at 60 ^{o}C water bath. Add 1ml CTAB buffer+ Proteinase K, optional) (10mg/ml, 30ul/1mlof CTAB buffer) + 0.2% β-mercaptoethanol v/v (1.6ul for 1ml of CTAB buffer), put it in water bath at 60 ^{0}c for 1hour and mix 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 add 200 ul 5M potassium acetate and put it in ice for 20 min and Add 700 ul chloroform:2-propanol (24:1) and invert it gently, put it undisturbed for 3-5 min. Centrifuge for 15 min at 10000 rpm and transfer the middle (interface) aqueous layer to properly labeled new 2ml tube using 1000ul tips.

Add 24:1 chloroform: isoamyl alcohol the same volume left in tube, mix it gently and leave it undisturbed for 3-5 min and then centrifuge for 10 min at 10000 rpm and remove the supernatant to properly labeled new 1.5ml tubs. Add 500 ul ice

cold isopropanol to get white flocculent (precipitate) and store it in -20c for 30 min. Centrifuge for 5min at 1000 rpm and remove the supernatant (do it carefully not to lose the pellet). Wash the pellet with 75% ice cold ethanol twice (the amount depends on the pellet size). Centrifuge for 5 min at 15000 rpm and remove the supernatant and leave the pellet to dry at room temperature. Add 500 ul water and leave it overnight at 4 ^{0}c.

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. Add 2ul RNase and put it in incubator (oven) at 37 ^{0}c for 1 hrs.

DNA qualification

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 was performed. 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 ul of primers (0.5 ul of forward primer, 0.5 ul reverse primer) and 1 ul genomic DNA µl containing 20 ng of sample, 6, 5 ul of water were added to make up 10 ul total volume. The PCR amplifications were performed in an Eppendorf Mastercycler (Techne TC-512) comprising an initial denaturation at 94°C 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 trans illuminator (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 makers 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 M'Barawa (susceptible parent) genomic DNA (Figure 2). Five SSR markers, CP333/334, CP115/116, CP7443/744, MA 62, and SSR1 were polymorphic between the two parents, representing 8% of the tested SSR markers (Table 1). They were selected to screen the genomic DNA of F2 segregating population.

Segregation of SSR markers in F2 segregating population

Among the markers who highlight the two types of alleles (susceptible and resistant), the marker CP743/744 (Figure 3) has the highest number of individuals (43) carrying only the allele from the resistant parent) followed by markers CP333/334 (Figure 4) and CP115/116 (Figure 5) with respectively 28 and 17 individuals carrying this allele. However, the highest number of individuals (46) carrying the alleles of the two parents is observed with a marker CP333/334 744 followed by has from the SSR marker CP743/744 and CP 115/116 with respectively 40 and 39 individuals who are heterozygous. The highest number of homozygous susceptible was obtained by SSR marker CP115/116 with 34 individuals followed by CP333/334 and CP743/744 markers with 17 and 16 Individuals respectively. For the two markers who reveal only one of the two types of alleles, the markers SSR-1 (Figure 6) amplified resistant bands in 80 genotypes out of 99 F2 plants and was absent in 19 susceptible F2 individuals. While SSR marker MA 62 (Figure 7) amplified resistant bands in 75 genotypes out of 99 F2 plants and was absent in 24 susceptible F2 individuals. The distribution of different genotypes according to the marker and the frequency of alleles are consigned in figure 8 and table2.

Chi- square tests ($\chi 2$) 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, CP333/334 and CP115/116 in F2 segregating progeny, and 3:1 (resistant/ susceptible) ratio among the genotypic data of the two dominant SSR markers in F2 segregating progeny. The genotypic data for the SSR marker CP115/116, CP743/744 showed that the genotypic data does fits the 1:2:1 ratio, chi square was highly significant $\chi 2 = 8{,}02^{*}$ and $\chi 2 = 18{,}37^{**}$ respectively. For the genotypic data for the SSR marker SSR1 and MA 62, chi square was highly significant suggesting that the distribution of the genotypic data does not fit Mendel's segregation of ratio 3:1 and with $\chi 2 = 150.00^{**}$ for SSR MA62 and $\chi 2 = 174.17^{**}$ for marker SSR-1 (Table 2). The data for markers CP333/334 genotyping are not significant what

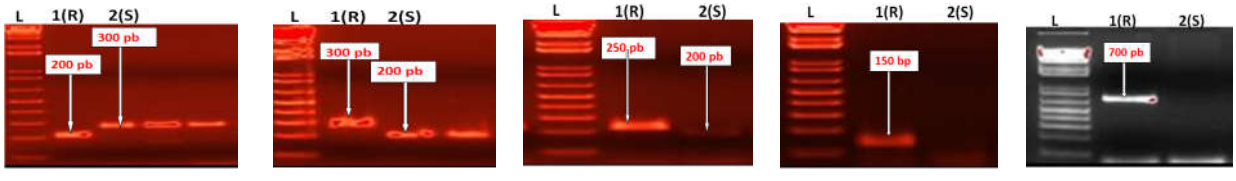

Figure 2 Simple sequence repeat profiles of five polymorphic markers CP333/334, CP115/116, CP744/745, SSR-1and MA 62 between IT97K-499-35 and M'Barawa. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa,

indicates that the distribution of the data of genotypic follows the law of Mendelian ratio of segregation data to 1:2:1 and a χ^2 = 2.67ns.

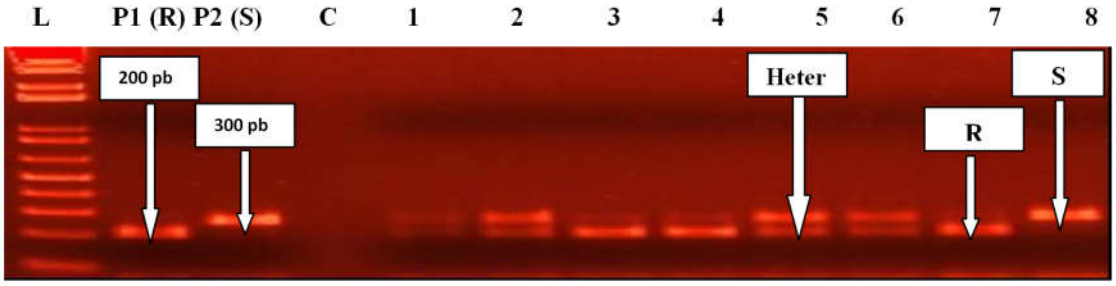

Figure 3 DNA bands from PCR amplification products of CP743/744 for F2 segregating population of cowpea (derived from IT97K-499-35 × M'Barawa) resolved in 2 % Agarose gel stained with ethidium bromide. The presence of 150bp band indicates resistant genotype and absence of this band indicate susceptible genotype. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa.

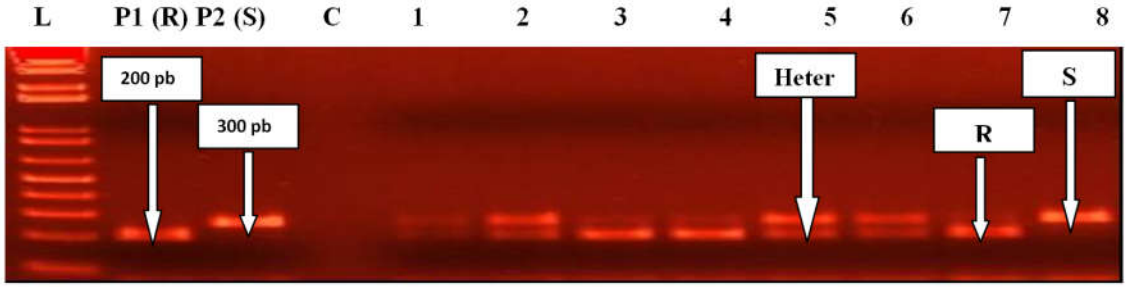

Figure 4 DNA bands from PCR amplification products of CPP333/334 for F2 segregating population of cowpea (derived from IT97K-499-35 × M'Barawa) resolved in 2 % Agarose gel stained with ethidium bromide. The presence of 150bp band indicates resistant genotype and absence of this band indicate susceptible genotype. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa,

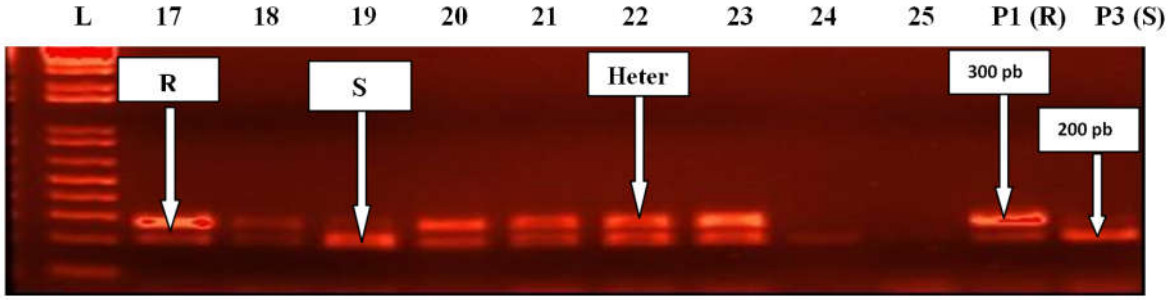

Figure 5 DNA bands from PCR amplification products of CP115/116 for F2 segregating population of cowpea (derived from IT97K-499-35 × M'Barawa) resolved in 2 % Agarose gel stained with ethidium bromide. The presence of 150bp band indicates resistant genotype and absence of this band indicate susceptible genotype. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa.

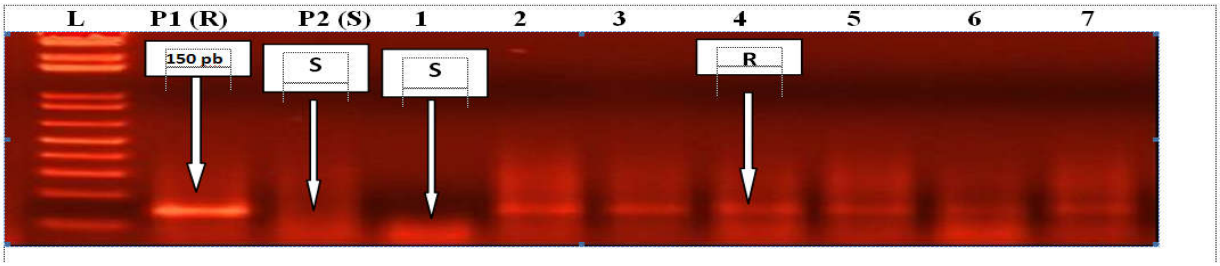

Figure 6 DNA bands from PCR amplification products of SSR-1 for F2 segregating population of cowpea (derived from IT97K-499-35 × M'Barawa) resolved in 2 % Agarose gel stained with ethidium bromide. The presence of 150bp band indicates resistant genotype and absence of this band indicate susceptible genotype. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa.

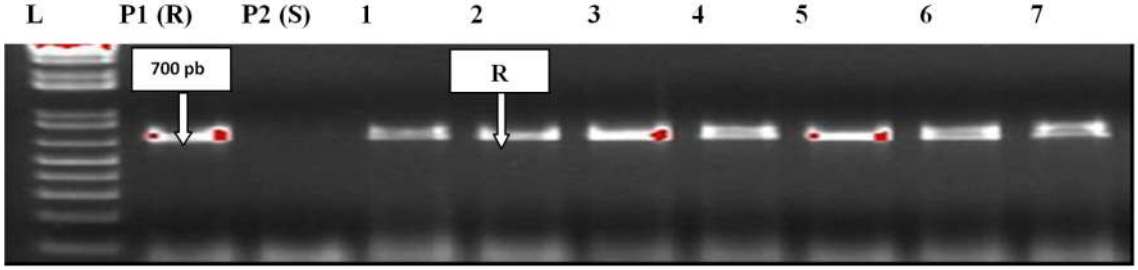

Figure 7 DNA bands from PCR amplification products of MA 62 for F2 segregating population of cowpea (derived from IT97K-499-35 × M'Barawa) resolved in 2 % Agarose gel stained with ethidium bromide. The presence of 150bp band indicates resistant genotype and absence of this band indicate susceptible genotype. L represents the standard 1kb ladder. P1 is IT97K-499-35 (150 bp), P2 is M'Barawa.

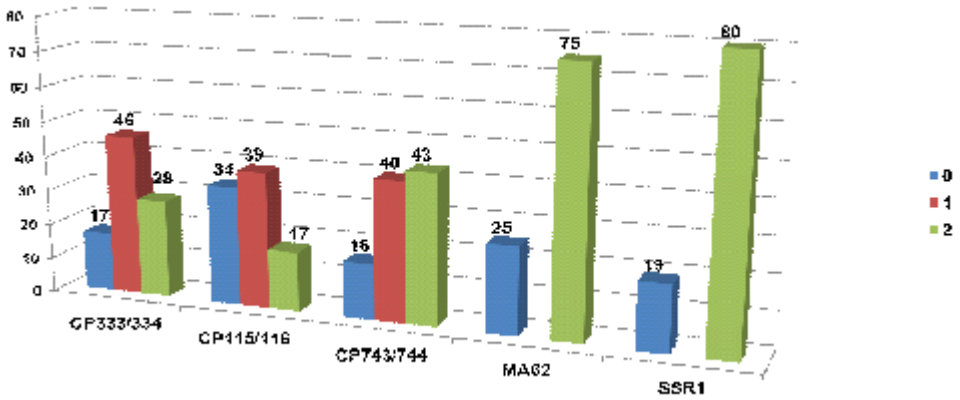

Figure 8 Segregation of CP333/334, CP115/116, CP743/744, SSR-1, and MA62 as susceptible, heterozygous and resistant, respectively, among F2 segregating population.

Table 2 Segregation pattern, allele frequency of the five polymorphic SSR markers among F2 progeny (IT97K-499-35 x M'Barawa).

Markers	[a]Progeny Segregation [b]Res/Heter/Susc	Allele frequency Resistant/Susceptible	[c]Chi-square $\chi 2$
CP333/334	28/46/17	0.813/0.186	2.67^{ns}
CP115/116	17/39/34	0.622/0.377	8.02^{*}
CP743/744	43/40/16	0.838/0.161	18.37^{**}
SSR-1	80/19	0.808/0.19	174.17^{**}
MA62	75/25	0.757/0.252	150.00^{**}

a = F2 segregating population derived from IT97-499-35 x M'Barawa, b = Resistant/ Heterozygote /Susceptible, c = Calculated Chi-square value as described by Steel et al. (1997) according to the expected Mendelian genotypic segregation ratio 1:2:1 and 3:1, ns=no significant, *= Significant, ***= Highly significant

Map Marker Analysis

To determine the degree of linkage between the three SSR markers and *striga* resistance gene race 2, 100 F2 individuals from the IT9K-499-35 x M'Barawa cross were analyzed using the five primer polymorphic identified. The CP333/334, CP115/116, and CP743/744 markers were codominant and, whereas the SSR-1 and MA 62 markers were dominant.

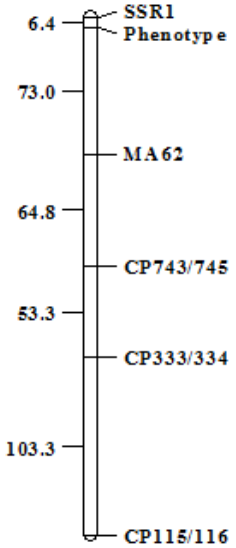

Figure 9 Map showing the linkage of SSR markers CP333/334, CP115/116, CP743/744, MA62, SSR-1 to S. gesnerioides race 2 resistance gene Rsg2–1 obtained by analysis of F2 progeny from the cross of IT82D-499-35×M'Barawa. Map distances are shown in centiMorgans

The linkage analysis showed that all markers codominant and dominant are ordered as shown in Fig. 9. Based on recombination frequency, the map distances between the SSR markers and Rsg2 were determined to be 6.5 cM for SSR-1, 73 cM for MA 62, 137.8 cM for CP743/744, 194.1 cM for CP333/334, and finally 294.3 cM for CP115/116. The primer SSR-1 with 6.5 cM seems to be tightly linked to the *Striga gesnerioides* race 2 resistance gene Rsg2 while the MA 62, CP743/744, CP115/116 and CP333/334 markers with distance flunked between 73 and 294.3 are not closely linked to the *Striga gesnerioides* race 2 resistance gene Rsg2.

Phenotypic and genotypic correlation

Table 3 reveals significant correlations between traits studied. Positive correlations were observed between genotypic data of SSR-1 and phenotyping data (r = 0.21, P< 0, 0001, & = 0,05). The correlation between the genotypic data of SSR marker MA62 and phenotypic data was negative (r = 0,21). Also, SSR marker CP115/116 was negative correlated to marker CP743/444 (r = 0,006), and marker SSR-1 (r = 0,003). A negative correlation was observed between markers CP115/116 (r=0,004), CP743/744 (r=0,006), and MA 62 (r = 0,045) and phenotypic data.

Table 3 Correlation matrix of Pearson

Variables	Phenotypes	CP333/334	CP 115/116	CP743/745	SSR1	MA62
Phenotypes	1					
CP333/334	0,009	1				
CP 115/116	-0,063	-0,096	1			
CP743/745	-0,077	0,074	-0,074	1		
SSR1	0,708**	0,041	-0,052	0,080	1	
MA62	-0,213*	0,324	-0,012	0,252	-0,030	1

*significant at 0.05 probability level

DISCUSSION

This study involved screening 159 SSR markers for polymorphism between the two distinct parents IT97K-499-35, Resistant and M'Barawa, Susceptible. The validation of the SSR markers result shows five (5) SSR markers of which three codominant (CP115/116, CP333/334, CP473/744 4) and two dominant (SSR-1 and MA 62) can differentiate the two parents IT97K-499-35, Resistant and M'Barawa, Susceptible and showed consistent polymorphic bands. Then, it was necessary to ensure that these markers segregated consistently with the gene of interest in the F2 segregated populations resulting from

the crossing of the two parents expressing the polymorphism. At the end of the test, 35 markers among the 159 (55.65%) showed a polymorphism. The sizes of the amplification products of these loci was comprise between 150 and 700 bp. These results are similar to those obtained by Ouedraogo *et al*., 2001 in their work of identifying AFLP markers related to the gene for resistance to *Striga gesnerioides* in cowpea. Validation of co-dominant and dominant polymorphic markers showed, of the 35 polymorphic markers, three (3) have a polymorphic profile characteristic of co-dominant markers because they can distinguish the heterozygotes parents from the homozygotes parents, and two (2) Showed a polymorphic profile characteristic of dominant markers because they make possible to distinguish resistant and sensitive homozygotes parents. Of the six informative SSRs, the one mapping closest to the *Striga gesnerioides* SG2 resistance gene present in the cultivar IT97K-499-35 was SSR1. 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 in 2001 by Ouédraogo *et al.,* is non-specific and reveals mass polymorphism, that is to say simultaneously from ten to one hundred polymorphic loci.

Indeed, the codominant 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 SSR1 with a distance of 6.5 cM is probably the marker closest to the gene and therefore consistently associated with the SG2 resistance gene of *Striga gesnerioides* present in the parent IT97K-499-35 (Li and Timko, 2009). According to Paterson *et al.,* 1996, the lower the number of recombinant, the closer the marker is to the gene, which is the case of SSR-1, on the other hand the higher the number of recombinant and the more the marker is removed from the gene. The SSR-1 marker can be used as resistance marker in the national breeding program.

The local varieties preferred by the farmers 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. However, the use of most resistant varieties is limited due to concerns about the potential adaptability and small or medium seed size as found in variety IT97K 499-35 (Omoigui, *et al.,* 2007). IT97K-499-35 is a derivative from B301, local landrace from Botswana, which produces small seeds but is a multi-race resistant genotype to both *S. gesnerioides* and Alectra vogelii (Singh, 2002). The SSR-1 primers distinguished between resistant and susceptible cowpea genotypes with different discriminating power. Indeed, the SSR-1 markers were found to co-segregate with *S. gesnerioides* race 3 or SG3 resistance gene (Li and Timko 2009; Omoigui, *et al.,* 2009). SSR-1 markers produced single bands of 150bp PCR products with amplification only in resistant genotypes which were absent in susceptible genotypes. According to Omoigui *et al*. (2009) SSR-1 identified resistant lines with a single band while the susceptible lines had no band. In the current study, the 150bp SSR-1 marker was more efficient at 92.6% discriminating ability compared to the others primers. The implication is that SSR-1 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*. In fact, the use of marker-assisted selection (MAS) and breeding has already substantially reduced the timeframe for delivery of superior cowpea cultivars (Timko and Singh, 2008).

CONCLUSION

The results described here provide a foundation upon which to begin working towards a molecular marker based breeding program for germplasm improvement in Mali. Only IT97K-499-35 could be used as a local donor for germplasm improvement. Since the resistance in this accession can be followed using the SSR-1 marker, it could be readily used in molecular breeding programs to improve local accessions with favorable agronomic traits.

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