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*Full Length Research Paper*

# Development of sequence characterized amplified region (SCAR) markers linked to race-specific resistance to *Striga gesnerioides* in cowpea (*Vigna unguiculata* L.)

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**An amplified fragement length polymorphism (AFLP) fragment, E-ACT/M-CAA$_{524}$, tightly linked to the *Striga gesnerioides* race 1 (SG1) resistance gene *Rsg-2-1* in cowpea (*Vigna unguiculata* L.) was isolated by polyacrylamide gel electrophoresis, cloned, and its nucleotide sequence determined. Based on the resulting sequence information, a pair of sequence specific primers were designed and used to isolate identical and similar fragments from cowpea genomic DNA of different cowpea lines by polymerase chain reaction (PCR) amplification. The primers amplified a ~500 bp fragment (SCAR marker designated as 61R) that was present in the resistant parent TVU14676, absent in susceptible parent IT84S-2246, and segregated with the resistance phenotype in an $F_2$ population, derived from a cross of these two genotypes. The same primers were used to isolate a fragment similar to 61R from another *S. gesnerioides* resistant line Kvx 61-1. The sequence of this fragment was used to design a new combination of primers that developed a second SCAR marker, designated as 61R-M2. Subsequent analysis of the three markers, E-ACT/M-CAA$_{524}$, 61R and 61M2 showed that they are linked to each other by 0.6 centimorgans (cM). The utility of these SCARs in marker assisted selection programs for cowpea was discussed.**

**Key words:** *Striga gesnerioides*, centimorgans (cM), race specific resistance, amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR), marker assisted selection (MAS).

## INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, is one of the most important warm-season legumes in the semi-arid tropics (Ehlers and Hall, 1997). The majority of cowpea is grown by subsistence farmers in west and central sub-saharan Africa, where its grain is highly valued for food, and the stover is used for animal forage (Langyintuo et al., 2003). In addition, because of its ability to fix nitrogen symbiotically with rhizobacteria when used in rotation with cereal crops, it helps to restore soil fertility (Carsky et al., 2002; Sanginga et al., 2003). Yields of cowpea grain are reduced by a variety of biotic pests like viruses, bacteria, fungi, insects, nematodes, herbivores etc., and abiotic stresses like drought, salinity, and heat (Timko and Singh, 2008). The major biotic constraints is attack by the root parasitic weeds, *Striga gesnerioides* and *Alectra vogelii*. *S. gesnerioides*, in particular, causes extensive damage to cowpea in the Sudano-sahelian belt of West and Central Africa (Parker, 2009) where its damaging effects are compounded by drought (Obilana, 1987).

The damage to host is already done before the *S. gesnerioides* shoots emerge from the soil. And at the end of the growing season, each plant produces thousands of

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seeds, which remain viable in soil up to 20 years. This makes it challenging to control the parasitic weed, though several control strategies have been developed for it, including improved cultural practices, selective breeding using wild and cultivated germplasm as sources of resistance, and the use of chemical controls. The use of resistant cultivars appears to be a generally acceptable, effective, economical and environmentally safe means of controlling this parasite. However, the rapid breeding of improved cultivars with pyramided desirable agronomic traits, including multiple disease and pest resistances requires effective screening as a part of selection procedure. Implementation of marker assisted selection (MAS) in cowpea breeding programs is severely limited by a general lack of information on gene structure and organization, and a paucity of trait-linked markers (Timko et al., 2007).

Heritable sources of resistance in cowpea to both *S. gesnerioides* and *A. vogelii* have been reported (Timko and Singh, 2008). However, most of these resistant lines have poor agronomic characteristics and therefore, their direct use is limited. These germplasms are being incorporated in local and regional cowpea breeding programs, but the advancement of improved varieties to the farmers is slow. Among the limitations to successful development of improved striga-resistant cowpea is the fact that *S. gesnerioides* is variable in its parasitic abilities, showing both host and cultivar-specific selectivity. At least seven distinct races of *S. gesnerioides* (designated SG1 through SG7) have been identified throughout West Africa (Lane et al., 1997a, b; Botanga and Timko, 2006).

Most cowpea plants are susceptible to striga parasitism, although some local landraces have been identified that show resistance to one or more of the known races (Timko et al., 2007) with resistance being conferred by single dominant genes (Aggarwal et al., 1984; Toure et al., 1997).

Using amplified fragment length polymorphism (AFLP) analysis, Vos et al. (1995) as well as others have been able to map several of the race-specific resistance genes to two linkage groups on the cowpea genome (Ouédraogo et al., 2001, 2002a, b; Boukar et al., 2004). The *S. gesnerioides* race SG1 and SG3 resistance genes Rsg2-1, Rsg1-1 and Rsg4-3, present in the resistant cowpea lines B301, IT82D-849 and Tvu14676, respectively, were mapped to LG1. Whereas, the *S. gesnerioides* race SG1 resistance genes Rsg3-1 and Rsg994-1, present in Suvita-2 (Gorom local) and IT81D-994, respectively, were located to LG6 (Ouédraogo et al., 2001, 2002a, b).

The availability of molecular markers tightly linked to *S. gesneriodes* resistance genes opens up the possibility of applying MAS to cowpea improvement. However, while AFLP analysis is powerful, highly reproducible, and allows rapid identification of markers, it does not allow rapid characterization of large number of populations with a minimal cost and skilled labor. It is also costly and requires either sophisticated equipment in case of fluorescent detection of DNA fragments in the gel or radioactive substances for detection of amplified products. These requirements are difficult to meet for most of the breeding programs in the developing countries. An alternative to this is the conversion of informative AFLPs into SCARs (Paran and Michelmore, 1993) that are specific, highly reproducible and efficient, and cost effective to use. Often, these markers are co-dominant, making scoring easy and reliable.

In the present study, we report the conversion of a 500 bp AFLP fragment (E-ACT/M-CAA$_{524}$) tightly linked to the *S. gesnerioides* SG1 resistance gene Rsg2-1 into PCR-based SCAR markers that tag both SG1 and SG3 resistance. We discuss the potential use of these SCARs in MAS and breeding for multirace striga-resistant cowpea in West Africa.

## MATERIALS AND METHODS

### Plant

The parental lines and plant populations used in this study, and their resistance/susceptible phenotypes were previously described (Ouédraogo et al., 2002b). Briefly, an $F_2$ population segregating for SG3 resistance was generated by crossing the *S. gesnerioides* susceptible cowpea line IT84S-2246 and a resistant cowpea line Tvu14676 (B203), and an $F_{11}$ Recombinant Inbred Lines (RIL) population of 94 individuals derived by single seed descent from a cross between cowpea accessions 524B and IT-84S-2049 was used to place SCARs 61R and 61R-M2 on the cowpea genetic map.

### DNA isolation, AFLP analysis, and DNA sequencing

Total DNA was isolated from the leaf tissues as described in Ouédraogo et al. (2002b). The precipitated DNA was resuspended in 10 mM Tris-Cl, pH 8.0, 1 mM EDTA (TE) buffer and the concentration determined spectrophotometrically on a ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Qualities of the DNA samples were accessed by electrophoresis through a 0.8% (w/v) agarose gel. Working solutions of 100 ng/µl were prepared for each of the samples. AFLP analysis was performed using the *CT EcoRI-ACT* (E-ACT) and *Mse*I-CAA (M-CAA) primer combination, as described by Ouédraogo et al. (2001). The ~500 bp E-ACT/M-CAA$_{524}$ fragment present in amplification products generated, using DNA from cowpea cultivar IT82D-849 was cut from the polyacrylamide gel, purified, subjected to a round of PCR amplification, and its nucleotide sequence determined as described in Ouédraogo et al. (2002b).

### Designing sequence specific primers and SCAR development

Based on the nucleotide sequence of the polymorphic E-ACT/M-CAA$_{524}$ fragment (Figure 1), a pair of sequence specific primers 24 nucleotides in length was designed and commercially synthesized (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA). The sequences are shown in Figure 1. PCR amplification reactions were then carried out using 100 ng genomic DNA template isolated from the SG1 resistant line Kvx 61-1, the SG3 resistant line Tvu14676,

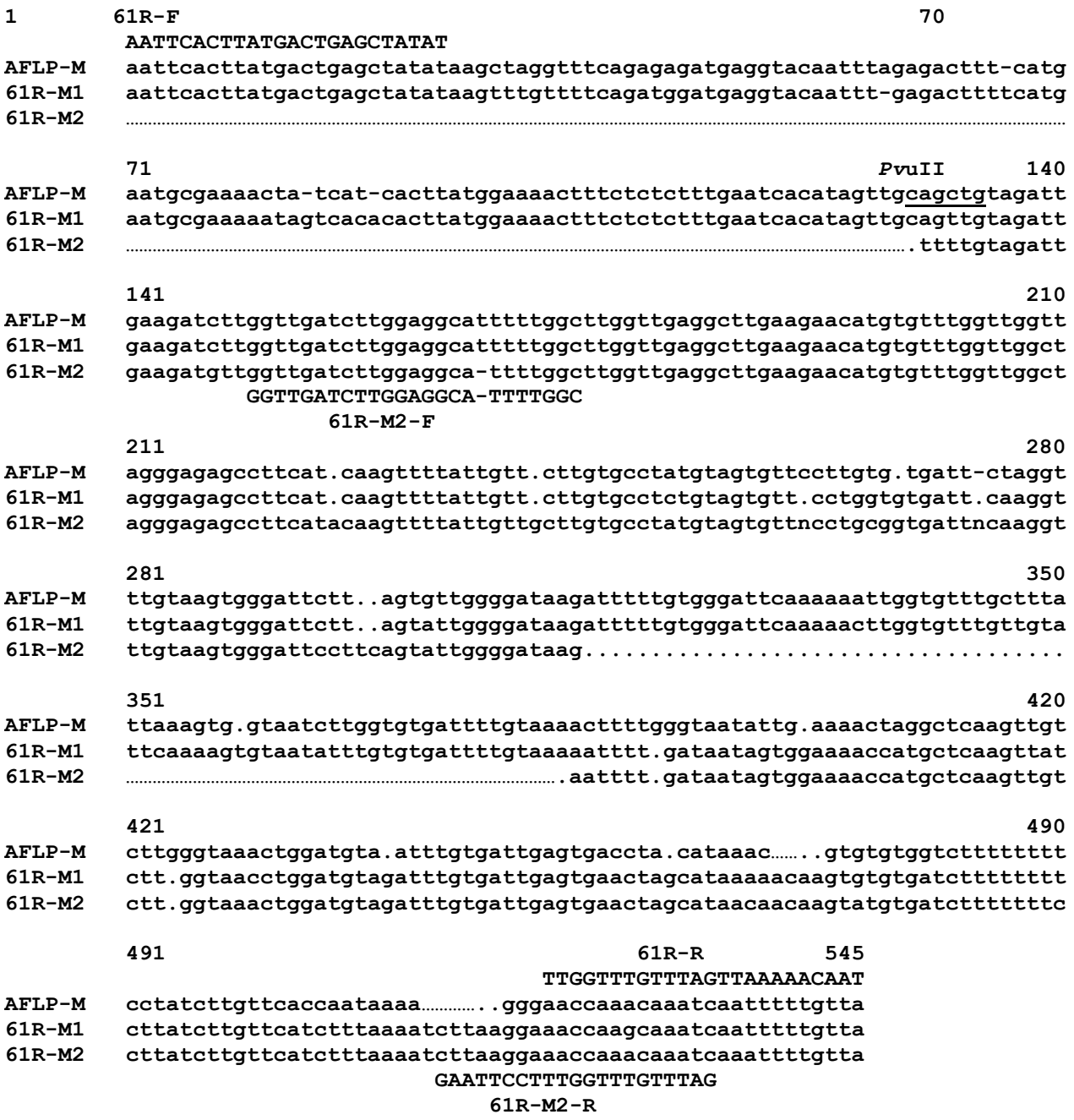


**Figure 1.** Comparison of AFLP-M (E-ACT/M-CAA$_{524}$), 61R-M1 and 61R-M2 nucleotide sequences. Primer sequences are shown just above/below the sequences in upper case letters with their names just above/below the sequences. Underlined letters indicate the *Pv*u II site.

and the susceptible lines 524B and IT-84S-2246 in 25 µL reactions, containing 0.4 µM of each primer, 1.5 units of Taq DNA polymerase (Roche Applied Science, Indianapolis, IN), 2.5 µL of the corresponding Taq 10× buffer, and 200 µM dNTP mixture. The amplification was carried out as above and the amplification products analyzed by electrophoresis on 2% (w/v) agarose gels.

DNA fragments amplified from 524B and Kvx 61-1 were excised from the gel, purified, and their nucleotide sequences were determined as mentioned earlier. The sequences were aligned, using DNASTAR (Lasergene, Inc., Madison, WI) and based on the nucleotide sequence (Figure 1), a pair of sequence specific primers was designed and commercially synthesized as mentioned earlier. Primer sequences are shown in Figure 1.

**Bulked segregant analysis and mapping of SCARs 61R and 61R-M2**

To determine whether SCARs 61R and 61R-M2 were linked to the SG3 resistance gene Rsg4-3, bulked segregant analysis

**Table 1.** Resistance response of different cowpea accessions to *Striga gesnerioides* races SG1 and SG3 and their molecular phenotype. Given in the table are the response of the various cultivars to attack by *S. gesnerioides* races SG1 and SG3. R and S represent resistant and susceptible, respectively.

| S/N | Cowpea accession | 61R | 61R-M2 | SG1 | SG3 |
|---|---|---|---|---|---|
| 1 | IT93K-693-2 | + | + | R | R |
| 2 | Tvu14675 | + | + | R | R |
| 3 | B301 | + | + | R | R |
| 4 | IT97K-499-35 | + | + | R | R |
| 5 | Aloka local | + | + | R | S |
| 6 | IT82D-849 | + | + | R | R |
| 7 | IT81D-994 | + | + | R | R |
| 8 | IT84S-2049 | - | - | S | S |
| 9 | IT84S-2246-4 | - | - | S | S |
| 10 | IT97K-461-4 | + | + | S | S |
| 11 | Suvita-2 | - | - | R | R |
| 12 | Tvu7778 | - | - | S | S |
| 13 | Tvu3236 | - | - | S | S |
| 14 | 524B | + | + | S | R |

The presence (+) or absence (-) of the molecular markers SCAR 61R and 61R-M2 as determined by agarose gel electrophoresis are also indicated.

(Michelmore et al., 1991) was carried out on the $F_2$ population, generated from the *S. gesnerioides* susceptible cowpea line IT84S-2246 and a resistant cowpea line Tvu14676 (B203), that had been previously scored for resistance to *S. gesnerioides* SG3 (Ouédraogo et al., 2001, 2002a). PCR amplification reactions were carried out using the 61R and 61R-M2 primer pairs and genomic DNA samples of parental (Tvu14676 and IT84S-2246) bulked pools and individual $F_2$ progeny, as described previously. The PCR products were resolved on 2% agarose gels and the presence or absence of the polymorphic 61R and 61R-M2 bands were scored.

To determine whether the 61R and 61R-M2 SCARs mapped to the same location as that of E-ACT/M-$CAA_{524}$ marker on the cowpea genetic map, PCR amplifications were carried out with 61R and 61R-M2 primers, using DNA isolated from 94 individuals of an $F_{11}$ RIL population, derived from a cross between cowpea accessions 524B and IT84S-2049 (Ouédraogo et al., 2002b). PCR conditions for the 61R and 61R-M2 were as mentioned earlier. Pictures taken from the PCR products resolved on agarose gels (stained with ethidium bromide) were used for scoring the bands. The presence and absence of the polymorphic bands associated with 61R and 61R-M2 was scored, and these data, along with segregation data for the E-ACT/M-$CAA_{524}$ (ACT/CAA-8) marker and some other markers surrounding the resistance locus were used to generate a partial map of linkage group 1 near the *S. gesnerioides* resistance locus, using JoinMap® 4.0 (Van Ooijen, 2006).

#### Correlation of SCARs 61R and 61-RM2 with field phenotypes

A set of cowpea accessions (Table 1) with known SG1 and SG3 resistance and susceptible phenotypes were analyzed for the 61R and 61R-M2 markers as described above.

## RESULTS

An approximately 500 bp AFLP fragment (E-ACT/M-$CAA_{524}$) was polymorphic between the *S. gesnerioides* susceptible cowpea line Tvx3236 and the SG1 resistant cowpea line IT-82D-849. The same fragment was also found to be tightly linked to the Rsg2-1 resistance gene in IT-82D-849 (Ouédraogo et al., 2001, 2002a). This fragment was isolated, following PCR amplification from IT-82D-849 genomic DNA and its nucleotide sequence was determined (Figure 1). The 545 bp fragment contained an *Eco*RI adapter at one end and the *Mse*I adapter at the other end. Basic Local Alignment Search Tool (BLAST) searches for similarities in the cowpea GSS database (Timko et al., 2008), legume Information system and Gene bank (Altschul et al., 1997) did not yield any closely similar sequences.

Two 24 nucleotide sequence-specific primers (61R forward and 61R reverse) were synthesized, based on the determined E-ACT/M-$CAA_{524}$ sequence (Figure 1) and used in PCR amplification reactions with genomic DNA from the SG1 resistant line Kvx 61-1, the SG3 resistant line Tvu14676 and the susceptible lines 524B and IT-84S-2246. The primers amplified two fragments of similar size to the AFLP fragment observed in IT82D-849; one that was present in both the resistant and susceptible genotypes and a slightly smaller fragment that was polymorphic, being present in resistant genotypes but absent in susceptible genotypes (Figure 2A). The polymorphic fragment was designated as SCAR 61R. Sequencing of both the monomorphic and polymorphic 61R amplification products from the different cowpea genotypes revealed that both had similar sequences, but that polymorphic fragment had a 69 bp deletion as compared to the monomorphic fragment (Figure 3).

Using the most conserved regions of the various sequences, a second set of sequence specific primers

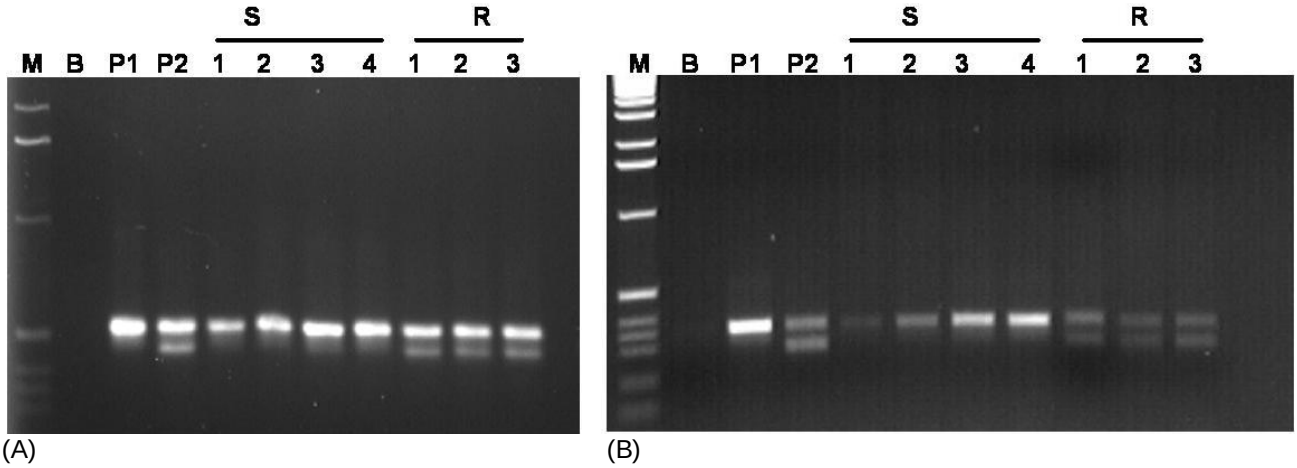


**Figure 2.** Bulked segregant analysis (BSA) of $F_2$ population derived from IT84S-2246 (P1) and Tvu14676 (P2) that was screened for resistance, using *S. gesnerioides* race SG3. In the figures: B = control (no DNA); S = Susceptible bulks; R = Resistant bulks, and M = 1 Kb ladder. Panel 2A: BSA carried out with SCAR 61R. Panel 2B: BSA carried out with 61R-M2.

```
        1                                                                   70
61R-T1  aattcacttatgactgagctatataagtttgttttcagatggatgaggtacaatttgagacttttcatga
61R-T2  aattcacttatgactgagctatataagtttgttttcagatggatgaggtacaatttgagacttttcatga

        71                                                                 140
61R-T1  atgcgaaaaatagtcacacacttatggaaaactttctctctttgaatcacatagttgcagttgtagattg
61R-T2  atgcgaaaaatagtcacacacttatggaaaactttctctctttgaatcacatagttgcagttgtagattg

        141                                                                210
61R-T1  aagatcttggttgatcttggaggcattttggcttggttgaggcttgaagaacatgtgtttggttggctag
61R-T2  aagatgttggttgatcttggaggcattttggcttggttgaggcttgaagaacatgtgtttggttggctag

        211                                                                280
61R-T1  ggagagccttcatcaagttttattgttcttgtgcctatgtagtgttcctggtgtgattcaaggtttgtaa
61R-T2  ggagagccttcatcaagttttattgttcttgtgcctatgtagtgttcctgcggtgattcaaggtttgtaa

        281                                                                350
61R-T1  gtgggattcttagtattggggataagatttttgtgggattcaaaaccttggtgtttgctttattcaaaag
61R-T2  gtgggattcttagtattggggataag-------------------------------------------

        351                                                                420
61R-T1  tgtaatcttggtgtgattttgtaaaaattttgataatagtggaaaaccatgctcaagttgtcttggtaaa
61R-T2  -------------------------aattttgataatagtggaaaaccatgctcaagttgtcttggtaaa

        421                                                                490
61R-T1  ctggatgtagatttgtgattgagtgaactagcataaaaacaagtgtgtgatctttttttccttatcttgt
61R-T2  ctggatgtagatttgtgattgagtgaactagcataaaaacaagtatgtgatctttttttccttatcttgt

        491                                          535
61R-T1  tcatctttaaaatcttaaggaaaccaagcaaatcaatttttgtta
61R-T2  tcatctttaaaatcttaaggaaaccaagcaagtcaatttttgtta
```

**Figure 3.** Comparison of SCAR 61R nucleotide sequences (T1 and T2) cloned from cowpea assession Tvu14676. Note that in the polymorphic fragment (T2) there is a 69 bp deletion compared to monomorphic fragment (T1).

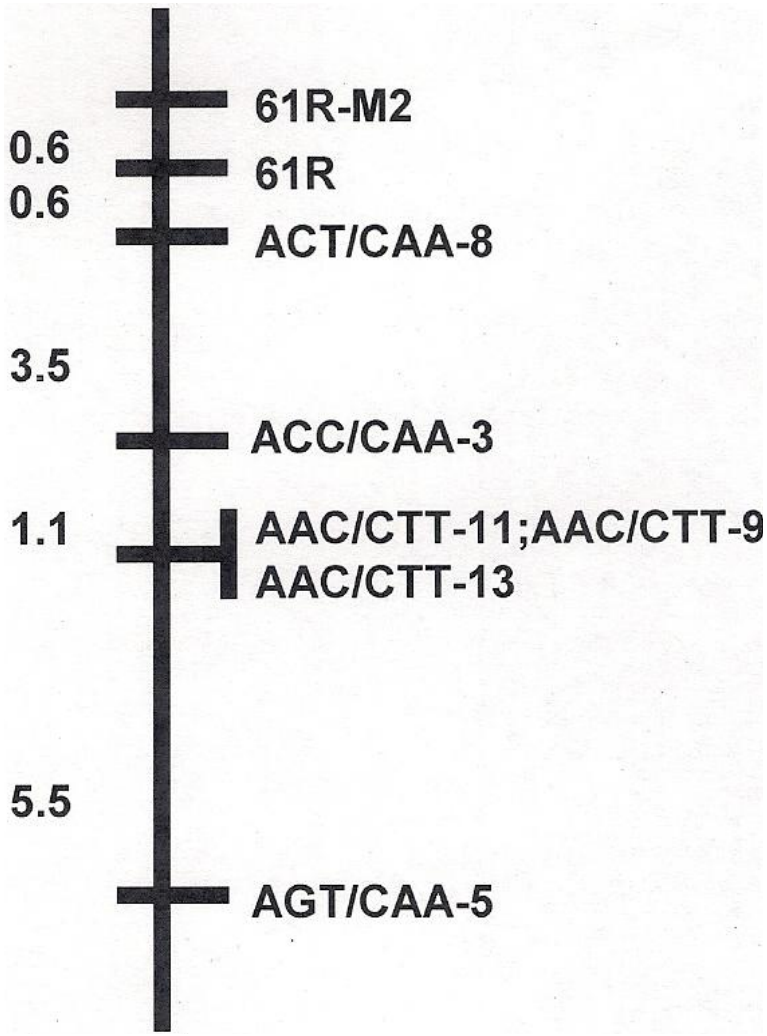


**Figure 4.** A portion of linkage group 1, showing the linkage relationships between SCAR 61R, 61R-M2 and ACT/CAA-8 (E-ACT/M-$CAA_{524}$). Horizontal bars represent the map positions of the markers and their names on the right side. Numbers on the left side indicate map distances between markers in centimorgans.

(designated 61R-M2F and 61R-M2R) were synthesized and tested for their ability to selectively amplify a SCAR in striga resistant versus susceptible parental and $F_2$ individuals. As shown in Figure 2B, the 61R-M2F/ 61R-M2R combination was equally as effective as SCAR 61R in detecting a polymorphism between resistant and susceptible individuals, and this SCAR was designated 61R-M2. However, 61R and 61R-M2 were linked to SG3 resistance gene by 3.5 cM and 10.6 cM, respectively in the $F_2$ population, derived from IT84S-2246 and Tvu14676 and the distance between these two makers was 7.1 cM.

Mapping of 61R and 61R-M2 loci in a population derived from 524B × IT-84S-2049 showed close linkage to their original AFLP locus (Figure 4). 61R mapped very close to the ACT/CAA8 (E-ACT/M-$CAA_{524}$), at the map distance of 0.6 cm, followed by 61R-M2 at 0.6 cM.

Genomic DNA from a set of 30 different selected cowpea breeding lines was screened for the presence of 61R and 61R-M2 PCR products (Table 1). All the cowpea accessions that had 61R also had 61R-M2 and if the 61R was absent, then 61R-M2 was also absent, irrespective of their resistance/susceptibility to SG3. Majority of the resistant accessions had both the bands, with few exceptions and majority of the susceptible accessions had single band with few exceptions.

## DISCUSSION

We have converted an AFLP marker E-ACT/M-$CAA_{524}$ into a SCAR marker that segregates with resistance to *S. gesnerioides* in the $F_2$ population, derived from a cross

between Tvx3236 × IT82D-849. This SCAR 61R was dominant marker, though there was a common PCR product in both parents. The common fragment was slightly larger than the polymorphic fragment and the polymorphism was due to 69 base pair deletion in the smaller fragment compared to that of a common product. Similar kind of deletions were also observed in SCAR markers derived from RAPD markers that were linked to *Cladosporium fulvum* resistance gene CF-6 in tomato (Wang et al., 2007) and from an AFLP marker for root knot nematode resistance gene N in pepper (Wang et al., 2009). But, both of these SCAR markers were co-dominant because of such deletion while in our case, both markers were dominant.

Though, there are few molecular markers available that show tight linkage to discrete race specific resistance genes for *S. gesnerioides* (Ouédraogo et al., 2001, 2002a; Boukar et al., 2004; Li and Timko, 2009). SCAR 61R is very tightly linked to the SG1 resistance gene and appears to be a promising marker for MAS. The SCAR 61R fragment linked to *S. gesnerioiedes* resistance has been amplified from other resistant cowpea accessions (Table 1) and it appears that this marker has broader application. Some of the resistant accessions tested did not show either 61R or 61R-M2 polymorphic bands, while some of the susceptible accessions did show both bands. One of the reasons could be that some of the SG1 resistance genes may be located on LG6, rather than on LG1 as in the case of the resistance genes found in Suvita-2 and IT-81D-994 (Ouédraogo et al., 2002a).

The identity of only one of the race-specific resistance genes to *S. gesnerioides,* present in cowpea is currently known (Li and Timko, 2009). Given that introduction of novel genetic material into cowpea by *Agrobacterium* mediated transformation is still labor intensive and time consuming (Popelka et al., 2006), rapid genetic improvement through genetic engineering is not practical at this point. Thus, the application of MAS appears to be the best and most immediately effective strategy to improve cowpea cultivars for *S. gesnerioides* resistance. The pyramiding of multiple resistances against *S. gesnerioides* by MAS appears to be the best available option. With appropriate molecular markers, augmentation of conventional breeding with MAS would allow the use of smaller populations, reduce the number of generations needed to reach the goal, and also increase the accuracy of evaluations (Timko et al., 2007). At present, the application of MAS in cowpea breeding is at the foundling stages, since the availability and number of informative molecular markers linked to traits of interest is still limited. The identification of tightly linked SCAR markers for genes of agronomic importance is an essential prerequisite.

Attempts to improve 61R marker yielded 61R-M2, a new marker which clearly distinguished the resistant from susceptible individuals. The latter one was found to be more specific than 61R, because PCR could be performed at a higher annealing temperature (55°C compared to 50°C for 61R) and better separation of polymorphic fragment from monomorphic fragment on agarose gels. Both 61R and 61R-M2 sequences were used to search in the cowpea GSS data base (Timko et al., 2008), legume information system and GenBank (Altschul et al., 1997). It did not yield any closely similar sequences, indicating that it could be a unique sequence and not related to or a part of a sequence that is similar to any genes reported so far.

Cowpea has a well developed map with about 440 markers distributed over 11 linkage groups, with an average map distance between markers being 6 cM (Ouedraogo et al., 2002a). Ouedraogo et al. (2001) reported four AFLP markers linked to Rsg2-1 (gene that confers resistance to SG1 from Burkina Faso) and six AFLP markers linked to gene Rsg4-3 (gene that confers resistance to SG3 from Nigeria). Based on this marker analysis, SG1 and SG3 resistance genes (present in B301, IT82D-849 and Tvu14676) were located on linkage group 1 of the improved cowpea map. Other markers linked to SG1 resistance Rsg3-1 and Rsg994-1, which are present in Suvita-2 and IT-81D-994, respectively located these genes on linkage group 6 (Ouédraogo et al., 2002a). On the genetic map of cowpea, 61R and 61R-M2 are located close to their original AFLP marker, E-ACT/M-$CAA_{524}$ (ACT/CAA-8). However, in another $F_2$ population derived from IT84S-2246 and Tvu14676, the map distance between 61R and 61R-M2 was 7.1 cM as compared to just 0.6 cM in the population derived from 524B and IT84S-2049. It appears that the map distance between the markers is cultivar dependent. The AFLP marker and 61R showed linkage to both SG1 resistance gene for *S. gesnerioides*, Rsg4-3 and SG3 resistance gene whereas; 61R-M2 is linked to SG3 resistance. It appears that these two resistance genes are closely linked indicating that race specific genes for *S. gesnerioides* may be clustered in cowpea genome.

Both classical and molecular genetic studies reported the clustering of disease resistance genes in plant genomes (Pryor and Ellis, 1993; Polzin et al., 1994; Witsenboer et al., 1995; Ashfield et al., 1998; Michelmore and Meyers, 1998; Ronald, 1998; Udupa and Baum, 2003; Kuang et al., 2005). Clustering may lead to new specificities by gene duplication, mutation and unequal crossing over during recombination, inter-allelic recombination or by gene conversion, thus resulting in gene evolution (Jones et al., 1997; Michelmore and Meyers, 1998; Hammond-Kosack and Parker, 2003).

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