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Full Length Research Paper

Introgression of *Striga* resistance gene into farmers' preferred cowpea varieties in Niger

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Striga gesnerioides* (Wild.) Vatke, a parasitic flowering plant is one of the main biotic sources of stresses that challenge cowpea production in drought-prone areas. At least seven races of *S. gesnerioides* with differential virulence on cowpea cultivars have been identified in West and Central Africa. This renders breeding effort very delicate. However the identification of molecular markers tightly linked to the various *Striga* races opened the way to the marker assisted selection for the resistance to *S. gesnerioides* in cowpea.The objective of this study was to introgress one *Striga* resistant gene (*Rsg1*) into susceptible and adapted cowpea genotypes. Marker assisted selection with backcross breeding was used to transfer *Rsg1Striga* resistant gene from the breeding line IT93K-693-2 into three farmers' preferred varieties; IT90K-372-1-2, KVx30-309-6G and TN5-78. The microsatellite marker SSR_1 was used to tract and introgress the resistant marker were selected in BC_2F_3, BC_3F_3 and F_6 populations derived from the crosses IT90K-372-1-2 x IT93K-693-2 and TN5-78 x IT93K-693-2. Further evaluations and improvement of these genotypes will accelerate the release of varieties combining farmers' preferred traits with stable resistance to *Striga.

Key words: cowpea, farmers' preferred varieties, introgression, *Striga gesnerioides*, *Striga* resistant gene.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L) Walp.) is one of the most important grain legumes in Africa. It is an essential supplement to the diet by its relatively high protein content and also, a valuable commodity that generates income to farmers. There has been a significant increase of cowpea worldwide production in the last few decades. However, *Striga gesnerioides* has become a serious biological constraint to the increase of production in smallholders' farms. *S. gesnerioides* is difficult to control and no single method can counter its injury on yield. Indeed, yield losses ranging from 83-100 % have been reported on susceptible cultivars (Cardwell and Lane, 1995). Thus, host plant resistance appears to be the most economically and environmentally sound strategy to control effectively *Striga* since it is affordable to small-scale farmers (Omoigui *et al.*, 2007). Unfortunately, resistant cowpea varieties identified or bred so far have mostly poor agronomic traits hardly accepted by farmers or end-users. The current focus in cowpea breeding and genetic improvement around the world is combining desirable agronomic characteristics such as: time to maturity, photoperiod sensitivity, plant type, and seed quality with resistance to the major diseases, insect pests or parasites that agronomically afflict adapted cowpea cultivars (Timko and Singh, 2008). Currently, depending on the type of trait being introgressed a decade would be required to breed a superior improved line through conventional breeding methods. Developing molecular marker-based tools for tracking single genes and quantitatively inherited traits linked to major disease and

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pest resistance, as well as the establishment of an array of protocols for marker assisted selection (MAS) can shorten the time frame. Indeed molecular markers used in 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 *S. gesnerioides* on cowpea genotypes (Singh, 2002) has serious impact on breeding and selection procedures. Therefore, the need of using race specific markers to complement conventional breeding methods for identification of cowpea resistant genotypes is essential. To date, at least seven races of *S. gesnerioides* have been identified based on host differential response and genetic diversity analysis within the cowpea growing regions of West Africa (Lane *et al.*, 1996, Botanga and Timko, 2006). Molecular markers linked with resistance genes to races SG1, SG2 and SG3 have been identified, and several sequence-confirmed amplified regions (SCARs) have been developed for use in MAS (Li et al, 2009).

A recently identified cowpea breeding line, IT93K-693-2, has resistance to all known *Striga* races (Singh, 2002) but lacks farmers' traits. It has a single dominant gene *Rsg1* that confers the resistance to the strain SG3 inherited from the cultivar B301 (Boukar *et al.*, 2004). This gene *Rsg1* present in the breeding line IT93K-693-2 and Botswana landrace B301 was found to be a dominant gene that could be introgressed into adapted cowpea genotypes through pedigree and backcross breeding (Tignegre, 2010).

In Niger, none of the landraces grown by farmers was found to be resistant to *Striga* and most of the introduced resistant varieties have poor agronomic traits. On the other hand some of the resistant genotypes show levels of breakdown of the resistance. The virulence of this parasitic weed and its rapid spread require an urgent need of varieties with multiple resistance (Boukar*et al*., 2004).

The objective of this research was to introgress the resistant gene *Rsg1* in three farmers' preferred varieties using backcross breeding and select resistant lines as basis for developing well adapted varieties.

MATERIALS AND METHODS

Plant Materials

Three farmer-preferred varieties (recurrent parents): IT90-K-372-1-2; KVx30-309-6G; TN5-78, susceptible to *Striga gesnerioides* were selected through Participatory Rural Appraisal (PRA) as parents for improvement to *Striga* resistance. The breeding line IT93K-693-2 was selected as the donor line. The choice of IT93K-693-2 was because it was resistant in the germplasm screening in Niger and it has been reported also to be resistant to all known *Striga* races (Table 1).The genotype IT93K-693-2 is a three way cross hybrid: [(IT88D-867-11 x IT90K-76) x IT89KD-374] that inherited the resistance gene *Rsg1* from B301 and the resistance to *Striga* race SG4z from the line IT88D-867-11.

Crosses were made between IT90-K-372-1-2 and IT93K-693-2; KVx30-309-6G and IT93K-693-2 and TN5-78 and IT93K-693-2 at Maradi INRAN station in 2013. The F1 generations were backcrossed to the recurrent parents (IT90-K-372-1-2; KVx30-309-6G and TN5-78) and also self-pollinated to generate both BC_1F_1 and F_2 populations. BC_1F_1 and F_2 generations were screened for *Striga* resistance in pot and the selected plants BC_1F_1 were again backcrossed to the recurrent parents (IT90-K-372-1-2; KVx30-309-6G and TN5-78) to produce BC_2F_1 and F_2 plants were self-pollinated to produce F_3 generations. The same procedure was used to generate BC_3F_1 and F_4 generations. BC_2F_1, BC_3F_1 and F4 were advanced by successive self-pollination to BC_2F_3, BC_3F_3 and F_6 generations respectively. The lines advancement was done by combining genotypic and phenotypic data for the backcross population while the selfed progenies (F_2 to F_6) were advanced based on the absence of *Striga* infestation in pots.

Plant culture and DNA extraction

Cowpea parental lines and the derived populations were grown in pots in a greenhouse at INRAN Maradi station from 2013- 2015. Each pot had a volume of seven liters filled with 5 Kg of a mixture of sandy soil, clay and farmyard manure to a ratio of 2:1:1 respectively. The mixture was previously sterilized. After soil infestation with about 1000 seeds per pot of one year-old *Striga gesnerioides*, the pots were watered for two weeks to precondition *Striga* seeds in order to break their dormancy and ensure optimum germination. Three seeds of cowpea were sown per pot. The seedlings were thinned to one per pot at 2 weeks after germination. The pots were watered every two days or when necessary in order to keep them moist.Genomic DNA was extracted from leaf tissues of 2 weeks old plants using the Fast Technology for Analysis (FTA) cards as described by Omoigui*et al*. (2012). The young leaf was placed on the FTA Plantsaver card covered with parafilm paper, pressure was applied with a pestle briefly until plant material was sufficiently transferred to the card. After air drying for about 1 hour, FTA cards were placed in a paper punch and stored at ambient temperature in a dry location. The samples were taken to Institut National de l'Environnementet de la RechercheAgronomique (INERA) Genetics and Plant Biotechnology laboratory at Kamboinse in Burkina Faso for the genotyping.

Preparation of samples for PCR Analysis

The samples were prepared as described by Omoigui*et al*. (2012). A disc from the dried FTA card was removed using a clean Haris micro punch and placed directly into

Table 1. Cowpea varieties used as parents in backcross breeding with their pedigree information.

Lines or cultivars	Pedigree	Resistance to *S. gesnerioides*
IT93K-693-2	(IT88D-867-11 x IT90K-76) x IT89KD-374.	*Rsg1* from B301 and SG4z from Benin
IT90K-76	(B301 x IT90K-2246-4) x IT90K-2246-4	
IT88D-867-11		SG4z from Benin
IT90K-2246-4		Susceptible to all races
IT89KD-374		Susceptible to all races
TN5-78	Selected and improved landrace	Susceptible to all races
IT90K-372-1-2	(IT87F-1784-2 x IT84S-2246-4) x IT87F-1784-2	Susceptible to all races
KVx30-309-6G	Not found	Susceptible to all races

a 1.5 mL Eppendorf tube. Precautions were taking in order to prevent cross contamination, by cleaning the Haris micro punch with a tissue dampened with 70% ethanol in between samples. The disc was washed twice with 200 µL of FTA reagent incubating for five minutes for each wash followed by a repeated wash with 200 µL of 70% ethanol, incubating for 5 mn at room temperature and the liquid was discarded. The tubes were inverted and drained on a paper towel and air dried for close to 1 h. After drying, the tubes were transferred for PCR analysis (Omoigui*et al*., 2012).

PCR analysis

One primer SSR_1 was used for the PCR analysis. Each PCR mixture (25 µL final volumes) contained, besides the purified 2 mm FTA DNA disc containing the DNA sample, 18 µL of sterilized water, 2.5 mM each of DNTPs mix and 10 x PCR buffer, 0.05 µL of Taq polymerase, and 1 µL of each of the forward and reverse primers. PCR reactions were performed on a heated lid thermal cycle (Biometra) operated at following conditions: 35 cycles of denaturation at 94 °C for 30 s, followed by annealing at 57.5 °C for 30 s and extension at 72 °C for 2 min. The repeat sequences of the primer are as shown below (table 2).

Electrophoresis

PCR product was electrophoresed on a 2% agarose gel stained with ethidium bromide. The gels were run for approximately 1 hour 30 minutes at 120 volt in 1X TAE buffer (45 mmol L -1 glacial acetic acid, 0.5 mmolL -1 EDTA, pH, 8.4). A 1 kb DNAstandardladder was loaded in the first well for band size determination of PCR products. The ethidium bromide-stained gel was visualized on an UV transilluminator and images photographed using a Polaroid camera.

Marker assisted selection for *Striga gesnerioides* resistance in segregating populations of cowpea

Plant genotyping was done using SSR_1 marker at BC1F1, BC2F1, BC2F2, BC2F3, BC3F1, BC3F3 and F6 generations. For all these generations, plant sampling, DNA extraction, PCR analysis and electrophoresis were done as described above. Data was scored by observing gels under UV light and recording the number of samples showing marker's single band. SSR_1 marker produced single bands of 150bp of PCR product with amplification only in resistant genotypes. Selection for advancement was done based on the presence of marker allele. No screening was done in field in all the stages; however, in order to confirm the effectiveness of marker assisted selection in introgressing*Striga* resistant gene, data on *Striga* emergence and dates to flowering was taken in pots for the genotyped BC_3F_3, BC_2F_3, F_6generations and their parents included as checks. The numbers of plants per generation were 3, 10 and 7 for BC_3F_3, BC_2F_3 and F6 respectively.

Marker validation

SSR_1 marker was validatedusing the basic generations: two contrasting parents, P_1(IT93K-693-2), resistant to *Striga* and P_2 (TN5-78), susceptible parent, 3 F_1 individual plants, a susceptible F_2 individual plant, a resistant F_2 individual plant, a susceptible BC_1F_1 individual plant and a resistant BC_1F_1 individual plant.

RESULTS

DNA was successfully extracted from leaf tissues using FTA cards. One primer SSR_1 linked to *Striga* resistance gene Rsg*1* was used to discriminate between resistant and susceptible lines in the populations.

Parents genotyping

The resistant parent IT93K-693-2 and the three farmers' preferred varieties were first genotyped in order to confirm the polymorphism of SSR_1 marker. The results showed the existence of a unique band with the resistant parent at 150bp of PCR products while the susceptible varieties had no bands (Figure 1).

Table 2. Structure of SSR1 primer used in MAS procedures.

Name	Repeat sequence
SSR_1 CP3 (F)	CAAGAAGGAGGCGAAGACTG
SSR_1 CP3 (R)	CCTAAGCTTTTCTCCAACTCC

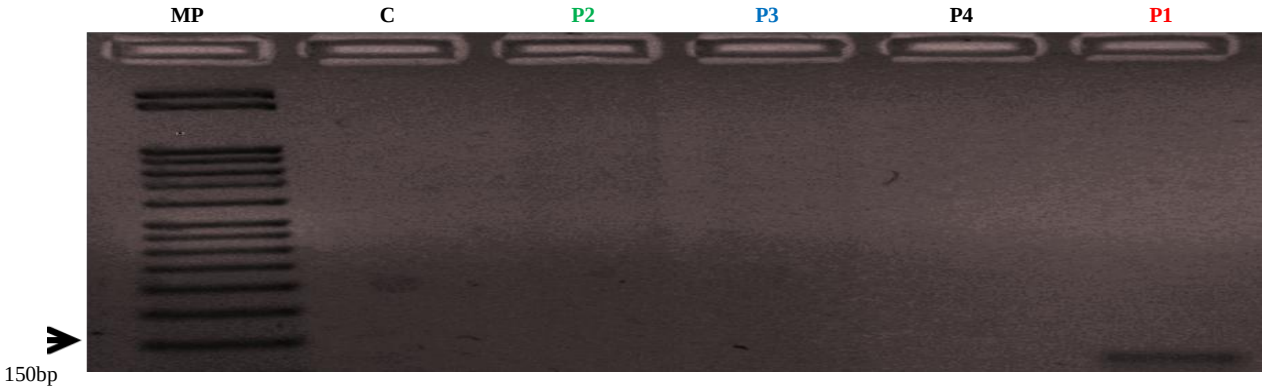

Figure 1:Results from PCR amplification of genomic DNA by SSR_1 for the parents. M1=1 kb Ladder; C=control; P1=IT93K-693-2; P2=IT90K-372-1-2; P3= KVx30-309-6G; P4=TN5-78 resolved in 2%agarose gel stained with ethidium bromide. Resistant line has the 150bp band.

BC_2F_3, BC_3F_3 and F_6 derived populations genotyping

DNA samples were taken from twenty three BC_2F_3, BC_3F_3, F_6 progenies and their parents for genotyping at the final stage of selection. Ten individual plants had the resistance marker as shown by the presence of single band at 150bp of PCR products (Figure 2 and 3). The different resistant individual plants showing the marker selected per population were as follows:

- 2 individual plant atBC_2F_3generation derived from IT90K-372-1-2 x IT93K-693-2
- 2 individual plant atF6 generation derived from IT90K-372-1-2 x IT93K-693-2
- 1 individual plant at BC_3F_3generation derived from TN5-78 x IT93K-693-2
- 5 individual plant atBC_2F_3generation derived from TN5-78 x IT93K-693-2.

Marker validation

The marker SSR_1 produced a monomorphic banding pattern that can be scored, red and reproduced. The single band at 150bp of PCR product was observed with the resistant parent, the F_1 plants, the resistant F_2 plant and the resistant BC_1F_1 plant while the bandwas absent with the susceptible parent, the susceptible F_2 plant and the susceptible BC_1F_1 plant as expected (Figure 4).Pots screening of BC_2F_3, BC_3F_3 and F_6 derived populations

Table 3 presents the results from the phenotyping of 23 genotyped individual plants. Dates to flowering varied from 34 days after planting (DAP) for the line BC_2F_3A1-1219-4 to 57 days for F_6A1-21. All the selected resistant progenies: BC_2F_3A1-1219-4, BC_2F_3A1-1219-1 and F_6A1-12 derived from the cross IT90K-372-1-2 x IT93K-693-2 with 34, 36 and 39 days respectively flowered before the recurrent parent IT90K-372-1-2 (40 days). The line F_6A1-24 with 45 DAP is the only resistant progeny derived from a same cross that flowered after the recurrent parent. The progeny BC_2F_3A1-1219-4 flowered before both parents. In the second cross, TN5-78 x IT93K-693-2, the progeny BC_3F_3C1-1 with 38 DAP was the only one that flowered before the recurrent parent TN5-78 which flowered at 42

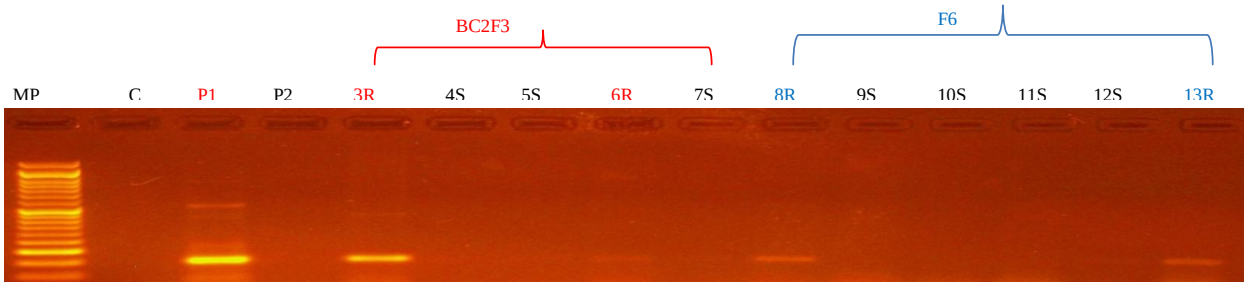

Figure 2. Results from PCR amplification of genomic DNA by SSR1 for the BC2F3 and F6progenies derived from IT90K-372-1-2 x IT93K-693-2. M = 1 kb ladder, C = control withoutgenomic DNA template, P1 = IT93K-693-2, P2 = IT90K-372-1-2 resolved in 2%agarose gel stained with ethidium bromide R and S indicate resistant and susceptible respectively.

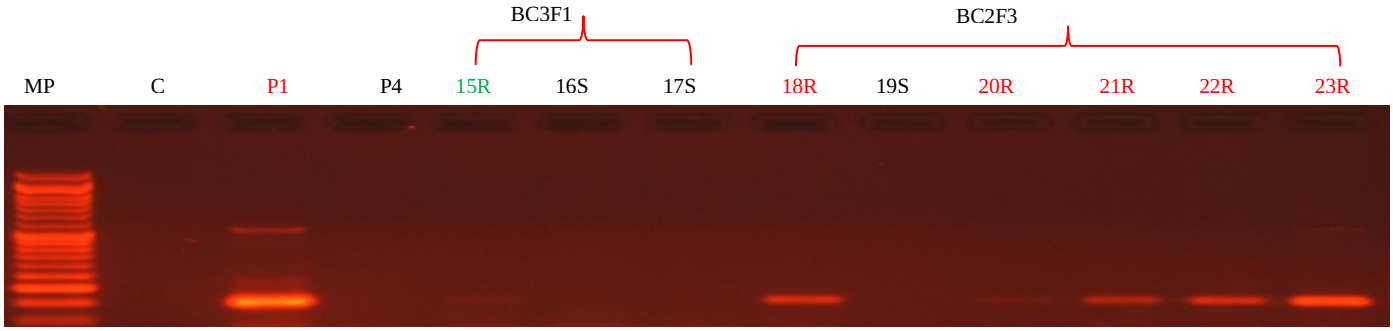

Figure 3. Results from PCR amplification of genomic DNA by SSR1for the BC2F3 and BC3F3 progenies derived from TN5-78 x IT93K-693 -2. MP = 1 kb ladder, C = control without genomic DNA template, P1 = IT93K-693-2, P4 = TN5-78 resolved in 2%agarose gel stained with ethidium bromide R and S indicate resistant and susceptible respectively.

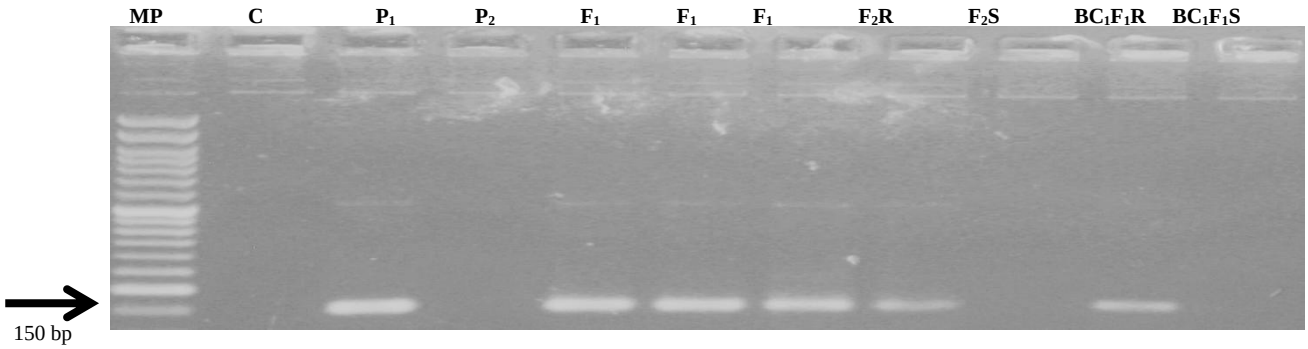

Figure 4. Results from PCR amplification of genomic DNA by SSR1 for marker validation.P1 = IT93K-693-2; P2 = TN5-78resolved in 2%agarose gel stained with ethidium bromide,R and S indicate resistant and susceptible respectively. MP represents 1 Kb ladder.

DAP. The number of *Striga* emerged shoots varied from 0 to 19 shoots. The resistant individuals that carried SSR1 marker were free of *Striga* emerged shoots. However 50% of the susceptible individuals without the resistant marker did not support *Striga* emergence.

The susceptible line F_6A1-21 had the highest number (19) of *Striga* emerged shoot.

DISCUSSIONS

In the present study, FTA cards technique was successfully used in DNA extraction from leaf tissues. As previously reported by (Omoigui *et al.*, 2012), this method was suitable for molecular analysis by PCR-based techniques similar to that obtained by classical methods using liquid nitrogen extraction. SSR_1 marker was used to discriminate between resistant and susceptible cowpea genotypes. This marker was found to co-segregate with *Striga gesnerioides* race 3 or SG3 resistance gene (Li and Timko, 2009). SSR_1 primer identified resistant lines by amplification of the 150 bp bands in only resistant genotypes as found by (Asare *et al.*, 2013). The marker was reliable since all the genotypes with SSR_1

Table 3. Reaction of cowpea lines: BC_2F_3, BC_3F_3 and F_6 populations derived from crosses IT90K-372-1-2 x IT93K-693-2 and TN5-78 x IT93K-693 -2 with their parents to *Striga gesnerioides* in pots.

Phenotypic reaction					**Genotypic reaction**
N^0	Genotypes	Dates to flowering	Pot trial	*Striga* emergence /pot	SSR_1marker150bp
1	IT93K-693-2	36	R	0	+
2	IT90K-372-1-2	40	S	6	-
3	BC_2F_3A1-1219-1	36	R	0	+
4	BC_2F_3A1-1219-2	45	R	0	-
5	BC_2F_3A1-1219-3	39	R	0	-
6	BC_2F_3A1-1219-4	34	R	0	+
7	F_6A1-11	40	R	0	-
8	F_6A1-12	39	R	0	+
9	F_6A1-13	41	R	0	-
10	F_6A1-21	57	S	19	-
11	F_6A1-22	37	S	1	-
12	F_6A1-23	37	R	0	-
13	F_6A1-24	45	R	0	+
14	TN5-78	42	S	4	-
15	BC_3F_3C1-1	38	R	0	+
16	BC_3F_3C1-2	39	S	1	-
17	BC_3F_3C1-3	40	S	17	-
18	BC_2F_3C1-220-1	52	R	0	+
19	BC_2F_3C1-220-2	53	R	0	-
20	BC_2F_3C1-220-3	54	R	0	+
21	BC_2F_3C1-220-4	52	R	0	+
22	BC_2F_3C1-220-5	44	R	0	+
23	BC_2F_3C1-220-6	55	R	0	+

did not support any *Striga* emergence in pots during the gene introgression process. Moreover, the resistant progenies could be traced back to their first parental resistant generations. The reproducibility of all the genotyping results and the consistency of the expected results for marker validation confirmed this reliability. In an earlier study (Asare*et al*., 2013), the 150bp SSR_1 marker was found to be efficient at 92.6% in discriminating ability. This implies the closeness of the marker to the resistance gene *Rsg1*. Pot *Striga* screening results revealed that IT93K-693-2 has the resistance to *Striga* race SG3 prevalent in Niger. Its resistance was characterized by neither emergence nor attachment of the parasite to the roots. The line IT93K-693-2 is a derivative of Botswana landrace B301. Earlier inheritance studies indicated the monogenic dominance of the resistance to *Striga* races SG1, SG2, SG3 and SG4 in some cowpea cultivars (Singh and Emechebe, 1990; Atokple*et al*., 1993; Moore *et al*., 1995; Tignegre, 2010; Asare*et al*., 2013; Omoigui*et al*., 2015). In this study, the segregation ratios were not analyzed in order to confirm previous work because the population size was low (10-20) for efficient handling of pots culture in limited facilities of a common screen house. The three farmers' preferred varieties used in this study showed high levels of *Striga* susceptibility during the gene introgression process, demonstrated by the high number of *Striga* emergence or attachments. All the three crosses between IT90-K-372-1-2 and IT93K-693-2; KVx30-309-6G and IT93K-693-2 and TN5-78 and IT93K-693-2 were compatible and able to generate progeny populations. The resistance gene *Rsg1*was tracked

and introgressed into 2 different adapted varieties IT90-K-372-1-2 and TN5-78 using marker assisted selection. The cross between KVx30-309-6G and IT93K-693-2 yielded poor seeds at BC_2F_2 and BC_3F_1generations resulting in the absence of resistant lines from these stages of gene introgression process. This could be attributed to the low compatibility of this particular cross. However, the combination of FTA technique of DNA extraction, a good source of *Striga* resistance, IT93K-693-2 having a single dominant gene and the use of a reliable marker contributed to transferring *Striga* resistance into farmer-preferred cowpea varieties. Marker assisted selection was used from early generations of backcrossing to BC_3F_3 generations while it was delayed to F_6 generations for the recombinant inbred lines. Phenotypic selection was applied in developing F_6 populations, but the precision of MAS enabled the identification of two individual resistant F_6 plants out of a total of seven genotyped. This result confirmed that conventional methods of breeding and DNA marker technology are complementary. In this study, some individual plants selected as resistant based on their phenotype were identified later on as susceptible after a progeny test. This can be explained by the escape from *Striga* infection which may be due to the growing conditions in pots. This could be why 50% of individuals without the marker were free of *Striga* emergence in the phenotyping in pots. It can also be explained by crossing over during meiosis where the gene separates from the marker. Those progenies without the marker but carrying the resistance gene will be resistant while the progenies without both marker and gene will be susceptible. Meanwhile, all the genotypes with SSR_1 marker were *Striga* resistant because they were free of *Striga* infestation. This confirmed the efficiency of MAS over the conventional method which is time consuming, laborious and lacks precision. The earliness of some progenies derived from the different crosses over the parents can be explained by the transgressive segregation of flowering characters from these parents. The donor parent IT93K-693-2 is an early maturing breeding line as compared to farmers' preferred varieties.

CONCLUSIONS

In this study, marker assisted selection with backcross breeding coupled with successive selfing was used to introgress*Striga* resistance gene *Rsg1* into two adapted and farmers' preferred cowpea varieties. The resistant genotypes were identified by the presence of single DNA band of 150bp by SSR_1 marker which was absent in the susceptible lines. The resistant BC_2F_3 and BC_3F_3 cowpea lines identified in the current study will have to be further advanced using marker assisted background selection to recover entirely the genome of the farmers' preferred varieties. After the 4^{th} backcross, the lines will be selfed before further evaluation and released to farmers. Also, the resistant F_6 lines will be advanced to F_8 before evaluation and release to farmers. Pyramiding of *Striga* resistance genes and other preferred traits identified as breeding priorities into elite cowpea varieties is becoming possible through MAS breeding which saves the breeder progeny testing. This will speed up the system of creating and delivery of new adapted varieties to farmers.

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