Full Length Research Paper

Genetic mapping of quantitative trait loci (QTLs) with effects on resistance to flower bud thrips (*Megalurothrips sjostedti*) identified in recombinant inbred lines of cowpea (*Vigna unguiculata* (L.) Walp)

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The first major insect pest of cowpea at reproductive stage is the flower bud thrips (FTh), which, if not controlled, is capable of causing significant grain yield reduction. Breeding for resistance to FTh in cowpea has been hindered by the quantitative nature of the resistance, and the breakdown of resistance under high insect infestation. The purpose of this study was to use molecular markers to identify genetic loci associated with the expression of resistance to FTh. A set of 92 recombinant inbred lines (RILs) was generated from a cross between susceptible and resistant lines. One hundred and thirty nine markers [134 Amplified Fragment Length Polymorphism (AFLP) and 5 cowpea derived microsatellites] were used to construct a linkage map using this set of RILs. The linkage map spans 1620 cM of the cowpea genome and markers were distributed in 11 linkage groups. Average distance between adjacent markers was 9.6 cM. There were significant associations between 23 DNA markers and resistance to flower bud thrips ($P<0.05$) using single marker analysis. QTLs with effects on resistance were detected in five linkage groups. The QTL on linkage group 3 explained 32.0% of the variation for resistance while all the five QTLs together explained 77.5%.

Key words: *Vigna unguiculata*, *Megalurothrips sjostedti*, quantitative resistance, molecular markers, gene mapping.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp.] is an important grain legume crop in sub-Saharan Africa where it serves as a rich and inexpensive source of dietary protein. Its grain yield potential is as high as 3.0 t/ha, but in farmers' fields, particularly in Africa and Asia, it is much less, averaging 0.2 – 0.4 t/ha (Singh et al., 1997). Insect pests are the major causes of these large yield deficits. The first major insect pest of cowpea at flowering stage is the flower bud thrips (FTh), which is capable of causing significant grain yield reduction in the crop. The yield reduction due to FTh ranges from 20 to 80% but under severe infestation, grain yield may be almost nil (Singh and Allen, 1980). Spraying appropriate insecticides can reduce the damage caused by FTh and other insect pests (Afun et al., 1991). Growing resistant varieties, however, appears to be the best option for the small-scale farmers of tropical Africa owing to its low cost, compatibility with other control methods and the low incomes realised by the farmers (Dent, 1991). Some cowpea land races have been identified with low levels of resistance to FTh but under high infestation the resistance in these lines succumb and the desired levels of resistance have not been identified or obtained among available cowpea landraces and improved varieties. To this end, concerted efforts are being made to develop cowpea varieties with higher levels of resistance to FTh.

A recent study (Omo-Ikerodah et al., 2000) has indicated that resistance to FTh may be controlled by three to five genes. Polygenes controlling metrical traits are usually distributed at several loci, which may not be

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Table 1. Primers, sequence information, repeat number and product size of cowpea derived microsatellite primers used for this study.

Primer	Primer sequence	Repeat	Product size
VM8	5' TGG GAT GCT GCA AAG ACA	(AG)16	285
VM10	5' TCC CAC TCA CTA AAA TAA CCA ACC	(AC)3(CT)10(AC)3	278
VM26	5' GGC ATC AGA CAC ATA TCA CTG	(TC)14	294
VM33	5' GCA CGA GAT CTG GTG CTC CTT	(AG)18(AC)8	270
VM36	5' ACT TTC TGT TTT ACT CGA CAA CTC	(CT)13	160

linked to one another. Such traits are therefore less amenable to conventional breeding methods in comparison with those controlled by single genes. However, the advent of DNA markers has made it possible to significantly upgrade information about the genetic basis underlying complex traits (Paterson et al., 1988). DNA markers can be used to develop saturated genetic linkage maps with full genome coverage suitable for locating and characterizing individual QTLs. Selection for these markers can be effected and this will lead to selection for the QTL with which they are associated. The usefulness of such detailed maps in locating and characterizing QTLs has already been demonstrated for seed weight, pod length, aphid and striga resistance in cowpea (Fatokun et al., 1992, 1993, 2000; Ouédraogo et al., 2002). Molecular markers associated with resistance genes controlling FTh would be extremely beneficial because plant breeders could use such markers during preliminary selection process to track the loci in existing population or to pyramid resistance into new populations. The purpose of this study was to identify DNA markers closely linked with genes for resistance to FTh in cowpea.

This paper reports on the development of a genetic linkage map consisting of AFLPs and microsatellite markers segregating in a recombinant inbred line population derived from a cross between resistant and susceptible cowpea lines. The genetic linkage map was used to identify and map QTLs associated with resistance to FTh in cowpea.

MATERIALS AND METHODS

Plant materials

One hundred and forty-five RILs (F_{10}) were derived from a cross between two cowpea lines, 'Sanzi' (resistant to FTh) and 'VITA7' (susceptible to FTh), by single seed descent (SSD) method. The RILs along with the two parental lines were evaluated in the screenhouse at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Testing of resistance to FTh

The RILs at F_{10} generation were evaluated for resistance to FTh in the screenhouse .The parents and RILs were planted in the screenhouse in 2001 in a randomised complete block design with two replications. One plant was maintained per pot of 21 cm diameter and 25 cm in dept, filled with 15 kg topsoil. Adult FTh were intro-

duced from the field into the screenhouse twenty-three days after sowing by dropping three flowers, each containing not less than 30 FTh in each pot. Subsequently, flowers loaded with FTh were introduced into the screenhouse on a daily basis until a high population of the insect was achieved. The plants were scored for damage caused by FTh first at 35 days after planting and subsequently at weekly intervals for four weeks thereafter. Plants were scored on a scale of 1 – 9 [1 = highly resistant, 9 = highly susceptible (Jackai and Singh 1988)]. Usually, cowpea lines with FTh damage score of 4.0 and less are classified as resistant to the pest.

DNA extraction, AFLP and microsatellite analyses

Newly expanded leaves were collected from each RIL and the parents for DNA isolation. Total genomic DNA was extracted using the procedure described by Dellaporta et al. (1983). AFLP analysis was carried out according to the procedure described by Vos et al. (1995). DNA samples of the two parents crossed to generate the mapping population were used to screen 48 AFLP primer combinations. The primers that detected polymorphisms between the parents were selected for AFLP analysis of 92 RILs. DNA was digested with *Eco*RI and *Mse*I to generate the template for AFLP reactions. The names of the restriction site were used to designate the Selective primers (E, *Eco*RI; M, *Mse*I) and nature of the additional nucleotides (e.g., E-ACA M-CAA, etc,). The 92 RILs were analysed with 18 (*Eco*RI+3 and *Mse*I+3) pairs of primers. Products of the selective amplifications were separated on 6% polyacrylamide gel. DNA fragments were transferred to Hybond N$^+$ membrane. Signals were detected using a digoxigenin (Dig) colorimetric detection (Roche) procedure as described by the manufacturers. The RILs were scored for presence or absence of bands.

One hundred and twenty-one cowpea derived microsatellite primers were screened for polymorphism between the two parental lines. Seventy-nine primers amplified DNA from the parental lines at annealing temperature of 64 and 54°C. Five primers, which amplified and showed clear polymorphic bands on the polyacrylamide gels, were used to analyze the 92 RILs. Their names, primer sequence, repeat types and predicted fragment length are given in Table 1. These primer sets were isolated from cowpea microsatellite – enriched libraries constructed from the DNA of the breeding line IT84S-2246-2 (Li, and Scoles, Unpublished data). The PCR reaction conditions were as described by Li et al. (2001). The gel was fixed, and stained using silver staining kit (Promega Corp., Madison, WI). The RILs were scored directly on the air-dried polyacrylamide gels for presence or absence of polymorphic bands, or as slow or fast bands.

Developing a cowpea genetic linkage map

Linkage order of markers was performed using Mapmaker/Exp 3.0 (Lander et al., 1987). A minimum LOD score of 3.0 and recombination fraction of 0.40 served as thresholds in inferring linkage between markers in the two-point analysis. Groups of markers
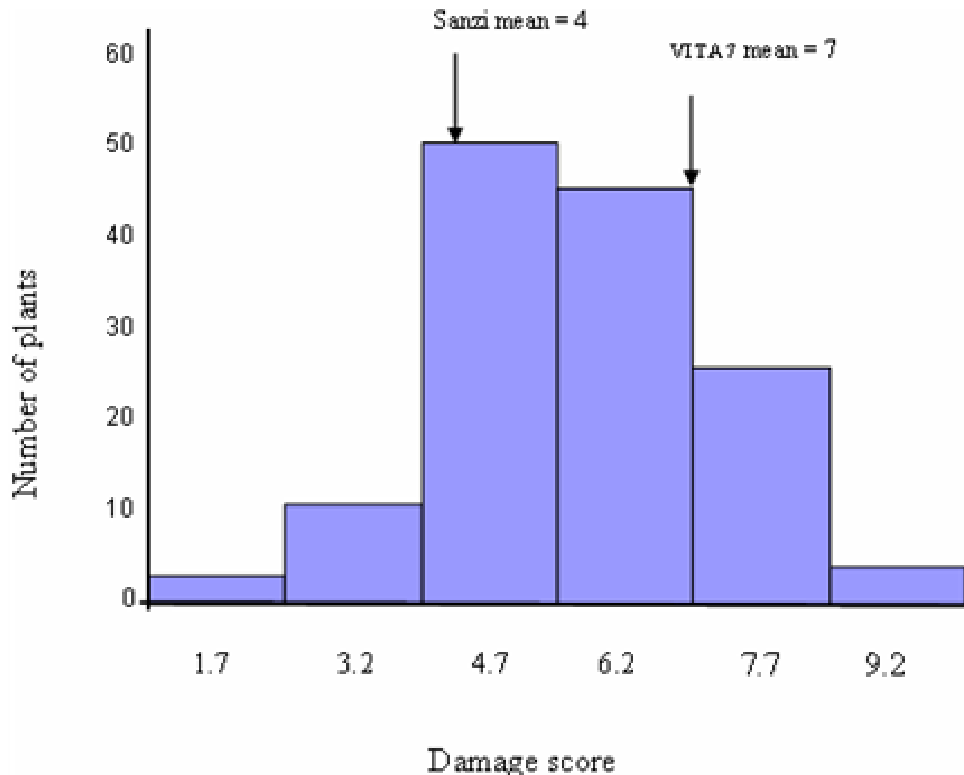
Figure 1. Frequency distribution of RILs for flower bud thrips damage in the cowpea cross Sanzi x VITA 7

obtained from the two point analysis were initially ordered using 'three-point' analysis and derived orders confirmed by 'multipoint' analysis using the 'multipoint | compare' function with a subset of not more than six most informative markers (that is, markers not linked by too small a recombination fraction or too low a LOD value). All remaining markers were tried in every interval using the 'multipoint | try' function. The frequencies of observed recombine-tion between two markers were converted to genetic distance, using the map function of Kosambi (1944). All maps were drawn to scale using the software program Mapchart (Voorrips 2002). To determine if the markers were randomly distributed within a linkage group, a chi-square goodness of fit test was used as described by Roupe van der Voort et al. (1997).

QTL mapping

Marker genotypic data from the linkage map and quantitative data for resistance to FTh were used for interval mapping using the program Mapmaker/Exp.3.0 and Mapmaker/QTL1.1 (Lander and Botstein, 1989; Lincoln et al., 1992). The LOD score of association between the genotype and the trait data was calculated using the free model of QTL effects (Paterson et al., 1988; Lincoln et al., 1992). A LOD score of 2.0 was chosen as the threshold level for presence of QTL. The Mapmaker/QTL1.1 program was also used to obtain estimates of the percentage of the total phenotypic variation explained (PVE) by the detected QTLs. The phenotypic effect of each QTL interval was also determined. Primary QTLs identified from the above step were fixed and the genome scanned again for other QTLs (secondary QTLs). Furthermore, the bilocus and multilocus models from the Mapmaker/QTL1.1 program were used to estimate the PVEs for various combinations of QTLs. The PROC

GLM procedure in SAS was used to detect significant associations between segregating markers and flower bud thrips damage scores as a trait (P<0.05). The coefficient of determination (r^2) from the regression, estimated the total proportion of phenotypic variation due to additive effects.

RESULTS

Phenotypic data

The characteristic symptoms of FTh damage include browning and dry stipules, stunted peduncles and flower bud abscission leading to no or very few pod production. These characteristics were expressed in varying degrees in both the susceptible checks and the RILs. The RILs displayed a continuous distribution of FTh damage scores averaged over four ratings taken at weekly intervals on each RIL in the screenhouse (Figure 1), indicating that more than two genes probably control the resistance of cowpea to flower bud thrips. The distribution of the phenotypic data was significantly different from normal (W statistic 0.86, P<0.01). Some RILs were characterised by lower damage ratings than the resistant parent thus suggesting transgressive segregation of the genes controlling this trait (Figure 1). Regression of the FTh damage score and the number of pods produced per plant showed negative relationship, $r^2 = 0.65$ (P < 0.01) (Figure 2).
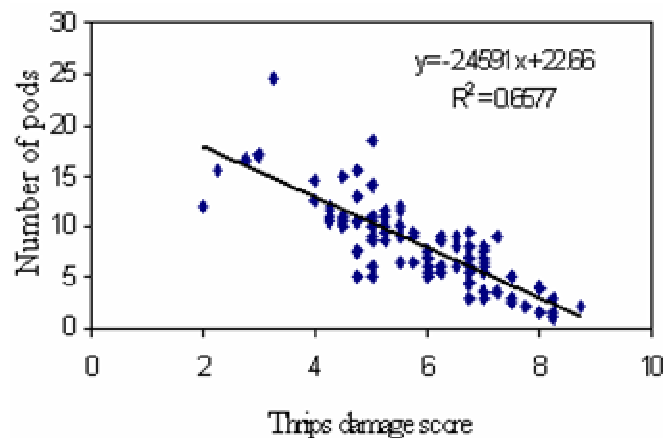
Figure 2. Effect of flower bud thrips on number of pods produced per plant observed in RILS from the cross Sanzi x VITA 7.

Polymorphism survey

All the 48 AFLP primer combinations that were tested amplified DNA from the RILS. However, 18 primer combinations were used to assess the RILs. The total number of polymorphic bands that could be scored confidently per primer combination ranged from 2 for E-ACC/M-CTC to 18 for E-AAC/M-CTC and E-ACT/MCAT. Only 16 of 121 (13%) cowpea derived microsatellite primers tested showed polymorphism between the two parents and five of these polymorphic primers were used to assess the mapping population.

Developing a cowpea genetic linkage map

A linkage map of the 134 AFLP and the 5 SSR markers scored in the mapping population was developed to facilitate QTL analysis for resistance to FTh (Figure 3). The map spans 1620.1 cM of the cowpea genome and the markers were distributed in eleven linkage groups. The number of map units per linkage group was correlated with the number of markers per linkage group ($r^2 = 0.97$). These linkage groups were designated 1-11. Table 2 gives a summary of the features of the genetic linkage map of 139 markers segregating among RILs. The number of markers placed in different linkage groups ranged from 2 to 44 while the distance between markers ranged from 0.2 to 37.0 cM. The average distance between adjacent markers was 9.6 cM. The linkage groups range in size from 2.5 – 512.5 cM. Thirty-five per cent of the intervals between loci were below 10 cM.

There were significant associations between 23 markers and resistance to flower bud thrips (P<0.05) [Figure 3 (markers in red)] using single marker analysis. These include 22 AFLPs and one microsatellite primer (VM36). These markers were located on linkage groups 1, 2, 3, 4, 6 and 7 (Figure 3). Both parents donated these markers. Two markers, E-ACA/M-CAC550 and E-ACT/M-CTG280 were significantly associated with resistance to FTh but are not yet assigned to any linkage group.

QTL mapping for resistance to flower bud thrips damage

Interval mapping procedure detected a total of three primary QTLs and two secondary QTLs using the single – QTL model in five regions along five linkage groups namely LG1, LG2, LG3, LG6 and LG7 (Figure 3 and Table 3). Significant peak values of LOD scores, the position of these peaks, and the percentage of phenotypic variance explained and estimated phenotypic effects are shown in Table 3. The primary QTLs with the most effects were located on linkage groups LG2, LG3, and LG6. The three QTLs jointly contributed 61.6% of the total phenotypic variance. When the primary QTLs were fixed, and the genome rescanned, two additional QTLs on LG1 and LG7 were identified that increased the total phenotypic variance explained from 61.6 to 77.5% with a LOD value of 14.72. QTLs can be arranged according to their contributions to resistance of flower bud thrips in descending order as follows LG3 (E-ACT/M-CAA376), LG2 (E-ACG/M-CTT2), LG6 (E-AAC/M-CTA120), LG7 (E-AAC/M-CAA155), and LG1 (E-AAC/M-CAA255). The QTLs are designated FTh1, FTh2, FTh3, FTh4, and FTh5 and the phenotypic variance explained by the QTLs, were 32.0, 18.4, 12.6, 11.9 and 9.5%, respectively. The results obtained with single marker analysis were similar to those obtained with interval mapping. However, despite the presence of several markers that showed significant associations with resistance to FTh on linkage group 4, no QTLs were detected on this linkage group following interval mapping.

DISCUSSION

Host plant resistance (HPR) is one of the most important strategies for crop improvement. Insect resistance genes have been introduced into several crop varieties and its importance is increasing as insecticides lose efficacy due to pest adaptation or are removed from use to protect the environment and human health (Eigenbrode and Trumble, 1994). In many cases, multiple genes are required for sustained resistance to counter pest adaptation. Thus maintaining agricultural productivity to meet world food needs depends on access by agricultural scientists, to many sources of HPR genes. Only low levels of resistance to FTh exist in different cowpea lines and there is need to bring these genes together in a line with good agronomic performance. The positive transgressive segregation observed among the RILs in this study indicated that both parents were contributing favourable factors towards resistance. Transgressive segregation for resistance to FTh has important breeding
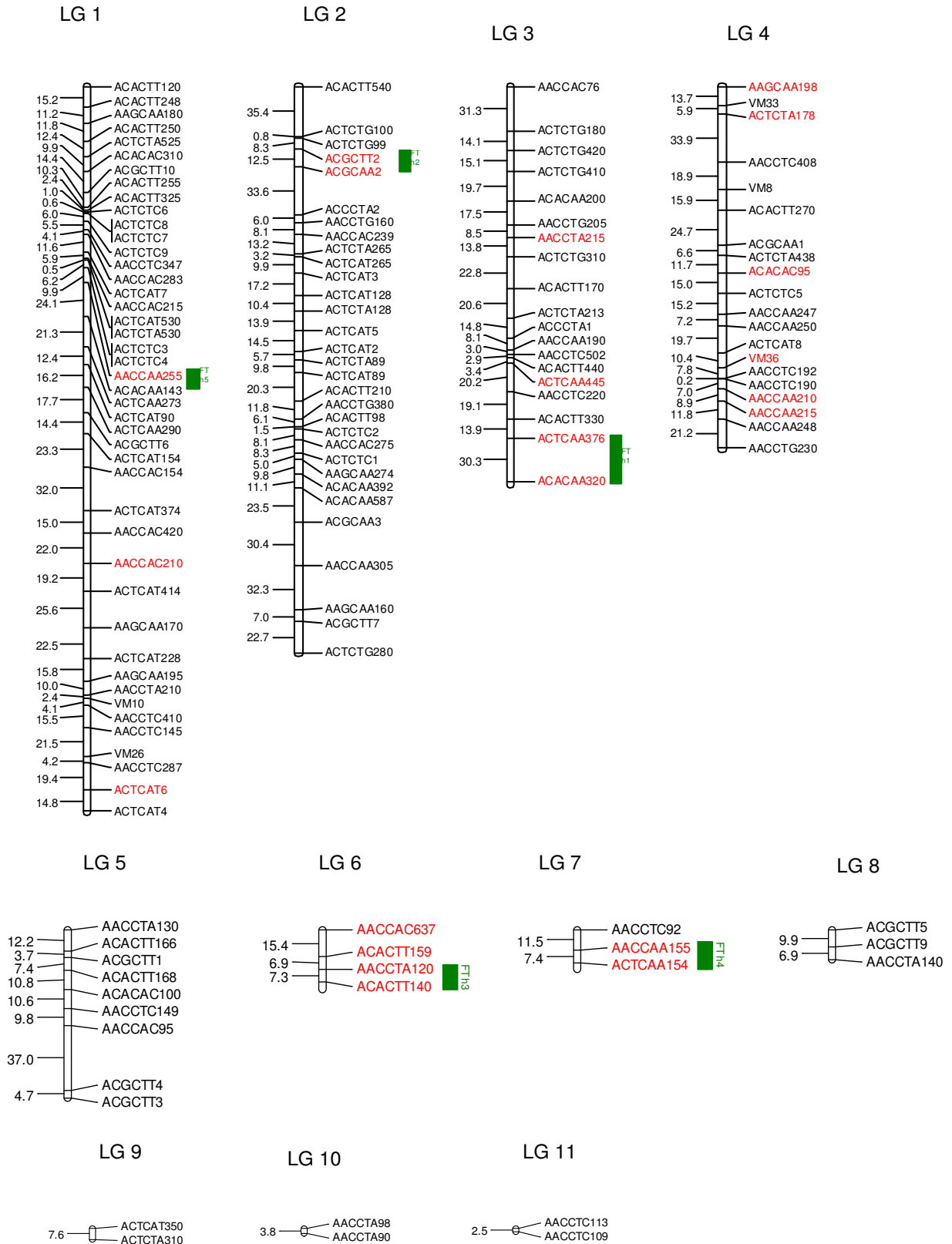
Figure 3. A genetic linkage map of cowpea showing the QTLs (in green) that is associated with the resistance loci for FTh. The map was developed using F_{10} RI population developed from the cross Sanzi x VITA 7 and Kosambi's (1944) mapping function. Markers in red are those closely associated to the resistance loci. Designations to the right represent marker names and to the left represent map distance in cM.

Table 2. Features of the genetic linkage map of 139 markers segregating among RILs derived from the cowpea cross Sanzi X VITA7.

Linkage group	No of Loci	No of loci segregating in ratio		Map distance cM	
	Total	1:1[a]	Distortion[b]	Total	Mean[c]
1	44	21	23	512.5	11.9
2	31	17	14	400.0	13.4
3	19	14	5	278.9	15.5
4	20	16	4	255.7	13.5
5	9	7	2	96.3	12.0
6	4	4	0	26.6	8.9
7	3	2	1	18.9	9.5
8	3	3	0	16.8	8.4
9	2	2	0	7.6	7.6
10	2	2	0	3.8	3.8
11	2	2	0	2.5	2.5
UM	31	19	12	-	-
Total	170	109	61	1620.1	-

[a]Number of loci segregating in ratio 1:1.
[b]Number of loci deviating from 1:1 (P<0.01).
[c]Mean distance between adjacent markers based on kosambi.
UM = Unassigned markers.

Table 3. Significant QTLs for resistance to flower bud thrips (FTh) in the cowpea cross Sanzi x VITA 7.

QTL	LG[a]	Single- QTL models			
		Locus	LOD	Variance explained (%)[b]	Additive efffect
FTh1	3	E-ACT/M-CAA376	3	32.0	2.30
FTh2	2	E-ACG/M-CTT2	3	18.4	1.83
FTh3	6	E-AAC/M-CTA120	2	12.6	1.49
FTh4*	7	E-AAC/M-CAA155	2	11.9	-1.45
FTh5*	1	E-AAC/M-CAA255	2	9.5	-1.47

[a] Linkage group.
[b]Phenotypic variation explained by a QTL.
*QTLs detected after the QTL position E-ACG/M-CTT2 was fixed and the genome was rescanned

implications because it is possible to obtain plants with resistance levels higher than those of the parental lines. The distribution of the phenotypic data was significantly different from normal and was skewed towards the susceptible parent. All phenotype analyses were however performed on untransformed data. Normalizing data through transformation may misrepresent differences among individuals by pulling skewed tails toward the center of the distribution (Okogbenin and Fregene, 2003). The RILs displayed a continuous distribution of FTh damage scores, indicating that more than two genes probably control the resistance of cowpea to flower bud thrips. This was consistent with the result obtained from the field test (Omo-Ikerodah et al., 2000).

One of the important advantages of the AFLP technique is the high number of markers that can be generated per experiment. In this study, AFLP analysis was able to show up to 18 scorable polymorphic bands in a single reaction. The average number of polymorphic bands per primer pair combination between Sanzi and VITA 7 was nine. This number is relatively low when compared with other crops such as populus (Cervera et al., 1996), tobacco (Ren and Timko, 2001) and hop (Jakše et al., 2001). A low level of polymorphism between Sanzi and VITA 7 is to be expected since cowpea is a highly self-pollinating crop. In addition, both belong to the same species (*V. unguiculata*). The more distantly related two sexually compatible individuals are taxonomically, the higher will be the frequency of polymorphism detected between them. In an earlier study, Fatokun et al. (1993) reported that polymorphism revealed by AFLP between an improved cowpea line and a wild relative was 22%.

The level of polymorphism between the two lines as revealed by the cowpea derived microstatellite primers in this study was also low as only 16 of 121 primers showed polymorphic bands (approximately 13%). Li et al. (2001)

observed that the level of microsatellite polymorphism among several improved breeding lines and cowpea germplasm lines was relatively high although this is much lower than in many other crops.

The markers are distributed in eleven linkage groups with an average distance of 9.6 cM between markers. The 11 linkage groups in this map probably correspond to the expected eleven chromosomes per haploid genome of cowpea. Ouédraogo et al. (2002) worked on different cowpea mapping populations and reported 11 linkage groups, which spanned a total of 2670 cM. However, Ubi et al (2000) and Menendez et al. (1997) in their studies each reported 12 linkage groups for cowpea. Ogundiwin (1999) reported the detection of 15 linkage groups for *Vigna vexillata*, a wild relative of cowpea. It should be noted that all members of the genus *Vigna* have 2n = 22 chromosomes except *Vigna glabrescens* which is the only known naturally existing tetraploid (Marechal et al., 1978). The marker loci were randomly distributed over linkage groups with an r^2 of 0.97 between the number of map units per linkage group (map length) and the number of markers per linkage group. Ubi et al. (2000) and Crouzillat et al. (1996) found an r^2 value of 0.89 in the cowpea genome and 0.43 in the cocoa genome respectively and concluded that the loci were randomly distributed in the respective genomes.

QTLs with effects on resistance to FTh were detected in five regions along five linkage groups. The QTL on LG3 explained 32.0% of the variation, which is an indication of the presence of a major QTL in this region. The allele inherited from the resistant parent contributed resistance to FTh at this locus. The five QTLs together explained 77.5% of the variation for resistance to flower FTh in this set of RILs. The identification of QTL accounting for this substantial fraction of phenotypic variability for resistance to FTh is a significant first step toward a more detail genetic characterisation of this important trait. The different locations of QTLs in this study emphasize the polygenic inheritance of this trait. This observation is consistent with reports from previous studies in cowpea and other species (Ubi et al., 2000; Lee et al., 1996; Agrama et al., 2002). Lee et al. (1996) observed different genomic locations of QTLs for plant height and maturity in soybean, which they attributed to the polygenic nature of inheritance of these traits. The phenotypic variance explained by individual QTLs was small. These results further support a model of quantitative inheritance (Paterson et al., 1991).

Despite the presence of several markers that showed significant associations with resistance to FTh on LG4, no QTLs were detected on this linkage group following interval mapping. It is possible that there were a few more QTLs of much smaller effects segregating in this cross that have not been detected either because they explain only a small proportion of the variation or because of epistatic effects. It may also be due to the gaps that exist in the present linkage map, which require additional markers to fill. If more markers are generated and placed on the map, these QTLs may become more readily detected. Consequently, estimates of QTL numbers should be considered as lower bounds. Thus, the number reported here represents the most significant QTLs. Future research should be focused on saturating the regions of possible QTL with more markers.

QTLs with positive as well as negative effects were detected. Three resistance-enhancing QTLs originated from the resistant parent Sanzi while two originated from the susceptible parent VITA 7. QTLs with effects opposite to the overall effect of the parents have also been reported in tomato (Tanksley et al., 1982).

The QTLs detected in this study have mainly additive gene effects. They can therefore be readily applied to breeding purposes. Though QTL-marker linkages have been found to remain reproducible across environment (Fatokun et al., 1992), specific QTLs are often only expressed under particular environmental conditions (Paterson et al., 1991). Replicated evaluations over years, which can be obtained from the RI population developed in this study, would provide insights into effects of the environment on these QTLs.

Identification of DNA markers associated with QTLs affecting resistance to FTh in cowpea is a key step in using molecular genetics for cowpea improvement for this trait. This study shows that DNA markers can be used to identify regions of cowpea genome that have genes for resistance to FTh. The use of RILs to characterize resistance to FTh permits genotypes to be replicated under different environments so that a standard measure of resistance can be developed. In this study five regions of the genome were shown to be specifically associated with resistance to FTh. The identified QTLs explained a large proportion of the total variation for thrips resistance indicating loci that impact resistance to FTh. A more detailed study of these loci should provide better understanding of this complex trait.

ACKNOWLEDGEMENT

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