



Mapping of AFLP loci linked to tolerance to cowpea golden mosaic virus

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ABSTRACT. AFLP markers combined with the bulk segregant analysis methodology was used for the identification of molecular markers associated with the cowpea golden mosaic virus (CGMV) resistance gene in 286 F₂ cowpea plants derived from the cross IT97K-499-35 x Canapu T16. Segregation data in the F₂ population demonstrated that tolerance to CGMV is controlled by a single dominant gene. Among the 196 combinations of AFLP primers tested, which generated approximately 3800 amplicons, three markers linked to the CGMV resistance gene were identified: E.AAC/M.CCC₅₁₅ at 4.3 cM, E.AGG/M.CTT₂₈₀ at 14.2 cM and E.AAA/M.CAG₃₅₂ at 16.8 cM, with 50.4, 24.4, and 28.7 LOD scores, respectively; the former two markers flank the CGMV loci. These markers could be used for the development of 'sequence characterized amplified region' type markers or for greater saturation of this region, to increase the precision of assisted selection for the development of cowpea strains tolerant to CGMV.

Key words: *Vigna unguiculata*; Bulk segregant analysis;
Genetic mapping

INTRODUCTION

Cowpea (*Vigna unguiculata* [L.] Walp.) is one of the principal crops of northeastern Brazil and is considered a source of income and basic food for its population (Oliveira et al., 2002). The crop is attacked by various pathogenic agents that reduce quality and grain yield (Sobrinho Athayde et al., 2000). Of these, the infections caused by viruses stand out because they can reduce production from 60 to 80% in more susceptible cultivars, predominantly the traditionally cultivated varieties. More than 20 viruses that naturally infect this crop have been reported (Thottappilly and Rossel, 1985). Among them, the cowpea golden mosaic virus (CGMV) stands out for causing excessive losses of 40 to 78% production (Santos and Freire-Filho, 1984).

Resistance to CGMV has been attributed to 2 dominant and independent genes by Sangwan and Rishi (2004), who worked with F₁ and F₂ populations and backcrosses of 2 resistant cultivars, CS 39 and CS 55, and 2 susceptible ones, GC 2 and HFC 42-1. Another study has indicated that resistance to the golden mosaic virus is controlled by a dominant gene (Kumar et al., 1994). In *Phaseolus vulgaris*, resistance to the golden mosaic virus has been attributed to 3 genes: a dominant gene that controls the reaction to dwarfism and 2 unlinked recessive genes responsible for resistance to chlorosis (Valez et al., 1998).

The use of molecular markers has contributed greatly to the development of genetic linkage maps for several plant species of economic interest, including *V. unguiculata*. Amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) have been used widely in the construction of genetic maps in cowpea (Menéndez et al., 1997; Quédraogo et al., 2001, 2002; Boukar et al., 2004). Owing to the unlimited number of polymorphisms that can be analyzed using the AFLP technique, specific regions of the genome can be saturated, a prerequisite for cloning based on target gene maps (Borém and Caixeta, 2009).

AFLP markers, combined with the bulk segregant analysis (BSA) methodology (Michellmore et al., 1991), have been used with success in the identification of molecular markers linked with genes of agronomic importance, such as those conferring resistance genes to pests and diseases. Quédraogo et al. (2001) have adopted AFLP and BSA methodologies to identify markers closely linked to 2 genes that confer resistance to 2 races of the pest *Striga gesnerioides* Willd. Other studies aimed at the application of molecular markers in the identification of resistance genes to several diseases caused by viruses have been carried out in plant species such as green pea (*Pisum sativum* L.) (Gao et al., 2004) and common bean (*P. vulgaris* L.) (Kelly et al., 2003).

Blair et al. (2007) have developed a sequence characterized amplified region (SCAR) marker, which was found at 7.8 cM from the gene *bgm-1*, which confers resistance to the golden mosaic virus in *P. vulgaris*. No studies on the mapping of resistant genes to the CGMV have yet been reported. The objective of the present study was to identify AFLP molecular markers linked to genes resistant to CGMV in an F₂ population derived from an IT97K-499-35 x Canapu T16 cross to facilitate the development of SCAR markers for assisted selection in the development of cultivars resistant to CGMV.

MATERIAL AND METHODS

Genetic material and cross

Two hundred and eighty-six F₂ plants from a cross between genotypes IT97K-499-35

and Canapu T16 were analyzed. IT97K-499-35 is a black-eyed cultivar developed by the International Institute of Tropical Agriculture. This cultivar presents multiple tolerances to several viruses, including CGMV (Singh et al., 2006). Canapu T16 belongs to the canapu group, a type largely grown in a region of the Vale do São Francisco that is susceptible to CGMV.

The cross was performed as described by Rachie et al. (1975). After planting the seeds that resulted from the crosses, the success of the hybridization using the seed coat phenotypic marker was observed: all of the F₁ seeds presented a black seed coat in contrast with the black-eyed pea type of the parental IT 97K-499-35 and the brown of the other parent.

Evaluation of CGMV resistance in the F₂ population

F₁ seeds from the cross IT97K-499-35 x Canapu T16 were planted in the experimental field of Embrapa Tropical Semiárido between 2 borders of Canapu, the variety susceptible to CGMV, which were planted 30 days earlier to increase the population of *Bemisia tabaci*, a vector of CGMV, and reduce the chances of escape. Normal cultural practices were adopted, but no insecticides were applied to avoid interference with the population of the insect vector. Plants that presented the typical symptoms of CGMV infection - golden yellow coloration on the leaf surface - were considered susceptible, whereas healthy plants that displayed no symptoms were considered resistant.

Extraction of genomic DNA and AFLP reactions

Young leaves of offspring from the cross IT97K-499-35 x Canapu T16 were collected, properly identified, and stored in a freezer at -80°C. The cetyltrimethylammonium bromide 2X protocol of Doyle and Doyle (1990) was used for the extraction of genomic DNA with the following modifications: 6000 and 10,000 rpm in the first and second centrifugations, respectively, and β-mercaptoethanol at 2% and incubation at 60°C for 30 min for all of the samples. After the addition of Tris-ethylenediaminetetraacetic acid buffer, the DNA solution was treated with RNase to remove co-isolated RNAs. The quantification of the DNA extracted was performed through visual comparison of the intensity of its bands with those obtained from phage λ DNA (10 to 100 ng) on 0.8% (w/v) agar gel stained with ethidium bromide. The samples were diluted to 50 ng/μL based on the estimated concentrations and stored at -20°C.

The methodology described by Vos et al. (1995) was used in the analysis of the AFLP markers. Initially, the genomic DNA was doubly digested with 2 restriction enzymes. For this, 200 ng DNA was doubly digested with 0.62 U of each endonuclease for 2.5 h in a thermocycler with the combinations between the restriction enzymes *EcoRI/MseI* and *PstI/MseI*.

The programming of the thermocycler for pre-selective amplifications consisted of 20 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min. Each pre-amplified reaction was diluted 20 times in ultrapure water. In the selective PCRs, 100 pairs of initiators *EcoRI* + *MseI* and 96 pairs of initiators *PstI* + *MseI* were used. The reactions were prepared for a final volume of 10 μL according to these concentrations: 0.2 μM *EcoRI* or *PstI* primer, 0.3 μM *MseI* primer, 0.2 mM deoxyribonucleotide triphosphates, 1X PCR buffer, 2.5 mM MgCl₂, 0.5 U *Taq* DNA polymerase, and 2.0 μL pre-amplified and diluted DNA. The pro-

programming of the thermocycler for the selective amplifications consisted of 2 steps: 1) a cycle at of 94°C for 30 s followed by 13 repetitions of 65°C for 30 s and of 72°C for 60 s, and an annealing temperature of 65°C diminishing 0.7°C in each subsequent cycle, and 2) 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

The amplification products were separated on 6% polyacrylamide gel (acrylamide:bis-acrylamide [19:1], 7.5 M urea, and 5X Tris/borate/EDTA buffer). The electrophoresis ran for approximately 3 h with a constant power of 40 W. The gels were stained with silver nitrate according to the procedure described by Creste et al. (2001).

Identification of AFLP markers associated with resistance loci

For BSA (Michelmore et al., 1991), 2 duplicate groups were created: one composed of CGMV-resistant plants and the other of CGMV-susceptible plant. Each group contained DNA from 5 individuals. Equimolar quantities of the DNA of these individuals were used for the preparation of 2 susceptible bulks and 2 resistant bulks (Figure 1). The primers that amplified candidate fragments - present in the susceptible bulk and absent in the resistant bulk and vice versa - were again evaluated in the sample of the 5 resistant and susceptible individuals and in a larger sample comprising 35 tolerant and 15 susceptible individuals (Figure 2). Only the primers that segregated close to the proportion of 3:1 were selected to phenotype the 286 F_2 individuals.

An estimate of the size of each AFLP fragment was made using the method of inverse mobility based on the regression of products of known size of the molecular marker of 50 bp (Fermentas, USA), applied in an extra well of the polyacrylamide gel.

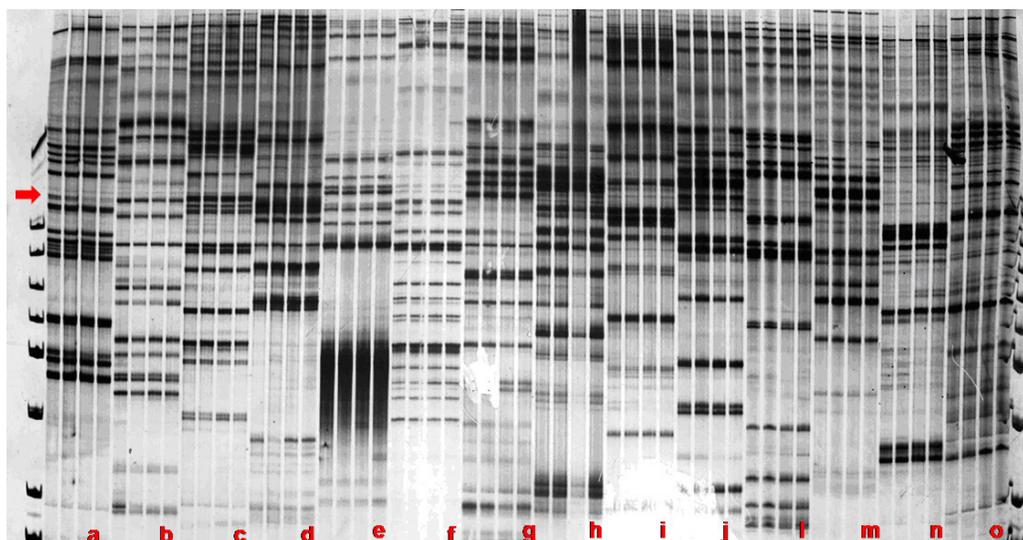


Figure 1. Polyacrylamide gel demonstrating 14 bulks of resistant and susceptible individuals (represented by the letters 'a' to 'o'), in duplicate with two resistant bulks and two susceptible ones, respectively. One polymorphism among the bulks was indicated by an arrow.

Statistical analysis

The data obtained using the AFLP markers and through visual evaluation of resistance/susceptibility were submitted to the chi-square test for verification of the Mendelian pattern of segregation of 3:1 expected in the F_2 population. The linkage analyses were performed using JoinMap version 2.0 (Stam and van Ooijen, 1995). The minimum logarithm (base 10) of odds (LOD) of 3.0, the maximum recombination frequency of 0.45, the “jump” limit of 3.0, and the “triplet” limit of 3.0 were adopted. The values obtained for the recombination frequencies were converted to genetic distance maps (centimorgans) using the Kosambi (1943) function for illustration in a diagram.

RESULTS

The visual identification of susceptible and resistant plants was easy to make, because the characteristic symptoms of CGMV are golden-yellow spots that contrast in the field. In the 286 plants evaluated in the F_2 population of the IT97K-499-35 x Canapu T16 cross, resistance was verified in 223 plants, whereas 63 showed susceptibility to CGMV.

The results obtained were submitted to the chi-square test that showed no significant result at the 5% level (1.35), which validated the Mendelian segregation of 3:1 (resistant:susceptible). These data suggest a segregation of 3:1, with dominance of the gene that confers resistance to the disease.

Of the 100 pairs of *EcoRI/MseI* initiators and 96 pairs of *PstI/MseI* initiators tested in the bulks of resistant and susceptible individuals of the cross IT97K-499-35 x Canapu T16, 3768 bands were amplified, with 2022 being the combination *EcoRI/MseI* and 1746 *PstI/MseI*. Thirty polymorphic bands were identified among the resistant/susceptible bulks, with 22 being in the pairs of initiators *EcoRI/MseI* and 8 in the pairs of primers *PstI/MseI*. Of the combinations of primers that showed polymorphism among the bulks, only 3 were consistent when they were analyzed in 35 resistant individuals and 15 susceptible ones, confirming the result of the initial evaluation with the resistant and susceptible bulks (see Figure 2).

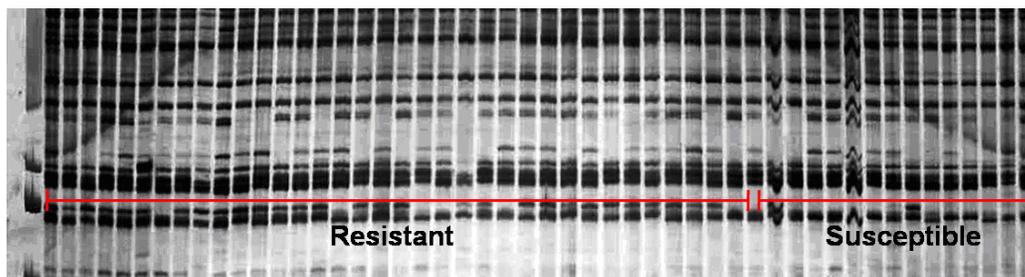


Figure 2. Polyacrylamide gel with 50 individuals. The first 35 individuals are resistant and the last 15 are susceptible to the golden mosaic virus analyzed with the combination E.AAA/M.CAG.

The markers E.AAC/M.CCC, E.AGG/M.CTT, and E.AAA/M.CAG correspond to amplified DNA fragments of 515, 280, and 352 bp, respectively. The linkage of these 3 markers with the resistance locus, which was called *Mdo-1*, the symbol of the resistance gene, was con-

firmed in the 286 individuals of the F_2 population given that the distances for the locus *Mdo-1* varied from 4.3 to 21.1 cM.

Recombination analysis estimated that the marker E.AAC/M.CCC₅₁₅ is located 4.3 cM from *Mdo-1*, with an LOD score of 50.4, whereas the markers E.AGG/M.CTT₂₈₀ and E.AAA/M.CAG₃₅₂ are positioned 14.2 and 16.8 cM away with LOD scores of 24.4 and 28.7 and markers E.AAC/M.CCC₅₁₅ and E.AAA/M.CAG₄₅₂, respectively, are flanking (Figure 3).

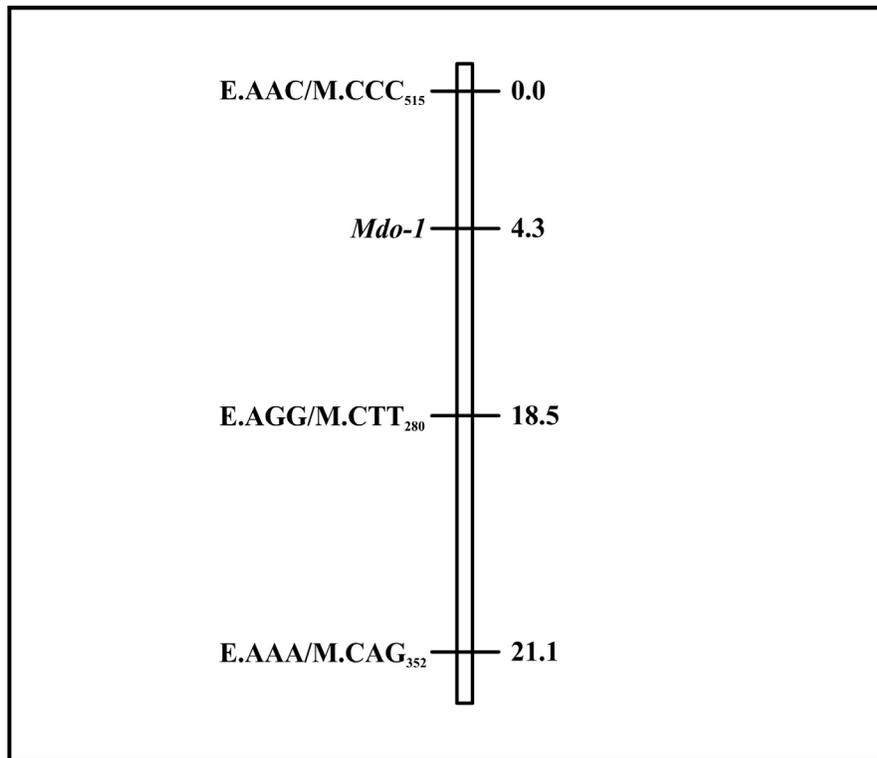


Figure 3. Linkage map of the resistance locus for golden mosaic (*Mdo-1*) with the positions of the amplified fragment length polymorphism markers identified and their respective distances (cM). Logarithm of the odds scores of 50.4, 24.4, and 28.7, respectively, for the markers E.AAC/M.CCC₅₁₅, E.AGG/M.CTT₂₈₀ and E.AAA/M.CAG₃₅₂.

The study of genetic inheritance performed in the segregating F_2 population for CGMV indicates that resistance to the disease is controlled by a dominant gene segregating in the proportion of 3:1 (resistant:susceptible). The results obtained differ from those reported by Sangwan and Rishi (2004), in which resistance to CGMV was attributed to 2 dominant genes segregating in the proportion of 15:1 in F_2 plants and 3:1 in backcrosses, indicating that resistance is conditioned by 2 dominant and independent genes. The authors cite 2 studies, in which resistance is attributed to only 1 gene (Bliss and Robertson, 1971; Kumar et al., 1994). Conversely, in the common bean (*P. vulgaris* L.), resistance to golden mosaic is conferred by 2 unlinked recessive genes (Valez and Bassett, 1998). Studies evaluating the segregating populations of backcrosses of these progenitors will be necessary to confirm the genetic inheritance to golden mosaic virus.

DISCUSSION

Our results showed that the combination of AFLP markers and the BSA technique was efficient for the localization of markers linked to the resistance gene to CGMV. Despite the fact that the great majority of published researches in this field have used 8 or more individuals to compose the bulks or groups (Quédraogo et al., 2001; Boukar et al., 2004), our use of only 5 individuals in each bulk did not compromise the results, because 3 markers linked to the resistance gene were identified, as adopted by Boiteux et al. (2000) and Brito (2007) in carrot and coffee, respectively.

The AFLP methodology has also been used satisfactorily in other studies with cowpea: in the identification of molecular markers linked to resistance genes to diseases and pests (Menéndez et al., 1997; Quédraogo et al., 2001, 2002; Boukar et al., 2004), in phenetic analyses and analyses of genetic diversity (Coulibaly et al., 2002), and in the localization of quantitative trait loci (Muchero et al., 2010). The ample use of AFLP markers in molecular analyses, especially in the genetic mapping of cowpea, demonstrates its efficiency in identifying loci linked to segregating characteristics of commercial interest. Despite its generation of dominant marks - in other words, being unable to distinguish heterozygote loci - the technique has become an important tool in the genetic improvement of cowpea as well as other species of the genus *Vigna*.

The combinations of the rare and frequent restriction enzymes *EcoRI/MseI* and *PstI/MseI* amplified 20.2 and 18.2 bands, respectively. These values are inferior to those found by Menéndez et al. (1997), who in analyzing a cross between 2 genotypes of *V. unguiculata* (IT84S-2049 x 524) developed in Nigeria and California and contrasting for several morphological traits and resistance to pests and diseases found an average of approximately 55 fragments/primer and 12% polymorphism per combination of AFLP primer using a fluorescent system and semi-automatic sequencer. Oliveira et al. (2001) obtained an average of 7.6 amplified bands using random amplification of polymorphic DNA markers in the genetic mapping and the selection of hybrids between *Citrus reticulata* Blanco and *Citrus sinensis* (L.) Osbeck. Boiteux et al. (2000) have reported an average of 2.4 amplicons of random amplification of polymorphic DNA in the mapping of a nematode in an F₂ population of carrot.

The enzymes *EcoRI/MseI* produced a superior number of total amplified fragments and polymorphic fragments between the segregating bulks: 53.6 and 73.3%, respectively. These values demonstrate that the combination *EcoRI/MseI* was more efficient than *PstI/MseI* in detecting polymorphic loci and was similar to those reported by Campos et al. (2005), in which the combinations of enzymes *EcoRI/MseI* produced greater numbers of bands and polymorphisms than did the combinations *PstI/MseI* in a study of genetic diversity among cultivars of *Citrus* ssp. The quantity of AFLP markers identified in this study is close to that obtained by Boukar et al. (2004), who identified 4 AFLP markers linked to the resistance gene to *S. gesnerioides* with genetic distances of approximately 3.2 (E.ACT/M.CTC₁₁₅), 4.8 (E.ACT/M.CAC₁₁₅), 10.3 (E.ACA/M.CAG₁₀₈), and 18.2 cM (E.AAG/M.CTA₁₉₀). The marker E-ACT/M-CAC₁₁₅ was sequenced and converted to an SCAR, and when tested in the F₂ population, was efficient in distinguishing susceptible and resistant individuals.

Quédraogo et al. (2002) have used AFLP markers to develop a linkage map that serves as a reference owing to its high density and ample coverage of the *V. unguiculata* genome. In this study, several markers linked to disease resistance genes were identified, including cow-

pea mosaic virus (CPMV), cowpea severe mosaic virus (CpSMV), Black-eyed cowpea mosaic virus, and Southern bean mosaic virus. Souframanien and Gopalakrishna (2006) have applied the inter-simple sequence repeat technique to identify the marker ISSR811₁₃₅₇, located at 6.8 cM from the resistance gene to the mung bean yellow mosaic virus in F₈ recombinant strains of genotypes of mung beans (*Vigna mungo* [L.] Hepper). ISSR811₁₃₅₇ was converted with success in an SCAR and was capable of distinguishing susceptible and resistant plants to the mung bean yellow mosaic virus in agreement with the phenotypic data.

In the present study, 3 markers linked to the resistance gene to CGMV were identified, with 2 markers flanking the resistance gene to the mosaic (E.AAC/M.CCC₅₁₅ 4.3 cM away and E.AGG/M.CTT₂₈₀ 14.2 cM away). Before this study, no marker linked to the resistance gene for CGMV had been identified, making this linkage map the first in a population of *V. unguiculata* segregated for resistance to the CGMV.

The marker E.AAC/M.CCC₅₁₅ obtained in the present study appears to be a strong candidate for use in assisted selection by molecular markers given its greater proximity to the resistance gene. It is also a strong candidate to be converted into an SCAR marker, which is superior to an AFLP marker. SCAR markers for genes that confer resistance to CGMV may assist breeding programs, increasing the accuracy of assisted selection for the development of CGMV-tolerant strains.

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