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Full Length Research Paper

Detection of RAPD markers-linked to resistance to cassava anthracnose disease

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Cassava anthracnose disease (CAD) is a major stem disease of cassava (*Manihot esculenta* Crantz) in the cassava growing areas of the world. To identify markers associated with resistance to CAD, F_1 progenies were produced from a cross between resistant genotype TME 117, and susceptible genotype TMS 92/0326, as the experimental population. The chi-square test gave a goodness of fit for the expected ratio of 1:1 for resistant to susceptible genotypes suggesting a monogenic dominant inheritance. Two hundred decamer primers were screened using both resistance and susceptible parent to CAD. Bulk segregant analysis was quickly used to search for Random amplified polymorphic DNA (RAPDs) linked to anthracnose resistance in F_1 derived from TMS 92/0326 and TME 117. The fragment linked to the gene was flanked on both sides by primers OPAF2 and OPF06 at 13.1 and 22.2 cM. To our knowledge this is the first report on this fungal disease of cassava and of molecular markers that is tag CAD resistance in cassava. We discuss the use of markers-linked to CAD1 for marker-assisted cassava breeding.

Key words: Bulk segregant analysis, cassava anthracnose disease, molecular marker-assisted selection, RAPD, host resistance.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important starchy staple of the lowland tropics and a mainstay of some of the most hard-pressed populations of the world (Cock, 1985; Best and Henry, 1992). It ranks as the sixth most important crop in the world (Mann, 1997). Cassava is native to South America, but very widely grown in sub-Saharan Africa. Africa produced 208 million metric tonnes of cassava in 2006 with Nigeria as the leading producer (FAO, 2006). The crop accounts for over 60% of the daily calorie intake of some 500 million people (FAO, 2000). It is used as a food reserve against famine and plays a major role in reducing food crisis since its production is season-less (Dixon et al., 1994; Osiru et al., 1997; Egesi et al., 2007).

Though there are reports of potential use of true cassava seeds in cultivation (Rajendran et al., 1993), cassava is mainly propagated from stem cuttings. In addition to revenue generated from roots, farmers realise substantial income from sales of healthy stems. Cassava is susceptible to several pathogens and pests, many of which are transmissible via stem cuttings. Diseases can cause up to 100% yield losses (Lozano and Booth, 1974) when susceptible varieties are grown (Lozano et al., 1981). This constitutes a great constraint to production, safe international germplasm exchange and conservation. These constraints have contributed to keeping the average cassava yield in Africa at 6.4 tons/ha, which is well below the world average of 8.8 tons/ha (IITA, 1990). The most important fungal disease of cassava is the anthracnose disease caused by the pathogen *Collectotrichum gloeosporoides f. sp. manihotis* (Fokunang et al., 2000). The pathogen attacks mainly the stem causing deep cankers some of which affect the nodes rendering the plant useless for propagation (IITA 1990); and thus

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reducing the amount of healthy stem cuttings available to farmers (Ikotun and Hahn, 1997). The disease symptoms consist of dieback of shoot and shallow cankers on the stems of the plant (Theberge, 1985). The typical high humidity of the tropics favours its spread. Cassava anthracnose disease was first detected in Dar Es Salam region (Tanzania – East Africa) in 1903, Brazil in 1904 (Chevaugeon, 1956). The disease was also recorded in Puerto Rico in 1939 and Madagasca in 1936 (Bouriquet, 1946) and in Nigeria and Zaire in 1953 (Goffart, 1982). Cassava anthracnose disease (CAD) can be controlled through the use of fungicide applications and improved phyto-sanitary practices, but the use of resistant varieties provides an effective and economic means of control. Development and wide distribution of varieties resistant to CAD will greatly alleviate the production problems being faced by poor cassava growers.

The conventional breeding methods used to select resistant materials involves field assessment of germplasm. However these methods may result in the selection of an otherwise susceptible type for resistant due to plasticity of disease expression in different environments. New molecular techniques will help facilitate an efficient selection of resistant germplasm through markers that are associated with resistant genes. Two cassava molecular maps are available (Fregene et al., 1997; Okogbenin et al., 2006) and these provide a vital framework for the use of marker-assisted selection in cassava breeding (Mba et al., 2001; Okogbenin and Fregene, 2002, 2003). A good number of molecular markers-associated with disease resistance genes in cassava are available for rapid screening of germplasm (Jorge et al., 2000, 2001; Akano et al., 2002). The Random Amplified Polymorphic DNA (RAPD) provides a cheap and rapid approach to screen a large number of accessions in a breeding population. The objective of this study was to identify DNA markers-linked to CAD resistance genes for application in molecular breeding of disease resistant cassava cultivars aimed towards improved cassava productivity.

MATERIALS AND METHODS

Planting materials and evaluation for CAD resistance

The planting materials used were an F_1 progeny from a cross between a susceptible improved genotype TMS 92/0326 (female parent) and a Nigerian landrace TME 117 (male parent) that represented the source of CAD resistance. The progeny, comprising 60 individuals, was established in the experimental field of the International Institute of Tropical Agriculture (IITA) Ibadan, Nigeria during 2000 and 2001 planting season. Ibadan is forest-savannah transition ecology, with coordinates 3º45´E; 7º26´N, an altitude of 210 MSL and an average bimodal rainfall of 1252.8 mm. The progenies were planted in an augmented randomized complete block design. Each row was planted to 10 cuttings in single-row ridges that were 10 m long, 0.75 m wide and 0.60 m high. The ridges were spaced 1 m apart. The progenies were screened visually and scored for susceptibility and resistance to anthracnose disease based on their response to natural infection seven months after planting as routinely done at IITA on a scale of 1 - 5, where 1 = no apparent symptoms and 5 = very severe symptoms (IITA, 1998).

DNA isolation and amplification

Extraction of DNA from the leaf samples of the parent and the progenies was according to Dellaporta (1983) while the 200 RAPD Primers from Operon Technologies, Alameda, Calif. USA were used in the PCR optimization experiments. The DNA from the two parental lines was amplified using 25 µl volume reaction containing 10.3 µl of distilled water, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10X buffer (1 M Tris-HCl, 1M KCl, pH 9.0), 2.5 µl of 5% (v/v) Tween-20, 1.0 µl of 2 µM primer, 1.0 µl of 2.5 mM dNTPs, 0.2 µl of 5 U Taq polymerase, 5 µl of 10 ng/µl DNA using MJR brand of Peltier thermal cycler which amplified the DNA using a cycling profile consisting of 3 min initial denaturation steps at 94°C, followed by 45 cycles of annealing and extension (94°C 30 s, 37°C 40 s, 72°C 1 min, respectively).

Bulk segregant analysis

A modified bulk segregant analysis (BSA) using two pools of 11 susceptible and 11 resistant progenies, from the progenies with the two parents and a set of 53 RAPD decamer primers were used to identify DNA bands associated with the CAD resistance gene. The PCR amplification products were mixed with 10 µl of loading dye, and loaded into a 24 well 1.4% agarose gel prepared in 0.5X TBE (2.5 mM Tris-HCl, 2.5 mM boric acid, 0.05 mM EDTA, pH 8.0) and electrophoresed at 102V/69Amp for 90 min, and the DNA was visualized by ethidium bromide staining according to standard laboratory procedures under ultra violet light. Bands found to be polymorphic in the two parents and the two bulks were selected as candidate markers.

Linkage analysis

Chi-square analysis was carried out using the SAS statistical package to determine possible association between the RAPD markers and the resistance gene (Anon, 1995). To develop a linkage group around the CAD-resistance locus, RAPD markers-associated with the CAD gene were subjected to linkage analysis using MAPMAKER 2.0 (Lander et al., 1987) on a G3 Macintosh computer. Thresholds for declaring linkage were a LOD score of 4.0 and a recombination fraction of 0.3 Map units (cM) were derived using the Kosambi function (Kosambi, 1944). Maximum-likelihood orders of markers were verified by the 'ripple' function and markers were said to belong to the framework map if the LOD value, as calculated by the 'ripple' command, was ≥ 2.0.

RESULTS

Reaction of F_1 progenies to CAD and identification of polymorphic markers

Based on the disease severity score of the F_1 progenies at eight months after planting results indicate 31 (51.6%) were resistant and 29 (48.3%) plants were susceptible. The chi-square test of the ratio of resistant and susceptible progenies gave a value of 1:1, which is not significantly different ($P > 0.05$). The disease syndrome consists of dieback of shoot and shallow cankers on the stems of

the plant (Figure 1).

The amplification of cassava genomic DNA, were used in the 25 µl-reaction cocktail and this optimal condition as it was mentioned in the materials and methods was chosen based on consistency and reproducibility of the results. Repeated amplification with a given primer yielded identical banding patterns in the two parental lines.

Two hundred random decamer primers from the A-Z series of Operon Technologies Inc. (Alameda, USA) were tested with the two parental genotypes. Out of these, 138 (69.0%) primers produced resolvable amplification products in which 53 (38.6%) of the decamer primers detected polymorphism bands between the parents. Twenty-eight (14.0%) primers showed no linkage with the gene, while 34 (17.0%) primers were partially linked with the primer, giving 31.0% of the total primer. These primers gave no or partial amplification products, indicating that they probably have no homology with cassava.

One hundred and thirty-eight (69.0%) primers showed linkage with the gene for there is a distinct amplification, indicating that they probably have homology with cassava DNA. Eighty-four (61.4%) of the 138 amplified primers were monomorphic which shows that they are not CAD gene-linked, while 53 (38.6%) primers were polymorphic in the parent genotypes, which is an indication that these primers are, CAD gene-linked (Table 1).

Genetic mapping of cassava anthracnose disease resistance gene

Out of the 200 decamer primers tested for polymorphism, 53 polymorphic primers were selected for bulk segregant analysis (BSA). Primers OPAC14, OPOF06, OPOO18, OPAF2, OPAF 17, OPAG 03 and OPJ10 had a band that was present in the resistant parent and the resistant progenies but absent in the susceptible parent and susceptible bulks. The primers that showed these distinct bands that were present in both the parent and bulks showed scoreable bands between the ranges of 81.8 to 100%. Primers OPAF2 and OPF06 had high distinct banding pattern during scoring, showing that both the resistant parent (TME 117) and resistant progenies had the candidate band present but absent in the susceptible parent (TME 92/0326) and susceptible progenies (Figures 2 and 3). OPF06 and OPAF2 markers evaluated segregated in 1:1 ratio ($P \leq 0.05$, chi-square test; Table 2). The markers OPAF2 and OPF06 was found located on the linkage group of the derived map of cassava which shows that CAD resistance is located at a distance of 12.0 cM (Figure 4). Results of single marker analysis showed that OPAF2 and OPF06 explained 33.7 and 27.4% ($P \leq 0.05$) of the CAD resistance, suggesting a single gene inheritance.

DISCUSSION

Stem cutting is the major sources of cassava propagation

Figure 1. F_1 progenies stem that is susceptible to cassava anthracnose disease.

Table 1. Number of decamer primers that are linked with the anthracnose disease gene of cassava.

S/No	Operon primers	No. of monomorphic bands	No. of polymorphic bands
1	OPAA	02	07
2	OPAB	11	06
3	OPOJ	10	05
4	OPOF	10	07
5	OPOO	02	06
6	OPAC	09	05
7	OPAE	05	07
8	OPAF	08	02
9	OPAG	13	05
10	OPAD	15	03

the genotype that do not have cankers on their stems are good planting materials, which will result in increased yields and better crop performance. Mature stems are used for propagation of cassava. Cassava genotypes that have cankers on their stems may cause reduction in planting materials. Large-cankered stems are also capable of increasing the number of infective propa-gules of the pathogen in the field. Kurt and William (1989) reported that variation in the expression of resistance is as a result of differences in the pathogenicity of the organism and differences in the gene governing resistance in the

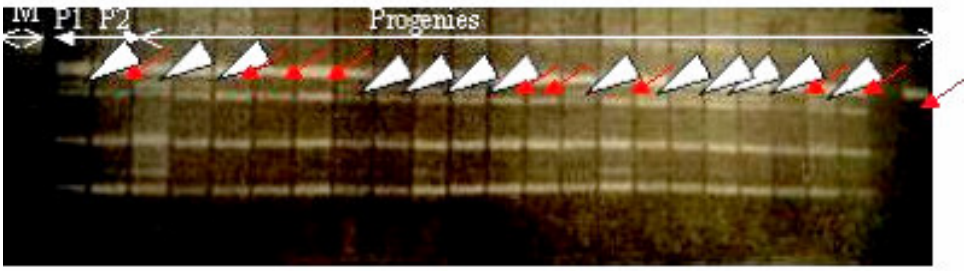

Figure 2. PCR amplification products with primer OPAF 2 in the bulk segregant analysis. M = 1 kb standard marker; P1 = Resistant Parent, P2 = Susceptible Parent, White arrow = Resistant band; and Red arrow = Absence of resistant band.

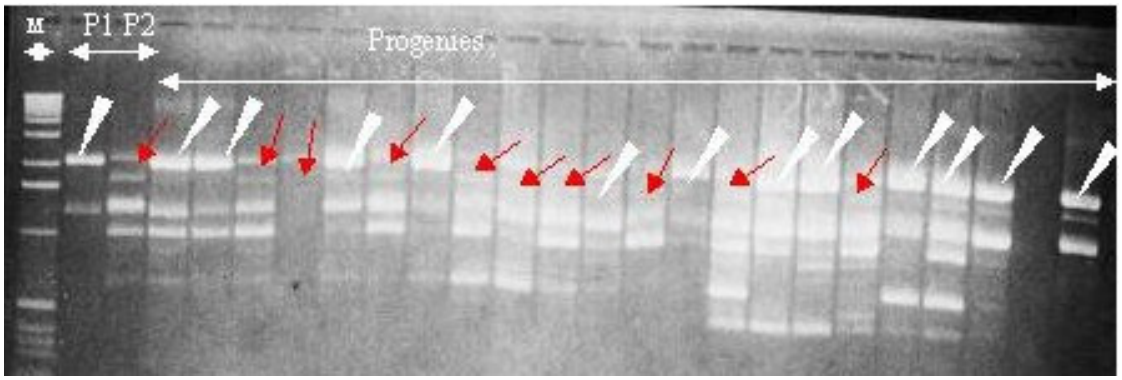

Figure 3. PCR amplification products with primer OPF 06 in the bulk segregant analysis. M = 1 kb standard marker; P1 = Resistant Parent, P2 = Susceptible Parent, White arrow = Resistant band; and Red arrow = Absence of resis-tant band.

Table 2. Segregation analysis of RAPDs DNA OPAF2 and OPF06 markers in the population of the cross TMS 92/0326XTME117 in the seedling nursery.

Locus Tested	Primer Sequences	Observed ratio	Expected ratio	X^2	P
OPAF2	5'-CAGCCGAGAA-3'	17:18	1:1	0.7	0.39
OPF06	5'-GGGAATTCGG-3'	17:18	1:1	0.7	0.39

host. Hence resistance cannot be accessed on the basis of any single component. Resistance to cassava anthracnose can be attributed to phenol metabolic changes, in particular with changes in peroxidase activity, tissue lignification and appearance of compound toxic to the fungus in the host tissue (Friend, 1981; vander Bruggen and Maraite, 1987).

The PCR-RAPD technique used provided means for genetic analysis for identifying a marker for cassava cultivar that is resistant to CAD. Molecular marker systems have proven to be effective in overcoming the limitations of traditional breeding methods. Candidate genetic markers that can distinguish between resistant and susceptible plants have been identified in the present study by the method of bulked segregant analysis. The 81.80 - 100% polymorphism observed in the arbitrary primers suggested the potential for DNA marker.

The advantage of marker-assisted selection is that it enables the breeder to eliminate at an early stage cassava anthracnose susceptible genotypes, which in the case of the heterozygous resistant genotypes could be 50% reducing the costs of disease evaluation by half and increasing selection efficiency (Akano et al., 2002; Fregene et al., 1997; Fregene, 2000). The elimination of inferior genotypes at an early stage increases the efficiency of selection by allowing the breeder to concentrate on fewer genotypes at the seedling and crucial single row trial stages where progenies are reduced by up to 90%. The CAD1 gene was flanked by OPAF2 on one side and OPAF06 on the other side. It explained 10 and 16% ($P < 0.001$) of the phenotypic variance of CAD resistance, which confirms a single gene inheritance hypothesis.

One of the primary objectives of gene tagging efforts in cassava is to provide tools that can increase the cost-eff-

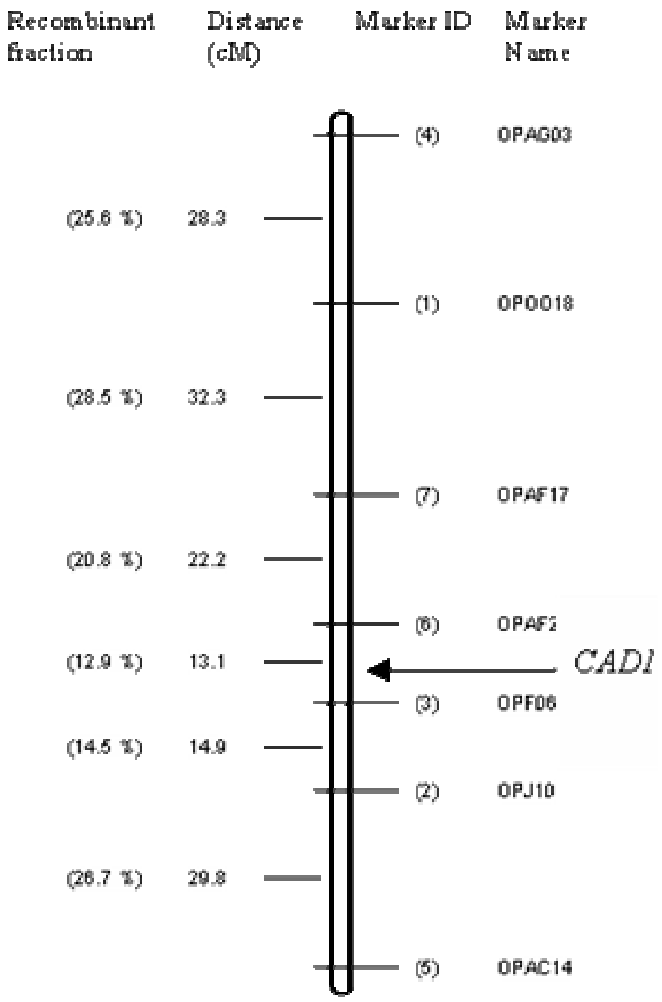

Figure 4. Relative position of all the RAPD markers linked to the putative anthracnose resistance locus identified in a segregating population derived from a cross TMS 92/0326 x TME117.

ectiveness and efficiency of cassava breeding program. These results demonstrate that RAPD analysis can still be a suitable tool for detection and mapping of major genes for marker-assisted selection in cassava breeding programmes. Even though reports of poor reproducibility exist, highly specific sequenced characterized amplified regions (SCAR) markers based on this RAPD marker could make for more efficient gene tagging in the future.

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