Molecular Marker Analysis and Validation of Resistance to Cassava Mosaic Disease in Elite Cassava Genotypes in Nigeria

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ABSTRACT
Cassava (Manihot esculenta Crantz), a highly heterozygous crop, is devastated by cassava mosaic disease (CMD). The discovery of the CMD2 dominant gene is helpful in the genetic analysis of CMD resistance. Molecular markers for CMD2 gene were used to introgress CMD resistance into Latin American cassava genotypes and validated in the field for 4 yr for stability of resistance conferred by CMD2. Field screening identified 64 Latin American genotypes with stable resistance to CMD. Resistance to CMD of two Nigerian cassava cultivars (TMS 97/2205 and TMS 98/0505) was analyzed with markers and in the field. Molecular data indicated that CMD resistance in the two Nigerian cultivars was mediated by the CMD2 gene. Results showed TMS 97/2205 to be highly resistant to CMD in three ecological zones in Nigeria. Further genetic analysis of this genotype as a source of high level of resistance to CMD using a segregating F1 population derived from a TMS 97/2205 × NR 8083 cross was initiated using 530 simple sequence repeat (SSR) markers to identify quantitative trait loci (QTL) for CMD resistance. A marker (NS198) associated with a QTL for CMD resistance, explaining 11% of the phenotypic variance observed, was identified. The combined effect of this QTL and CMD2 may account for the high level of resistance of TMS 97/2205. The resistance profile of the evaluated CMD2 genotypes in growth cycle was not uniform and was affected by genetic background. The discovery of a new QTL (CMD3) for CMD resistance in TMS 97/2205 offers new opportunities for pyramiding CMD genes for enhanced durability of CMD resistance in cassava.

Cassava is widely grown and is one of the most important food staples in sub-Saharan Africa. Africa accounts for most of the cassava root harvest worldwide with more than half of the world’s total production (FAOSTAT, 2010) followed by Asia and Latin America. Apart from its importance as animal feed and industrial raw material, cassava has emerged as an important biofuel resource (Egesi et al., 2007a); it is, therefore, a cash source for resource-poor farmers.

In Africa, one of the most important diseases of cassava is the cassava mosaic disease (CMD), and it is a major constraint to stable root production in the region. Yield loss estimates in susceptible cultivars vary from 20 to 95% (Fauquet and Fargette, 1990). The

**Abbreviations:** BSA, bulked segregant analysis; CGM, cassava green mite; CMD, cassava mosaic disease; dNTP, deoxyribonucleotide triphosphate; EACMV, East African cassava mosaic virus; EDTA, ethylenediaminetetraacetic acid; MAP, month(s) after planting; MAS, marker-assisted selection; NRCRI, National Root Crops Research Institute; PCR, polymerase chain reaction; QTL, quantitative trait locus or loci; SCAR, sequence characterized amplification region; SSI, symptom severity index; SSR, simple sequence repeat.

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CMD is transmitted by whitefly (*Bemisia tabaci*) and by the planting of cuttings derived from disease-infected plants (Egesi et al., 2007b). Host-plant resistance is an effective long-term strategy to tackle the disease (Hahn et al., 1980; Nnodu and Ogbe, 1994; Otim Nape et al., 1994; Akano et al., 2002). The aim of breeding for resistance is to produce cultivars with improved resistance that is durable under a wide range of environmental conditions. However, cassava has a long growth cycle and it is highly heterozygous, and heterozygosity is the reason that an F1 population segregates. Quantitative trait loci (QTL) mapping has been done in cassava using F2 and backcross populations, which normally reveal a high level of segregation (Jorge et al., 2000, 2001; Okogbenin and Fregene, 2002, 2003; Fregene et al., 2006; Akinbo et al., 2012). It takes 8 to 10 yr to develop and release a new cassava cultivar. A typical breeding scheme starts with seedling evaluation and clonal evaluation with primary emphasis on screening for resistance to CMD and other major diseases of cassava before complex characters, such as agronomic traits and yield, are evaluated. It therefore requires 2 to 3 yr to screen for CMD resistance to select resistant lines under conventional breeding before the commencement of advanced yield trials. The discovery of close flanking markers to the CMD2 gene facilitates the use of molecular markers in CMD resistance breeding in cassava (Blair et al., 2007).

The dominant gene CMD2 was discovered in a farmer-preferred Nigerian landrace, TME 3, and mapped with the aid of simple sequence repeat (SSR) and sequence characterized amplified region (SCAR) molecular markers. Molecular markers facilitate the analysis and discovery of genes for disease resistance and other traits. The CIAT in collaboration with cassava breeding programs in the African National Agriculture Research System has been exploiting CMD2 derived from Nigerian landraces (Akano et al., 2002) for the development of CMD-resistant Latin American germplasm in the center of origin. By introgressing CMD resistance into Latin American germplasm, the germplasm can be efficiently deployed for breeding and gene pool development of strategic novel traits in CMD endemic areas such as Africa. The ability to effectively broaden the genetic base of cassava using Latin American germplasm largely depends on transferring CMD resistance into this germplasm, which is otherwise highly susceptible to CMD (Okogbenin et al., 1998, 2007), given that the pathogen is absent in Latin America.

Under the Generation Challenge Program project, Latin American germplasm with CMD resistance was developed and identified via marker-assisted selection (MAS) and finally introduced from the center of origin to Nigeria. Preliminary evaluation has resulted in the identification of elite resistant Latin American genotypes. The Latin American genotypes developed through MAS were evaluated in Nigeria, leading to the selection of about 64 genotypes representing 29 families in the breeding program of the National Root Crops Research Institute (NRCRI) in Nigeria. However, the successful use of these materials as cultivars and in breeding programs is highly dependent on the stability and durability of their disease resistance. It is therefore important to validate the stability of disease resistance in these genotypes. Equally important is the need to evaluate and assess the CMD resistance pattern of these Latin American CMD2 genotypes under different genetic backgrounds. The introduced Latin American genotypes have different pedigrees and sufficiently represent the diverse genetic backgrounds for broadening the genetic base of cassava.

Because of the high level of susceptibility of the Latin American germplasm to CMD, its use for breeding and possible release as cultivars in Africa has been highly impaired for several decades. Given that cassava is vegetatively propagated, disease buildup in planting materials could also rapidly increase, leading to, across time, high incidences of disease in susceptible genotypes in the field. It is therefore necessary to evaluate the disease for a couple of years in the breeding scheme to assess the effectiveness of the CMD2 gene in conferring resistance to Latin American cassava as well as the stability and durability of this resistance. No prior information on the phenotypic response pattern in Latin American germplasm for this source of resistance has been reported. New information will be useful in determining the selection and adaptation response of the introduced germplasm in cassava breeding programs in Africa.

One of the major challenges in cassava breeding is the quest to overcome the evolutionary capacity of the disease by identifying additional sources of resistance to the disease. The need to identify other sources of CMD resistance is critical in building durable and stable resistance to CMD through gene pyramiding. Some elite breeding genotypes have been intensively used to breed for CMD resistance in Nigeria, but the genetic basis of resistance is yet to be determined. It has also not been ascertained if resistance is governed by genes other than the CMD2. Lack of knowledge of the genetics of resistance of these breeding lines is a constraint to their effective use in breeding activities. Molecular markers can be effectively used to genetically analyze and identify genes involved in CMD resistance in these materials. By screening parent genotypes using CMD2 gene markers, it can be easily discerned if the resistance is mediated by the CMD2 gene or other genes or by a different source of resistance. Such knowledge should therefore rapidly permit the identification or discovery of new alleles for CMD resistance.

Among the breeding lines used for breeding for CMD resistance are two newly released cultivars in Nigeria, TMS 97/2205 and TMS 98/0505. TMS 97/2205 has exceptionally high CMD resistance with good stability in different ecologies, including high CMD pressure zones, with near
immunity to the disease. Molecular markers, including the CMD2 markers, were used to analyze CMD resistance in TMS 97/2205 to understand the genetic basis for the high resistance associated with the cultivar. Identifying new genes for CMD resistance is a major objective of our cassava breeding program to enhance disease resistance and durability. TMS 98/0505 was also analyzed to determine if resistance is conferred by CMD2 or another source. The two cultivars (TMS 97/2205 and TMS 98/0505) are being widely deployed as parental lines to develop new superior genotypes with high yield and disease resistance. The knowledge of the type of CMD resistance existing in elite germplasm is very useful and strategic in the efficient deployment and use of such elite lines as well as in selection of cross combinations or choice of crossing designs in breeding programs.

The objectives of this study were therefore to validate stability of CMD resistance in Latin American genotypes in different genetic backgrounds, to conduct molecular marker analysis of CMD resistance in resistant parental breeding lines at NRCRI, and to identify new alleles for CMD resistance in TMS 97/2205. This paper also reports on the importance and significance of the results in CMD resistance breeding for the development of superior genotypes toward increased cassava productivity in Nigeria.

MATERIALS AND METHODS

Evaluation of Latin American Cassava Genotypes for Cassava Mosaic Disease Resistance

Latin American genotypes were developed at CIAT, Colombia, for CMD resistance through F1 crosses and a modified backcross scheme using markers to introgress the CMD2 gene from TME 3, a Nigerian landrace, into the Latin American genotypes. Two series of families (CR and AR) were developed based on the crossing scheme used to generate the germplasm. The CR series families were generated from F1 crosses whereas those of the AR series were developed through the modified backcross scheme, combining CMD resistance (introgressed from TME 3) and cassava green mite (CGM) resistance (from wild Manihot species). The Latin American germplasm was subjected to marker analysis using the CMD2 markers to select for CMD resistance in the populations. Details of the MAS process have been described by Okogbenin et al. (2007).

Based on the molecular data, genotypes with the favorable allele of the CMD2 gene were selected. More than 200 genotypes selected via MAS using CMD2 markers were then introduced into Nigeria, following routine plant quarantine procedures as described by Okogbenin et al. (2007), and evaluated in the field for CMD resistance. Based on the 68% MAS success achieved with the markers for the CMD2 gene (Okogbenin et al., 1998), genotypes with CMD resistance were identified after a 2-yr evaluation. Given the need to integrate the best Latin American genotypes into the cassava breeding scheme at NRCRI, Latin American cassava clones combining good CMD resistance with good vigor were further evaluated for additional 2 yr to validate stability of resistance conferred by the CMD2 gene. The Latin American genotypes (i.e., 64 clones) came from 29 families of different pedigrees and therefore represented diverse genetic backgrounds for the assessment of CMD resistance profile. The 64 clones were evaluated, in a randomized complete block design with three replications, for 4yr between 2005 and 2010. Evaluation for CMD was done on the basis of a severity index score across a 12-mo growth cycle each season at Umudike (with bimodal rainfall, mean annual precipitation of 2200 mm, altitude 120 m, mean temperature of 22 to 31°C, coordinates 05°29’ N, 07°33’ E, luvisol soils, and humid forest environment). The NRCRI headquarters at Umudike in southeast Nigeria is a high CMD pressure location (Egesi et al., 2007a) and well suited for CMD disease screening.

Symptom severity index (SSI) of CMD has been used solely to classify cassava genotypes for their reaction to CMD (Terry, 1975; Nnodu and Ogbe, 1994). The CMD symptom severity was rated on a scale of 1 (highly resistant) to 5 (highly susceptible). Plants with no symptoms were scored as 1 (Terry, 1975; IITA, 1990). Symptom severity index of 2 was assigned for mild symptoms, SSI 3 for moderate symptoms, SSI 4 for severe symptoms, and SSI 5 for highly severe symptoms with chronic distortion of four-fifths or more of the leaflets, including leaf strapping (Ogbe et al., 2006). The genotypes were scored five times at 1, 3, 6, 9, and 12m after planting (MAP). The highest severity score at the peak of the disease was used to assess the CMD resistance potential of each genotype. NR 8082, TMS 30572, and TMS 4(2)1425 were used as susceptible check cultivars.

Multilocational Evaluation of Cassava Mosaic Disease Resistance in TMS 97/2205 and TMS 98/0505

Two elite cultivars (TMS 97/2205 and TMS 98/0505) were evaluated for CMD resistance profile in the NRCRI breeding program in three locations for 2yr. The locations were Umudike (see location description above), Ishiagu (mean annual rainfall of 1500 mm, altitude 319 m, mean annual temperature of 24 to 35°C, coordinates 8°45’ N, 7°20’ E, ferric luvisols, and southern Guinea Savanna ecology), and Otobi (mean annual rainfall of 1800 mm, altitude 150 m, mean annual temperature of 24 to 32°C, coordinates 5°56’ N, 7°31’ E, Dystric luvisol soils, and forest–savanna transition ecology). Two of the susceptible check cultivars used in the NRCRI breeding program for CMD evaluation and other key breeding traits, TMS 30572 and TMS 4(2)1425, which were readily available in sufficient quantities, were included in the trials.

The experiment was conducted using a randomized complete block design with four replications planted in each of the three sites used in the study under rainfed conditions. Clean planting materials of relatively uniform size were selected for the trials with each plot consisting of 36 plants arranged in a six by six (column by row) arrangement. Planting was done on ridges at a plant-to-plant spacing of 1 m to give a plant population of 10,000 ha−1.

Data were collected from the inner 16 plants within each plot. Data on CMD severity score were taken at 1, 3, and 5 MAP using the scale described above (Terry, 1975; IITA, 1990). Disease incidence, which is described as the proportion (expressed in percentage) of infected plants to the total number of plants per cultivar, was also calculated.
Screening for CMD2 Marker Alleles in TMS 97/2205 and TMS 98/0505

Genomic DNA was prepared from the three parental genotypes using about 3 g of young leaves according to Dellaporta et al. (1983). The extracted DNA was resuspended in 200 μL of Tris-ethylenediaminetetraacetic acid (EDTA) (10 mM Tris HCl and 1 mM EDTA) (pH 8.0). These clones were screened with four flanking markers of the CMD2 gene (Okogbenin et al., 2007). TME 3, a resistant landrace and source of the CMD2 gene, was included as a positive control (of the CMD2 allele) for polymerase chain reaction (PCR) amplification. Two additional cultivars of Nigerian origin, TMS 30555 (CMD susceptible) and NR 8083 (CMD moderately resistant), were also included as controls. Two SSR markers (NS158 and NS169) and two SCAR markers (RME1 and RME4) were used for the screening. The product size and other marker information are provided in Table 1. The reaction mixture for SSR contained 2.5 μL of 10x thermobuffer (Promega), 2.5 μL of MgCl₂ (25 mM), 2.0 μL of deoxyribonucleotide triphosphates (dNTPs) (2.5 mM), 0.5 μL each of forward and reverse primers (5.0 μM), and 0.4 μL of DNA per reaction tube. The thermocycles were as reported by Mba et al. (2001) and were performed in PerkinElmer GeneAmp PCR system, model 9600 (PerkinElmer). The PCR products for SSR markers were denatured and electrophoresed on 6% polyacrylamide gels using Bio-Rad sequencing apparatus (Bio-Rad Inc.). The PCR amplification products were mixed with 4 μL of loading dye (98% formamide, 10 mM EDTA, pH 8.0, bromophenol blue, and xylene cyanol), heated for 2 min at 96°C, and chilled on ice for 3 min. Of this mixture, 4 μL were loaded onto a 6% denaturing polyacrylamide gel. Electrophoresis was in 1x Tris-borate-EDTA at 40 V cm⁻¹ for 2 h, and DNA was visualized following silver staining according to the manufacturer's manual for the polyacrylamide gel electrophoresis (PAGE) gel silver-staining kit (Promega) (Akano et al., 2002). The reaction mixture for SCAR per tube contained 2.5 μL each of 10x Thermobuffer, 2.5 μL of MgCl₂ (25 mM), 2.0 μL of dNTPs (5 mM), 0.5 μL each of forward and reverse primers (5.0 μM), and 0.4 μL of DNA per reaction tube. The temperature cycling profile was an initial denaturation step for 2 min at 95°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, and primer extension at 72°C for 1 min; a final extension cycle of 5 min at 72°C was added. The thermal cycles were also performed in the PerkinElmer GeneAmp PCR system, model 9600. Between 2 and 3 μL of PCR reaction for SCAR markers were separated by electrophoresis in an ethidium bromide (0.5μg mL⁻¹)-stained 1% metaphor agarose gel at 80 to 90 V and 60 mA for 1 h. The DNA bands were observed using the UVP (model UVP GDS 8000) gel documentation and analysis system (Ultra Violet Product Ltd.). The marker data for the two cultivars and check clones were then read for the favorable marker allele of the CMD2 gene for resistance.

The F₁ Segregating Population with TMS 97/2205 as Parent

Based on the high level of CMD resistance of TMS 97/2205, it was further selected for molecular marker analysis to identify CMD resistance QTL or genes in this cultivar that has near immunity to the disease. To conduct marker–trait association studies for CMD resistance involving this cultivar, a biparental F₁ segregating population having TMS 97/2205 as one of the parents was therefore selected from existing crosses in the cassava breeding seedling nursery of NRCRI. The F₁ population selected was a cross between TMS 97/2205 and NR 8083, which showed good segregation in reaction to the CMD; hence its suitability for effective genetic analysis of CMD resistance. NR 8083, with a moderate symptom expression level to CMD, was used as the susceptible parent instead of a highly susceptible genotype because of the adverse effect of CMD on flowering in cassava.

Phenotypic Evaluation for Cassava Mosaic Disease in the F₁ Population

The progeny in the F₁ segregating population was planted at Umudike and evaluated in two seasons (2006 and 2007) for CMD. The F₁ seeds were germinated in a prenursery for 2 mo and later transplanted to the field in the first year (2006). Because the F₁ population was raised from seeds, each genotype was evaluated for CMD using only one plant in the first year. The genotypes were randomly planted in the field in the seedling nursery in 2006.

In the second year (2007), 6 to 10 cuttings were generated per genotype depending on the vigor of each genotype. The clonal evaluation trial in the second year was planted in a randomized complete block design with three replicates (each replication consisted of 2–3 plants in a row). The cuttings were planted on ridges with 1 m between rows and within rows. The experiment was weeded and fertilizer was applied 2 MAP at the rate of 600 Kg ha⁻¹ to boost growth and development in accordance with recommendation for cassava cultivation for this region.

The genotypes in the F₁ population were scored for CMD between May and October in 2006 on a monthly basis; this period coincided with high disease pressure and whitefly (vector of the virus) activity. In the second season, evaluation was repeated on a monthly basis but only from May through August. Two highly susceptible cultivars (NR 8212, and TMS 30555) and two moderately susceptible cultivars (TMS 30572 and TMS 4(2)1425), commonly used as checks in our breeding program, were planted to assess CMD pressure in the field. To effectively determine the genetic makeup of each genotype relative to CMD, the highest severity score observed for each genotype, combined across two seasons (i.e., the highest symptom severity index from both seedling nursery and clonal trial), was used to classify the genotypes as either resistant or susceptible. Disease symptom evaluation was done between 1 and 5 MAP when CMD is often prevalent, reaching its peak normally at 5 MAP in the test sites used. Genotypes with SSI 1 to 2 were classified as resistant whereas those with SSI of 3 to 5 were classified as susceptible.

Marker–Trait Association for Cassava Mosaic Disease Resistance

The F₁ population was analyzed to identify markers linked to a gene or genes associated with resistance to CMD via bulked segregant analysis (BSA) as described by Michelmore et al. (1991). Two bulked (pooled) DNA samples corresponding to the extreme groups of resistant and susceptible individuals, based on the phenotypic data from a 2-yr evaluation, were prepared. Polymorphic markers between the two pools are expected to be genetically linked to the trait used to construct the pools (Michelmore et al., 1991).
The two contrasting bulks were made by mixing equal volumes of DNA solution from each of the genotypes in each bulk (resistant vs. susceptible). The resistant bulk contained 24 individuals and the susceptible bulk 12 individuals. The DNA extraction and genotyping were conducted as described above. For the BSA, 530 SSR markers with a wide coverage of the cassava genome were used for genotyping. The scheme used for the BSA is illustrated in Fig. 1. The parents of the selected cross were screened for polymorphic markers. The polymorphic markers between the two parents were then used to analyze the contrasting bulks to identify SSR markers with possible association with genes (QTL) for resistance.

**Data Analysis**

Phenotypic data scores for CMD in 2006 and 2007 were correlated using Pearson correlations computed via Microsoft Excel (Microsoft Corporation, 2010). Phenotypic association was done to assess disease expression and variation under high CMD pressure. Segregation ratio of the F1 population was analyzed by chi-square analysis. Polymorphic markers selected after screening of contrasting bulks and individuals in the population were analyzed by correlating the phenotypic and genotypic data of each selected marker. The selected candidate markers from correlation analysis were further subjected to t test using Genstat Discovery edition 3 (VSN International, 2007) to identify markers significantly associated with genes linked to CMD resistance. Each candidate marker was scored for the presence or absence of band for all individuals in each phenotypic group (resistant and susceptible bulks). Both groups were then compared via t test for significant differences for each candidate marker. Statistical significance was declared at \( P \leq 0.05 \). Genstat was used to calculate phenotypic variance explained by the significant markers linked to genes or QTL for CMD resistance through regression analysis.

**RESULTS**

**Cassava Mosaic Disease Profile of Latin American Genotypes**

The CMD response profiles of the Latin American genotypes evaluated for CMD resistance for 3 to 4 yr to validate their CMD resistance profile are shown in Fig. 2 and 3. Resistance levels and response patterns varied among the genotypes. While some genotypes were relatively stable in CMD resistance, as they maintained a low symptom expression (SSI 1 or 2) throughout the growing season, some genotypes showed a significant increase (to SSI 3) in symptom expression during the peak disease period and later recovered from the disease rapidly with less symptom expression levels.
The highly resistant genotypes often showed good resistance at the peak pressure of the disease (often midway into the season) and therefore were relatively stable.

Of the 64 genotypes validated, 48 genotypes possessed good resistance whereas the other 18 genotypes showed moderate resistance to CMD (Table 2). The number of genotypes with good resistance per family ranged from 1 to 8, with the best families being CR52 with eight genotypes and CR15 with six genotypes. The highly resistant genotypes (e.g., CR 36-5) were often without symptoms of the disease for most of the season and did not exceed SSI 2 at the peak of the disease (Fig. 2). The resistant genotypes (e.g., CR 52A-41) notably had SSI 2 for most of the period of the disease pressure in the growth season, which often declined by the time of harvest (at 12 MAP; Fig. 2).

The 18 genotypes identified as moderately resistant had a maximum SSI of 3 at the peak of the disease even though they were often low in symptom expression for most part of the season as they tended to recover quickly from the disease, with a mean score below SSI 3 for the season. The best lines with good disease response, especially those categorized as highly resistant or resistant, showed good stability in disease response across 4 yr, for example, genotypes CR 100-15, AR 14-10, and CR 100-215 (Fig. 3).

**CMD2 Screening in Elite Nigerian Cultivars**

The results of molecular marker screening of elite Nige-rian genotypes using CMD2 multiple flanking markers (NS158, NS169, RME1, and RME4) are presented in Fig. 4 and Table 3. Two of the cultivars (TMS 98/0505, which is resistant, and NR 8083, which is moderately resistant) were positive for the CMD2 alleles and therefore consistent with their disease response ratings. TMS 97/2205, a highly CMD-resistant cultivar, was positive for three (75%) of the CMD2 markers except for RME1, with the marker data (Fig. 4; Table 3) more or less supporting a CMD2-based resistance in this cultivar.

TMS 97/2205 was evaluated at three locations representing three agroecologies with good disease pressure. This cultivar showed high disease resistance (Fig. 5) with no apparent symptoms for the disease at Umudike and Ishiagu. At Otobi, the incidence of the disease was relatively low (1%). This cultivar showed better response than TMS 98/0505 at these locations. However, both were better in CMD resistance response than other elite
cultivars, such as TMS 30572 and TMS 4(2)1425, which had high disease incidence ranging from 10 to 38% in these ecologies (Fig. 5). Two other susceptible cultivars selected as negative checks (TMS 30555 and NR 8212) were shown to be negative for all the markers, confirming the absence of the favorable CMD2 alleles, which was consistent with their CMD phenotypic classification in the breeding program (data not shown).

Phenotypic Analysis of Cassava Mosaic Disease in F1 Population

Cassava is highly heterozygous and the cross between the highly resistant cultivar TMS 97/2205 and a moderately resistant cultivar NR. 8083 resulted in segregation as expected (Fig. 6). Segregation resulted in the distribution of the genotypes in all five phenotypic classes, indicating the suitability of the cross for genetic analysis of CMD resistance or susceptibility. The proportion of resistant genotypes in the population was 65%, which according to chi-square goodness of fit test supported a 3 resistant to 1 tolerant or susceptible phenotypic ratio.

The 2-yr results showed that CMD mean severity scores increased slightly in the second year compared with mean symptom scores obtained in the first year, indicating that results were fairly similar and that high disease-pressure location used for the CMD evaluation was effective. The mean severity symptom index increased from 2.18 to 2.42. Correlation of CMD response for the 2 yr of evaluation was highly significant ($r = 0.72; P = 0.001$).
Identification of a New Marker Linked to Cassava Mosaic Disease in TMS 97/2205

The F1 population was subjected to BSA to identify markers linked to resistance for CMD other than the CMD2 resistance. Results obtained from parental screening with 530 SSR markers revealed 169 polymorphic markers between the two parents. Screening of the contrasting bulks with these polymorphic markers followed by further analysis of the individuals in each bulk of the population, based on the correlation of phenotypic and genotypic data, resulted in the selection of candidate marker associated with CMD resistance. The \( t \) test analysis showed that the marker NS198 was highly significantly associated with CMD resistance \( (P = 0.015) \) (Table 4). The allele associated with CMD resistance was, as expected, from the female parent (TMS 97/2205). The phenotypic variance explained by NS198 was 11% and the marker was not linked to the CMD2 gene region, indicating that it represented a new source of CMD resistance. This QTL was, therefore, designated as CMD3. The marker data information is provided in Table 1.

DISCUSSION

Cassava has assumed an important role in the agricultural economies of African countries given its strategic role as a food security crop and its immense potential to grow in diverse agroecologies (El Sharkawy, 1993). The development of cassava cultivars resistant to pests and diseases, especially CMD, is critical in boosting crop productivity for enhanced income and improved livelihood of farmers in sub-Saharan Africa as well as serving as the engine for rural development and growth in farming communities.

Access to Latin American (center of origin and genetic diversity) germplasm, which is a reservoir of useful genes (Akinbo et al., 2011), is crucial to the new emergent role of cassava as a cash and industrial crop in the African continent. Latin American cassava genotypes are highly susceptible to CMD in Africa although the disease does not exist in Latin America. Genetic mapping of the CMD2 gene in previous genetic mapping studies resulted in the discovery of four markers, which are now regularly used in MAS for CMD2 gene (Akano et al., 2002; Fregene et al., 2006; Okogbenin et al., 2007). The use of CMD2 gene, through markers linked to it, facilitates breeding of CMD-resistant genotypes in Latin American germplasm in the absence of the pathogen. In this study, the 64 best genotypes identified based on CMD resistance response and good establishment, earlier developed through crosses having elite Latin American cassava clones and TME3 in their pedigree and selected with markers for CMD2 allele at CIAT (Okogbenin et al., 2007), were evaluated for CMD resistance in Nigeria. The CR and AR families were developed for CMD resistance except that in the latter families, CGM resistance was also incorporated (Fregene et al., 2006) for adaptation to the dry ecologies where CGM is severe under drought conditions. The AR families, therefore, have resistant Latin American parent lines in their pedigree.

### Table 3. Marker data of cassava clones screened for CMD2 gene.

<table>
<thead>
<tr>
<th>Markers</th>
<th>TMS 97/2205</th>
<th>TMS 30555</th>
<th>NR 8212</th>
<th>NR 8083</th>
<th>TMS 98/0505</th>
</tr>
</thead>
<tbody>
<tr>
<td>RME1</td>
<td>–↑</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RME4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NS158</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NS169</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

↑Presence (+) or absence (–) of favorable marker allele for CMD2 gene.

### Table 4. Student \( t \) test analysis for significant association to cassava mosaic disease (CMD) resistance in F1 segregating population.

<table>
<thead>
<tr>
<th>Family</th>
<th>Markers</th>
<th>Band presence</th>
<th>Band absence</th>
<th>( t ) test ( (P ) level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COB6</td>
<td>NS198</td>
<td>1.591</td>
<td>2.611</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Figure 5. Disease incidence (proportion of infected plants to total number of plants) of cultivars evaluated in three agroecologies.

Figure 6. Histogram of segregating F1 population into the different cassava mosaic disease (CMD) severity classes.
The 64 genotypes evaluated showed good resistance to the disease although with differential response. Three types of disease response were observed in the CMD2 genotypes, namely, (i) highly resistant genotypes, (ii) resistant genotypes, and (iii) moderately resistant genotypes. Both the highly resistant and resistant genotypes are generally low in disease symptoms with the former almost symptomless for nearly the entire growing season and hence lower severity index than the latter. The moderately resistant genotypes had SSI 3 at the peak of disease pressure but recovered quickly, with the genotypes showing low disease symptoms for most part of the season. The disease response profile of the moderately resistant genotypes is indicative of a good and efficient recovery mechanism in the disease response pattern of the CMD2 gene. The results also indicated that resistance status of these genotypes was influenced by their different genetic backgrounds, considering that the 64 genotypes evaluated were from different cross combinations representing 29 families. However, disease symptom expression observed among the CRs was not distinctly different from that of the ARs.

Marker data evaluation of elite Nigerian genotypes revealed that CMD2 markers effectively predicted disease susceptibility in TMS 30555 and NR 8212, which were negative for the favorable marker alleles. Interestingly, all the resistant elite lines evaluated for CMD resistance were positive for almost all or all of the favorable marker alleles for the CMD2 gene. Therefore, the resistance in the three elite CMD-resistant cultivars, consistently used in crosses in the NRCRI breeding program primarily as sources of resistance to the CMD (and other good breeding attributes), was largely mediated by the CMD2 gene. The results tend to show that the resistance in a good number of the recently released cultivars in Nigeria was largely based on the CMD2 gene.

Based on a countrywide survey on begomoviruses conducted in Nigeria (Ogbe et al., 2006), African cassava mosaic virus (ACMV) is the most common virus in Nigeria, with a mean incidence of 74.1%. The East African cassava mosaic virus (EACMV) occurrence was reportedly 0.3% whereas mixed infections of both viruses were about 24% in the field (Ogbe et al., 2006). In East Africa, where the EACMV-Ug2 is prevalent, genotypes with the CMD2 resistance have been observed to respond better to CMD than local cultivars with good recovery response pattern to infection (Martin Fregene, personal communication, 2010). The discovery of CMD2 gene has opened up opportunities for MAS for CMD resistance breeding in Africa. Several Latin American germplasm lines have been introduced from other countries, such as Ghana, Tanzania, and Uganda, into Africa through CIAT (Fregene et al., 2006; Blair et al., 2007).

While the CMD2 resistance has been relatively effective in the field in Nigeria, this may not be very desirable. Monogenic or single-gene resistance (Leach et al., 2001) is generally nondurable; that is, it can be easily overcome by plant pathogens, in most cases, because of the generally high mutation rate among plant pathogens (Kiyosawa, 1982; Kloppers and Pretorius, 1997; Takken and Joosten, 2000). Mutation can lead to evolution of new pathogen strains, which are able to overcome the effect or effects of an individual resistance gene. Additionally, the probability of disease resistance breakdown is increased by the large-scale and long-term cultivation of cultivars carrying single genes, enabling the pathogen to overcome the resistance (Singh et al., 2001). Therefore, the need for the identification and use of several resistance genes (horizontal resistance) is crucial for ensuring durability and stability of resistance.

TMS 97/2205, developed at the International Institute of Tropical Agriculture (IITA), is a highly resistant cultivar, as demonstrated by its low disease incidence (proportion of infected plants to total number of plants), which was 0% in two agroecological zones and 2% in the third ecology, thus confirming its high resistance profile. Phenotypic data (disease response) indicated that TMS 97/2205 was better than other resistant cultivars evaluated in the three ecologies, suggesting the possibility of additional genetic factors underlying resistance in addition to the CMD2 gene, which it possesses. Of the four CMD2 markers used to screen for CMD resistance allele in TMS 97/2205, three were positive and one (RME1) was negative for the CMD2 allele. When the linked markers used to select or screen CMD resistance are at a distance from the gene, recombination (crossover) could lead to false negatives. RME1 is at a distance of 4.0 cM from the CMD2 gene (Okogbenin et al., 2007).

African germplasm lines are sources of resistance to CMD, given that cassava in Africa has evolved with the virus for more than 300 yr since the introduction of cassava by the Portuguese in the 16th century. The discovery of high resistance in TMS 97/2205, therefore, provided a strong basis for its genetic analysis to identify a gene or genes associated with CMD resistance.

The segregating population developed from a cross involving TMS 97/2205 revealed good segregation for CMD resistance. Disease evaluation data of the F1 population in the first year did not significantly change from results obtained across time of the experimental years. The slight increase in the severity index in the second year was mainly attributed to increased SSI recorded for susceptible genotypes, which may be because of both primary and secondary infections that occur with passage of time in the field, as compared to resistant genotypes, which did not change in disease ratings and remained resistant. This result showed that under high CMD pressure, phenotyping could be rapidly achieved to differentiate CMD-resistant lines from CMD-susceptible ones. The location used was therefore sufficiently high in CMD pressure to allow for adequate reaction and expression of F1 population to the disease in the first
year and confirmed its suitability as a screening site for CMD resistance breeding in Nigeria (Egesi et al., 2007b). Phenotypic segregation indicated 65.5% of the individuals in the population showed resistance to the disease, which was therefore consistent with a single, dominant gene expression, as revealed by the molecular data.

Bulked segregant analysis, a gene tagging technique (Collard et al., 2005), has been successfully used to identify markers associated with disease resistance (Chantret et al., 2007). The present study, using this approach, has identified a marker linked to the QTL (CMD3) for CMD resistance. The SSR marker is located on the same linkage group as the CMD2 gene but is at least 36 cM away from the CMD2 marker loci (SSRY28, SSRY158, and NS169) (Whankaew et al., 2011). Simple sequence repeat marker SSRY106 in the genomic region with NS198 and in the interval with CMD2 markers was not significantly linked to the CMD2 gene, indicating that NS198 is associated to a different QTL. The synergistic effect of the CMD2 gene and this QTL (CMD3) may thus have accounted for the high resistance to CMD observed in TMS 97/2205.

The Latin American genotypes validated in this study for their resistance were developed using the Nigerian landrace TME 3 as a donor parent for CMD2 gene resistance. To increase durability of CMD resistance in the Latin American genotypes, TMS 97/2205 should be used to introgress CMD3 in the Latin American cassava to improve their utility in the Nigerian and African breeding programs. By phenotypic selection alone, it appears to be very difficult or even impossible to reliably combine and detect multiple resistance genes in one genotype because of dominance and epistatic effects (Fredt and Oerdon, 2007). Our results indicated that in the presence of a dominant gene, a QTL could easily be masked and may be undetected in conventional breeding based on phenotypic data assessment alone. This further corroborates the importance of molecular markers in understanding the genetic basis underlying oligogenic and polygenic characters. Identifying different disease resistance genes is critical in providing stable and durable resistance (Mohan et al., 1997; Fregene et al., 2000) and efforts in this direction to identify additional QTL for CMD resistance are expected to continue in the African germplasm. The use of molecular markers in national breeding programs in Africa is therefore likely to rapidly increase in the years ahead. The genetic improvement of cassava for CMD resistance through marker-assisted breeding is largely expected to result in rapid development of superior cultivars with good productivity.

References


Fredt, W., and F. Oerdon. 2007. Molecular markers for gene


