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Full Length Research Paper

Evaluation of cassava varieties for cassava mosaic disease resistance jointly by agro-inoculation screening and molecular markers

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The whitefly-transmitted cassava mosaic disease (CMD) has become a potential threat to cassava (*Manihot esculenta* Crantz) cultivation in ASEAN countries because of its devastating impact on cassava and overgrowth of whitefly vector regionally. To reduce the risk caused by the disease, it is necessary to evaluate the capacity of major cassava germplasms for CMD resistance to guide local farmers in adopting CMD-resistant cultivars once CMD epidemics occur. After agro-inoculation mediated infection of plantlets of collected cassava cultivars from China, Thailand and other ASEAN countries, the 18 cultivars tested developed various levels of CMD symptoms, indicating a lack of resistance to CMD. There was a positive association between symptom severity scores and accumulation levels of viral DNA in the different cultivars tested. The molecular markers RME1, SSRY28 and/or NS158, which link with the CMD resistance loci CMD2 in cassava, were found in only three cultivars (11Q, T7 and N13) with moderate resistance to CMD. Our study suggests that CMD-resistance germplasms should be introduced from Africa.

Key words: *Manihot esculenta* Crantz, cassava mosaic disease, agro-inoculation, molecular marker, screening.

INTRODUCTION

Cassava mosaic disease (CMD) is one of the major constraints in cultivation of cassava (*Manihot esculenta* Crantz), an important tropical and subtropical root crop (Fauquet and Fargette, 1990) . It is caused by several cassava mosaic geminiviruses and is the most important disease of cassava in Africa and the Indian subcontinent (Legg and Fauquet, 2004). Although there are no reports of cassava mosaic virus in ASEAN countries yet, there is a potential and serious threat on cassava production due to epidemics of the whitefly (*Bemisia tabaci*) vector in the cassava growing regions, which makes the producers and breeders to be at alert.

Cassava, native to South America, was introduced to South China from surrounding South-East Asian countries countries 200 years ago (Zhang et al., 1998). In the last two decades, millions of cassava hybrid seeds provided by collaborators from the Centro Internacional de Agricultura Tropical (CIAT) with the support of Nippon Foundation were used for breeding new cultivars suitable for Chinese ecological environments. For example, cultivars SC5, SC8, GR891 and GR911 were bred in the farmer participatory crop improvement program (Howeler, 2001). Recently, importation of core cassava germplasms from CIAT diversified the genetic background of cassava in China, allowing cassava breeders to produce new varieties with more desirable traits. Nevertheless, most of these germplasms are expected to be susceptible to CMD due to their South American origin. High CMD resistant cassava had been developed through integration of resistance trait from *Manihot glaziovii* by interspecific hybridization, which becomes the major resistance source dominating CMD resistance in pan-Africa (Fargette et al., 1996). Classical genetic analysis and genetic mapping of some resistant landraces showed that a major dominant gene, *CMD2*, confers resistance to

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CMD and important molecular markers associated with *CMD2* have been identified (Fregene et al., 1997; Akano et al., 2002). Another CMD resistance gene *CMD1* was also identified and mapped on the cassava genetic linkage map by CIAT (Akano et al., 2002). *CMD2* is located on linkage group R, whereas *CMD1* is on linkage group D of the cassava molecular map (Fregene et al., 1997). The action of the two genes is also different: *CMD2* is dominant, whereas *CMD1* appears recessive in that its effect is detected only in backcross progeny and not in the F1 (Akano et al., 2002). *CMD2* is on linkage group R of the male cassava framework map 8 cM from the marker SSRY28. Later, the group at CIAT identified NS158 to be closely linked to CMD resistance. The dominant *CMD2* gene for CMD resistance was introgressed into Latin American germ- plasms through maker-assisted selection (MAS). After two years of evaluation, 14 genotypes with high CMD resistance and good rooting yield potential were selected (Okogbenin et al., 2007).

In parallel, transgenic approaches of engineering CMD resistance in cassava have been reported (reviewed by Vanderschuren et al., 2007a). For example, increased ACMV resistance in cassava have been developed in transgenic cassava plants expressing antisense RNA or dsRNA targeting the viral mRNAs of *Rep* (*AC1*), *TrAP* (*AC2*) and *REn* , or the viral untranslational common region (Zhang et al., 2005; Vanderschuren et al., 2007b, 2009).

Currently, a dozen of elite cassava varieties, for example KU50 and NZ199, are grown by local farmers in ASEAN countries. Evaluation of their capacity for CMD resistance is of importance due to devastating impact of CMD on cassava production. In order to screen cassava genotypes for CMD resistance efficiently, a platform that could integrate virus infection tests and marker-assisted selection would be useful. Most of ACMV inoculations were conducted by biolistic viral DNA delivery (Briddon et al., 1998; Zhang et al., 2005) and only recently, a very efficient agro-inoculation protocol with ACMV-N*Og* infectious clones in the laboratory was developed (Vanderschuren et al., 2009). In this paper, we developed a screening system, which combines the infection assay and CMD resistance-associated molecular markers to identify CMD resistant or susceptible cassava varieties. To our knowledge, this is the first report of screening CMD resistance using cassava varieties from ASEAN region.

MATERIALS AND METHODS

Plant material

Cassava (*M. esculenta* Crantz) varieties used in the present study were grown in a greenhouse condition under 25 ± 2°C and 16 h/8 h photoperiod. Cassava varieties SC5, SC8, SC124, SC205, NZ199 and ZM9781 were provided by Chinese Academy of Tropical Agricultural Science. Cultivar KU50 (Thailand) and GR911 were provided by Guangxi Institute of Subtropical Crops. Other cultivars including N13, Q12, R9, T7, V5, 3C, 5E, 11Q, 16P, 18R and TMS60444 were from the *in vitro* germplasm bank established in Shanghai Center for Cassava Biotechnology, Chinese Academy of Sciences. Besides the varieties mentioned above, two other varieties were also used in molecular marker analysis: TME3 (a Nigerian landrace displaying strong CMD resistance, as the positive control) and TMS30555 (a CMD-susceptible line, as the negative control) (Akano et al., 2002), kindly provided by International Institute of Tropical Agriculture (IITA).

Virus isolate and bacterial strain

The infectious clones of ACMV-N*Og* DNA-A and DNA-B (Liu et al., 1997) harboring in the *Agrobacterium tumefaciens* strain LBA4404 were used for agro-inoculation of all cassava varieties. The Genbank accession numbers of the published ACMV-N*Og* DNA-A and DNA- B sequences used in this study are AJ427910 and AJ427911, respectively. The construction of infectious clones of ACMV-N*Og* had been reported by Vanderschuren et al. (2009).

Agro-inoculation

Clones of *A. tumefaciens* strain LBA4404 containing the infectious clones of ACMV-N*Og* DNA-A and DNA-B (Vanderschuren et al., 2009) were separately cultured on YEB plates supplemented with streptomycin (100 mg/l), rifampicin (25 mg/l) and kanamycin (50 mg/l) at 28°C for 72 h. Then the bacteria of DNA-A and DNA-B were mixed together in equal proportion. One-month-old cassava plantlets with 5 - 6 leaves were needle-punctured 6 times with the mixed bacteria suspension at the tip of the shoot axis using a fine syringe needle (Ø 0.5 mm). A minimum of three plants per variety were inoculated.

Molecular marker analysis

Genomic DNA was isolated from fresh and young leaves of all uninfected cassava varieties grown in the greenhouse according to Dellaporta et al. (1983) . SSR markers SSRY28 and NS158, and a SCAR marker RME1 associated with CMD resistance were provided by Dr. Martin Fregene, CIAT, and were used in genotyping the DNA samples (Akano et al., 2002). Each 50 l PCR reaction contained 50 ng genomic DNA, 0.4 M of each forward and reverse primers, 10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP and 1 U Taq DNA polymerase. The PCR profile involved an initial denaturation for 5 min at 95°C; then 30 cycles at 95°C for 40 s, 56°C for 1 min, 72°C for 30 s; and a final extension at 72°C for 10 min. For SSR marker, 2.0 l of the PCR product was electrophoresed on 8% polyacrylamide gels for 5 - 6 h at 100 V, and DNA was visualized by standard silver staining using the Promega's silver staining kit (Promega, Shanghai, China). The SCAR marker was scored on a 1.5% agarose gel and visualized by ethidium bromide.

Virus detection in infected plants using Southern blot analysis

Total DNA was extracted from the top leaves of three infected plants per variety at 60 days post infection (dpi) according to Soni and Murray (1994). Aliquots of 5 ug of total DNA from each sample without digestion were analyzed using standard protocol for Southern blot (Sambrook et al., 1989). Accumulated viral DNA was hybridized with a DIG-labeled probe specific to the ACMV-N*Og* AC1 gene. Labeling, hybridization and chemiluminescent detection were performed according to the instruction of the manufacturer (Roche

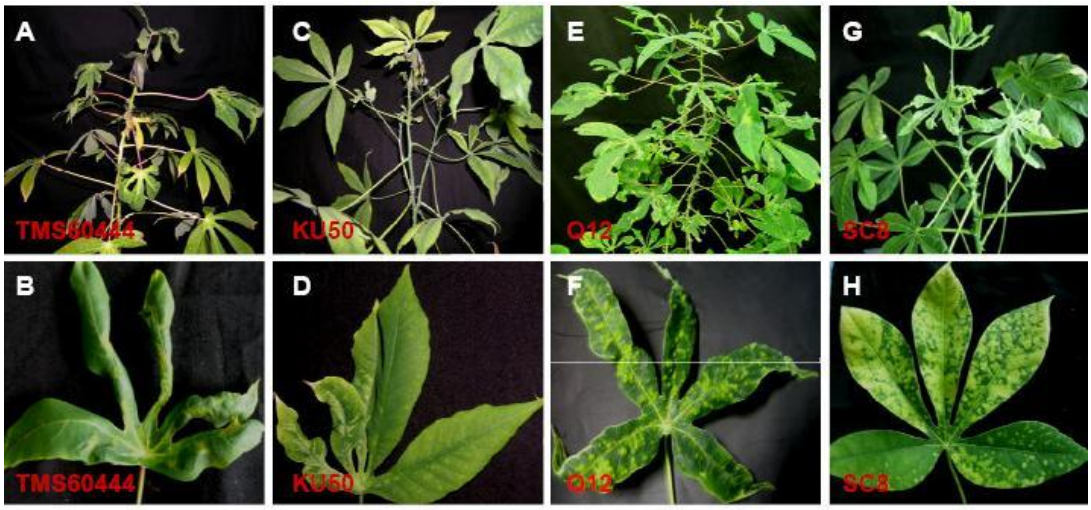

Figure 1. Cassava mosaic disease symptoms on plant and leaves of different cassava varieties after agro-inoculation with African cassava mosaic virus infectious clone ACMV-N*Og*. A, B: TMS60444; C, D: KU50; E, F: Q12; G, H: SC8.

Applied Science). The intensity of bands was quantified by using Eagle Eye® II still video system (Stratagene).

CMD symptom assessment

After agro-inoculation, CMD symptom development on new emerging leaves was assessed weekly. The symptoms were recorded from 10 dpi to 60 dpi till each plant has developed more than 20 new leaves. Disease symptom severity on fully expanded leaves was recorded on a scale of 0 - 4 (0, no symptom; 1, faint mosaic; 2, yellow mosaic, malformation, 5 - 10% size reduction; 3, severe mosaic, distortion, up to 50% size reduction; 4, severe mosaic, severe distortion, leaf reduced to veins with 50 - 80% size reduction), as described by Zhang et al. (2005).

RESULTS

Screening of cassava varieties for CMD resistance by agro-inoculation

After agro-inoculation of 18 cassava varieties with ACMV-N*Og* in a greenhouse, symptom development was monitored regularly. Various foliar mosaic symptoms were displayed by all plants tested of TMS60444, KU50, Q12 and SC8 at 60dpi (Figure 1). Among these varieties, Q12 and SC8 showed more severe symptoms than TMS60444 and KU50 at the whole plant level (Figure 1 A, C, E, G). Also, leaves of TMS60444 showed downward curling in most cases (Figure 1B) and KU50 displayed certain upward curling (Figure 1D). Yellowing, mosaic symptoms and reduced size were observed on the leaves of Q12 (Figure 1E, F) whereas SC8 displayed foliar mosaic symptoms with different degrees of malformation vary from faint to severe (Figure 1G, H). These four varieties showed varied degrees of foliar symptom severity based on leaf position (Figure 2). Symptoms were most severe on leaves in positions of 7 - 9 for TMS60444, KU50, Q12 and SC8. For TMS60444, the average level of severity score had never reached 2.0 on newly developed leaves while the average level remained high for new leaves of Q12 and SC8. Symptoms observed on SC8 and Q12 are more severe than those of TMS60444 and KU50. The symptom trend lines of other varieties are provided in the supplementary data (Suppl. Table 1).

Total symptom severity scores of the 18 varieties are presented in Figure 3 in the order from lowest to highest (Figure 3, upper panel). Cultivar TMS60444, the model cultivar for genetic transformation frequently used in virus resistance study (Zhang et al., 2005; Vanderschuren et al., 2007b, 2009), showed a moderate resistance to ACMV- N*Og* infection. This is in consistent with results from a previous study on AFLP analysis of CMD resistance (Fregene et al., 2000). In this study, all varieties were categorized into three groups: moderately resistant (MR) varieties R9, V5, 16P, TMS60444, N13, T7 and 11Q with total severity scores less than 40; moderately susceptible (MS) varieties 3C, SC205, KU50, ZM9781, SC5, 18R and GR911 with total severity scores from 40 - 50; and highly susceptible (HS) varieties Q12, SC8, SC124 and NZ199 with severity scores more than 50.

Viral DNA accumulation in infected plants

The accumulation of ACMV DNA was detected by Southern blot analysis in apical leaves of inoculated plants at 60 dpi (Figure 4). In the six varieties analyzed, the accumulation of ACMV DNAs, including double-stranded (ds), single-stranded (ss) DNA and open circular (oc) forms, was detected. The level of viral DNA accumulation was lower in N13, T7 and TMS60444 than

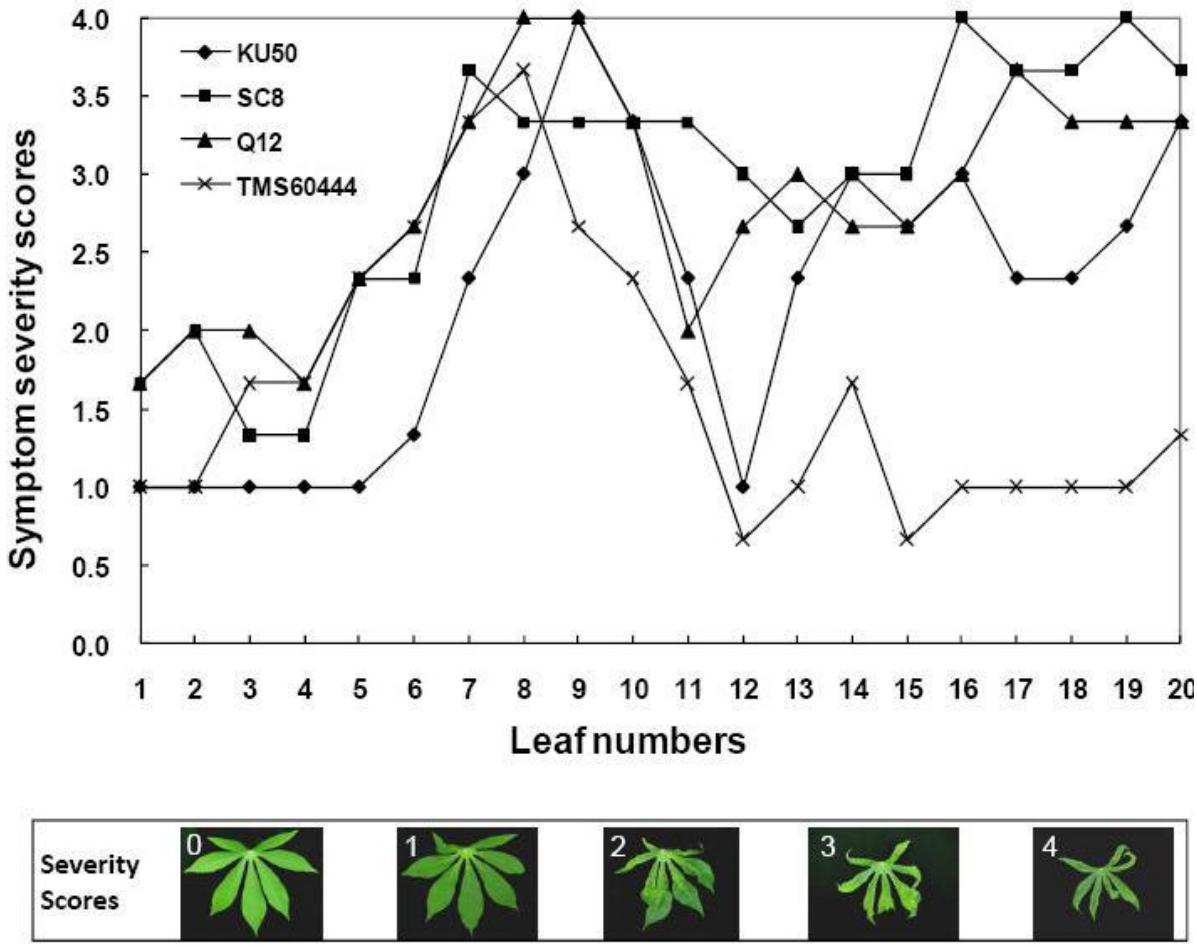

Figure 2. Cassava mosaic disease symptom severity scores on new emerging leaves 1 to 20 of cassava varieties KU50, SC8, Q12 and TMS60444 after ACMV-N*Og* agro-inoculation. The bottom panel illustrates representative leaves showing different degrees of symptoms for the evaluation of symptom severity scores (0 = no symptoms, 4 = severe symptoms).

in KU50, SC8 and Q12. This result reflected a correlation between symptom severity and level of viral DNA accumulation.

Molecular marker analysis

The 18 cassava varieties, together with the CMD resistant line TME3 and the susceptible line TMS30555, were evaluated by three markers associated with the CMD resistance gene *CMD2*, including sequence-characterized amplified regions (SCAR) RME1 and simple sequence repeats (SSR) SSRY28 and NS158 (Lokko et al., 2007). For the SCAR marker RME1, only varieties 11Q (Suppl. Figure 1A, lane 11) and T7 (Suppl. Figure 1A, lane 18) showed the expected bands, similar to the CMD resistant line TME3 (Suppl. Figure 1A, lane 21). Resistance-associated bands were obtained using primers of SSR marker SSRY28 in varieties N13, 11Q and T7 (Suppl. Figure 1B; lanes 9, 11, 18, respectively); whereas varieties 11Q (Suppl. Figure 1C, lane 11) and T7 (Suppl. Figure 1C, lane 18) showed the same pattern as the resistant line TME3 by using SSR marker NS158. Using SCAR-RME1 and SSR-NS158, variety 18R (Suppl. Figure 1A and C, lane 7) showed a similar pattern as the susceptible line TMS30555 (Suppl. Figure 1A and C, lane 22).

From the molecular marker analyses and agro-inoculation screening, varieties T7, 11Q, and N13 showed resistance-associated bands and low symptom severity scores compared to varieties NZ199, SC124, SC8 and Q12 (Figure 3). All varieties showing the resistance associated bands as the resistant line TME3 were grouped under MR type. Resistance- associated bands were not detected in any varieties of either MS or HS classes. But the only variety 18R, which come under MS group showed same band pattern as the susceptible line TMS30555. Moreover, there are also some varieties in MR group such as R9, V5 and 16P, which come under the lower symptom severity scores, did not show any

resistance-associated bands.

DISCUSSION

Successful agro-inoculation of cassava with ACMV was reported recently (Vanderschuren et al., 2009) and proved useful for CMD studies in cassava. Using agro-inoculation, 18 cassava varieties for CMD resistance under greenhouse conditions were screened. According to the average total symptom severity scores, cassava varieties were grouped into three classes: MR, MS and HS, respectively. In parallel, analysis of *CMD2* associated molecular markers RME1, SSRY28 and NS158 showed resistance-associated banding pattern in varieties N13, T7 and 11Q. The result suggests that other resistance loci or genes related to CMD resistance might exist in the other cassava cultivars from the MR group.

Most of previous screening studies for CMD resistance in Africa were conducted in field conditions under high CMD epidemic pressure (Akano et al., 2002; Lokko et al., 2005; Okogbenin et al., 2007). In this paper, agro- inoculation of ACMV-N *Og* infectious clones was used for the first time to screen cassava varieties from ASEAN countries for CMD resistance in a greenhouse. An agro-inoculation mediated screening system has several advantages compared to field screening studies. First, conditions for cassava growth and virus infection such as temperature, humidity and light can be controlled under greenhouse conditions; this could minimize the draw-backs of field evaluations. Second, populations of whitefly vectors vary frequently with variable environmental conditions such as rainfall distribution, light intensity and temperature (Hahn et al., 1980); thus, field evaluation for CMD-resistance screening that relies on vector -transmission of the virus can be unpredictable. Third, CMD resistance screening experiments in a greenhouse are safer than in the field in ASEAN countries, where CMD does not occur in cassava field yet. Finally, agro-inoculation of cassava with ACMV infectious clones can facilitate the study of virus infection, transmission and pathogenesis.

Nevertheless, there are some limiting factors for the agro-inoculation mediated screening system. Expression of CMD in different cassava genotypes is known to be dependent on the

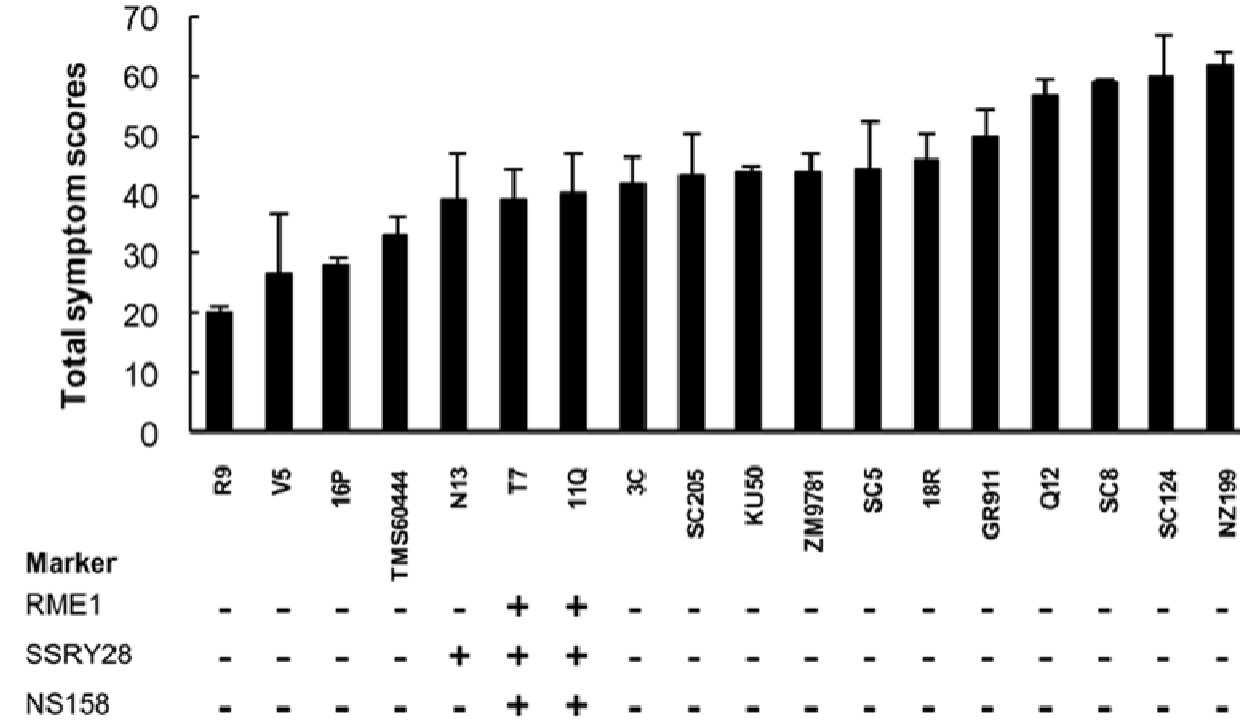

Figure 3. Evaluation of cassava mosaic disease resistance in combination of agro-inoculation screening and the molecular marker analysis. The upper panel shows the average total symptom severity scores of cassava varieties and the lower panel shows the results of molecular marker analysis using RME1, SSRY28 and NS158. + means having the expected bands similar to the cassava mosaic disease resistant line TME3; - means no expected band detected.

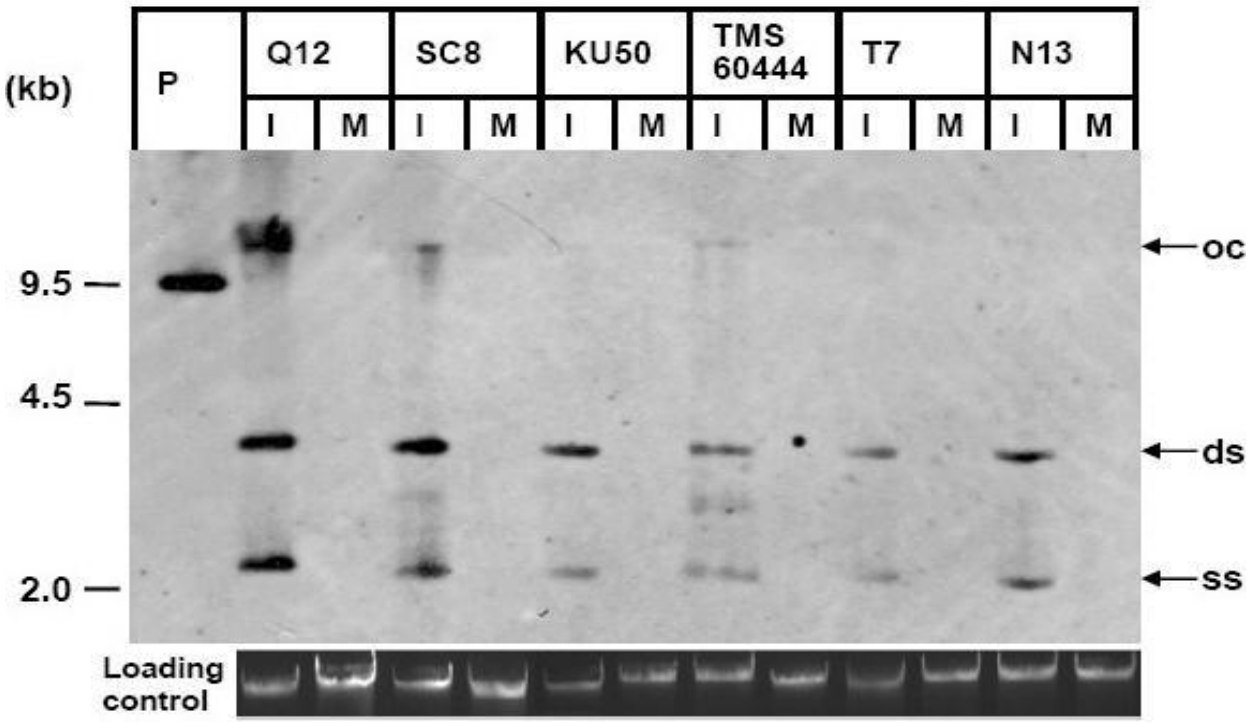

Figure 4. Southern blot analysis of ACMV-N*Og* replication and accumulation in leaves of different cassava varieties after agro-inoculation. P, plasmid control; I, ACMV- N*Og* infected plants; M, mock inoculated control. The positions of viral single-stranded (ss), double-stranded (ds) and open circular (oc) DNA forms are indicated.

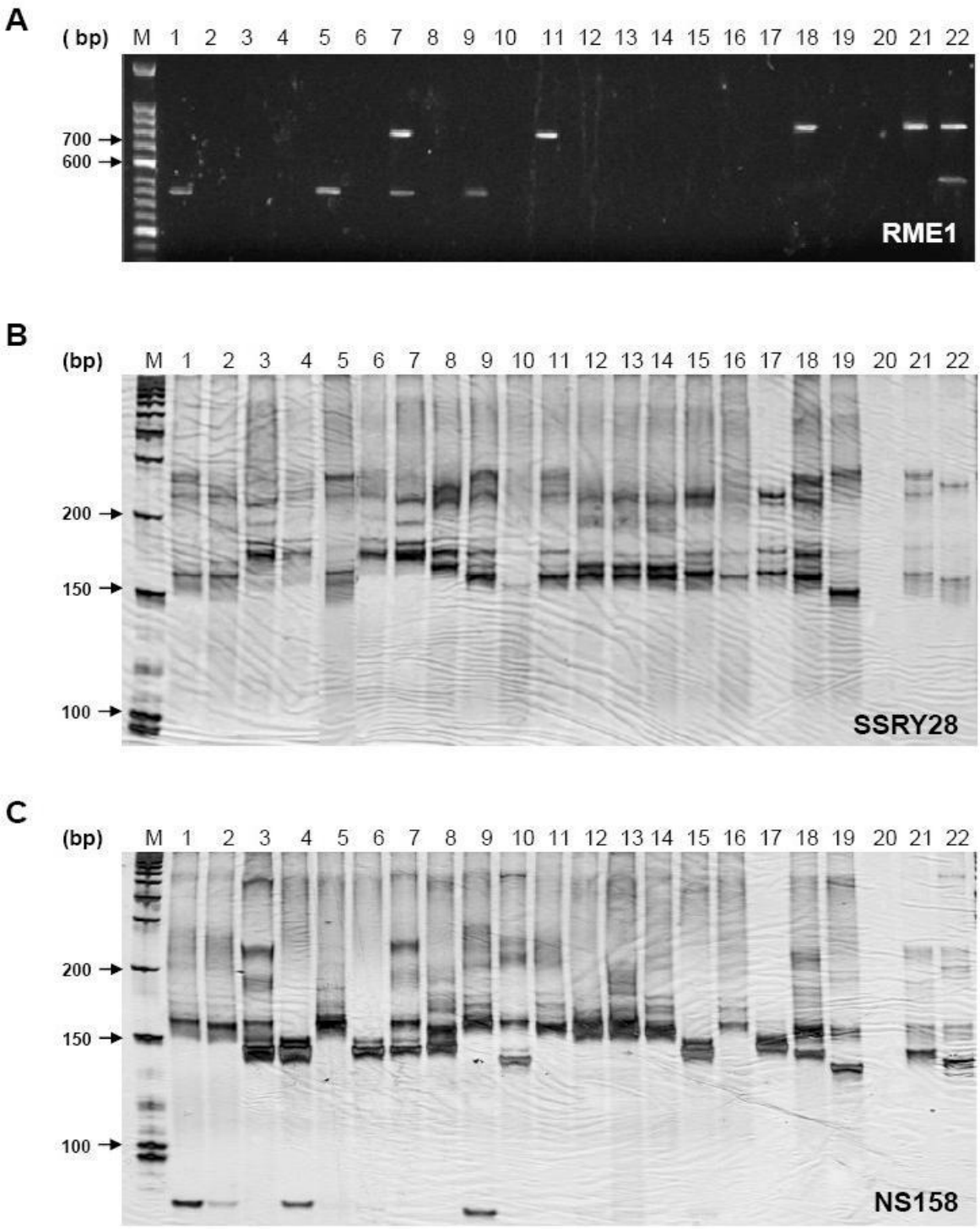

Figure 1. Molecular marker analysis of cassava varieties. A: SCAR marker RME1; B: SSR marker SSRY28; C: SSR marker NS158. M, molecular marker; 1, KU50; 2, V5; 3, GR911; 4, SC205; 5, NZ199; 6, SC8; 7, 18R; 8, SC5; 9, N13; 10, 5E; 11, 11Q; 12, 16P; 13, 3C; 14, SC124; 15, Q12; 16, ZM9781; 17, R9; 18, T7; 19, TMS60444; 20, H_2O; 21, TME3 (resistant line); 22, TMS30555 (susceptible line).

environment, and a strong relationship between the range of symptoms and genotype x environment interaction has been reported Fargette et al., 1994).

CMD evaluation requires evaluation tests in multiple environments to confirm the resistant genotypes (Lokko et al., 2005). Resistance screening in the green-house

does not reflect field conditions but proves to be a rapid and reliable monitor system to evaluate CMD resistance capacity of varied cassava germplasms. In this study, all inoculated plants developed CMD symptoms with various degree of severity. One reason might be due to the lack of CMD resistance genes in the varieties tested as confirmed by the molecular marker analysis.

After the mapping of two CMD resistance genes *CMD1* (recessive) and *CMD2* (dominant) on the cassava genetic linkage map established by CIAT (Akano et al., 2002; Fregene et al., 2001), three molecular markers associated with *CMD2*, namely RME1, SSRY28 and NS158, were developed (Lokko et al., 2007). Through cassava breeding programs, these markers hold great promise in fast tracking the CMD-resistant germplasms. For example, with the aim to introduce Latin American germplasm to Africa, these markers were used to preselect neotropical cassava genotypes for CMD resistance (Okogbenin et al., 2007). In this study, the varieties holding the *CMD2* markers are possibly from an African origin but other varieties belonging to the MR group did not have the markers. One possible reason is that other genes may contribute to CMD resistance besides *CMD2.* This is supported by the identification of five additional sources of resistance to CMD (Legg and Fauquet, 2004).

In this agro-inoculation screening studies, none of the cassava varieties tested showed immunity to virus infection but CMD resistance was associated with suppressed levels of viral DNA accumulation. Based upon virus accumulation and symptom severity scores, cassava varieties were categorized into different groups. However, symptom severity is not always correlated with virus concentration (Ogbe et al., 2003).

In conclusion, 18 cassava varieties from ASEAN countries were screened for CMD resistance using agro-inoculation and molecular markers associated with the *CMD2.* This study indicated that most cassava varieties tested are susceptible to CMD, suggesting that those germpl- asms originated directly or indirectly from Latin America as they lacked the CMD resistant genes/ mechanism. It is necessary to introduce African cassava germplasms that harbor CMD resistance genes such as *CMD2* to assist breeding for CMD resistance.

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