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Diversity of begomoviruses associated with mosaic disease of cultivated cassava (*Manihot esculenta* Crantz) and its wild relative (*Manihot glaziovii* Müll. Arg.) in Uganda

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Cassava (*Manihot esculenta*) growing in Uganda during 2001–2002 has been screened for the presence of begomoviruses using PCR-RFLP, cloning full-length genomic components and nucleotide sequence analysis. In contrast with a recent survey in neighbouring Kenya, which identified three distinct strains of East African cassava mosaic virus (EACMV, EACMV-UG and EACMV-KE2) as well as East African cassava mosaic Zanzibar virus and the new species East African cassava mosaic Kenya virus, only EACMV-UG and, to a lesser extent, African cassava mosaic virus (ACMV) were found associated with cassava in Uganda. The integrity of the cloned genomic components of representative virus isolates was confirmed by demonstrating their infectivity in *Nicotiana benthamiana* and cassava using biolistic inoculation, providing a convenient means to screen cassava varieties for disease resistance. Both EACMV-UG and ACMV were also associated with *Manihot glaziovii*. Infectivity studies using cloned components confirmed that viruses from one host could infect the other, suggesting that this wild relative of cassava might be a reservoir host for the disease. The relatively low level of diversity of begomoviruses associated with cassava mosaic disease in Uganda is consistent with reports that EACMV-UG has displaced other begomovirus species and strains during the recent epidemic that swept through the country.

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important staple food crops in the tropics, particularly in Africa (Nassar & Ortiz, 2007). Cassava mosaic disease (CMD), the major constraint to cassava production in Africa, is caused by a number of distinct begomoviruses (family *Geminiviridae*) (Thresh & Cooter, 2005). To date, seven begomovirus species have been identified in association with CMD, namely African cassava mosaic virus, East African cassava mosaic virus, East African cassava mosaic Cameroon virus, East African cassava mosaic Kenya virus, East African cassava mosaic Malawi virus, East African cassava mosaic Zanzibar virus and South African cassava mosaic virus (Stanley *et al.*, 2005; Bull *et al.*, 2006). Although cassava begomoviruses are naturally transmitted by the whitefly *Bemisia tabaci* (Storey & Nichols, 1938; Dubern, 1994), CMD is widely disseminated by the distribution of stem cuttings used for vegetative propagation. Furthermore, cassava is often infected with more than one begomovirus that can increase symptom severity (synergism) and encourage virus diversification as a consequence of recombination. For example, the severe outbreak of CMD that was first reported in Northern Uganda in the 1990s and that has since spread throughout the country and to neighbouring countries in central and eastern Africa (Gibson *et al.*, 1996; Legg *et al.*, 2001, 2004; Neuenschwander *et al.*, 2002; Bigirimana *et al.*, 2004; Legg & Fauquet, 2004; Were *et al.*, 2004; Bull *et al.*, 2006) has been attributed to a synergistic interaction between African cassava mosaic virus (ACMV) and the East African cassava mosaic virus (EACMV) recombinant

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A supplementary table showing the primers used in this study is available with the online version of this paper.

strain EACMV-UG (Deng *et al.*, 1997; Zhou *et al.*, 1997; Pita *et al.*, 2001) coupled with an increase in the populations of the vector *B. tabaci* (Legg *et al.*, 2002; Colvin *et al.*, 2004).

Cultivated cassava is believed to be the principal reservoir for CMD-associated begomoviruses because of its perennial growth and scale of production (Fauquet & Fargette, 1990). However, alternative hosts have been identified (Bock *et al.*, 1978, 1981), including *Manihot glaziovii* Müll. Arg., a wild relative of cassava native to Brazil (Allem, 1999) that was introduced into Africa as a potential source of resistance to pests and diseases (Allem, 2001, 2002). *M. glaziovii* is common in Uganda, where it is used by farmers as a source of latex and shade in crop plantations and many homesteads because of its tree-like and bushy growth. Although considered to be epidemiologically unimportant (Fauquet & Fargette, 1990) there is a high incidence of a severe mosaic disease in *M. glaziovii* (Fig. 1) closely resembling that associated with CMD in cassava, suggesting begomovirus aetiology. Hence, it is possible that the begomoviruses which have caused such a devastating epidemic in the highly susceptible varieties grown in Uganda can also infect *M. glaziovii* causing severe CMD-like disease. Despite this, there have been no reports of begomoviruses isolated from *M. glaziovii* or related wild species, and their possible role in the epidemiology of the disease remains unclear. Without empirical data on the begomoviruses associated with this disease and their phylogenetic and pathogenic relationships, it is impossible to assess the potential risk to cassava cultivation posed by the relatively small number of diseased *M. glaziovii*.

Previous studies of the diversity of CMD begomoviruses and the severity of the disease in Uganda have focused exclusively on viruses isolated from cultivated cassava (Harrison *et al.*, 1997; Zhou *et al.*, 1997; Otim-Nape *et al.* 2001; Pita *et al.*, 2001; Owor *et al.*, 2004; Sseruwagi *et al.*, 2004a). Recently, a comprehensive survey of cassava begomoviruses associated with CMD was undertaken in neighbouring Kenya (Bull *et al.*, 2006, 2007). Here, we report a similar survey carried out in Uganda over the same period and have extended the investigation to include *M. glaziovii* as well as cassava. Cloned genomic components of representative begomovirus isolates have been constructed and used to confirm infectivity in cultivated cassava and *M. glaziovii* and to screen cassava cultivars for susceptibility to the disease.

METHODS

Virus isolates. Cassava stem cuttings [15–30 cm in length, taken from the middle section of each symptomatic shoot according to Otim-Nape *et al.* (2001) and Sseruwagi *et al.* (2004b)] were obtained from naturally infected plants showing symptoms of CMD. Samples were taken from farmers' fields in 28 of the major growing districts in Uganda between December 2001 and December 2002. *M. glaziovii* plants with either white or red petioles, showing severe and mild CMD-like symptoms, respectively (Fig. 1), were sampled from farmers' fields, homesteads and from the wild in Rakai and Wakiso districts of Uganda in July 2003. Cuttings were planted in compost and maintained at 25 °C with supplementary lighting to provide a 16 h photoperiod under containment at the John Innes Centre (UK). Virus isolates and clones were maintained and manipulated in accordance with DEFRA licence PHL 185A/4538 (7/2003).

Fig. 1. CMD-like symptoms in *M. glaziovii* plants with white (a) and red (b) petioles growing in a field at Namulonge (Wakiso district).

Viral DNA extraction, cloning and analysis. DNA was extracted using a Nucleon PhytoPure kit (Amersham) and the presence of begomovirus DNA was confirmed by PCR amplification using universal primers specific to the DNA-A component (Briddon & Markham, 1994). RFLP patterns were established for the PCR fragments using restriction enzymes *Dra*I, *Eco*RV and *Mlu*I as described by Bull *et al.* (2006). Specific primers were subsequently designed to PCR-amplify full-length copies of the genomic components (Supplementary Table S1, available in JGV Online). PCR products were cloned into TOPO pCR2.1 according to the manufacturer's recommendations (Invitrogen). For *M. glaziovii* samples, extracted DNA was analysed initially by blot hybridization using full-length genomic components of EACMV-UG and ACMV as probes, labelled using a Random Primers DNA Labelling kit (Gibco-BRL) and a NucTrap Probe Purification Column (Stratagene). This was to ascertain the presence of begomovirus prior to PCR-RFLP analysis and cloning. Nucleotide sequences were established using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and primers designed to the vector and specific to the viral DNAs. Reaction mixtures were resolved either on an ABI 3700 automated sequencer at the John Innes Centre or commercially (Lark Technologies). Nucleotide sequences were assembled and analysed using version 7 of the Genetics Computer Group (GCG) package (Devereux *et al.*, 1984) and aligned using CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic analyses were performed using the neighbour-joining option of PHYLIP (version 3.57c) (Felsenstein, 1995) and

phylogenetic trees were viewed and manipulated using TreeView (Page, 1996).

Infectivity of cloned viral DNA components and transmission of progeny virus. Infectivity of cloned begomovirus components was assessed using full-length DNA-A and DNA-B excised from the cloning vector with the appropriate restriction enzyme. Combinations of DNA components were coated onto gold particles (Garzón-Tiznado *et al.*, 1993) and introduced into *Nicotiana benthamiana*, cultivated cassava varieties from NACRRI, Uganda (Ebwanateraka, Nase 4, Nase 12, Njule Omumyufu, Njule Omweru and TME 14), and CIAT, Colombia (N Mex 55), and *M. glaziovii* by biolistic inoculation using a hand-held gun as described previously (Briddon *et al.*, 1998). Cassava and *M. glaziovii* plants used for infectivity assays were produced from cuttings of healthy stock plants shown to be free of begomovirus infection by PCR analysis. Inoculated plants were maintained at 25 °C with a 16 h photoperiod.

RESULTS

Only ACMV and EACMV-UG were detected in cassava and *M. glaziovii* in Uganda

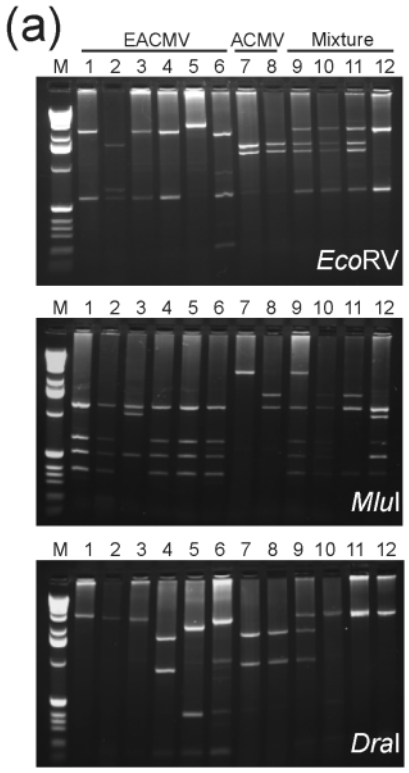
RFLP analysis of PCR products amplified from symptomatic cassava plants using universal primers for begomovirus DNA-A was used as a preliminary screen to assess genetic diversity of the begomoviruses throughout the major cassava-growing regions of Uganda. The analysis revealed six polymorphic groups (EA1–EA6), subsequently shown by sequence analysis to correspond to EACMV isolates, two groups (A1 and A2) corresponding to ACMV isolates and mixtures of isolates of both begomovirus species (Fig. 2a). The RFLP analysis demonstrated that the vast majority of isolates (347) corresponded to EACMV, 19 isolates corresponded to ACMV and 19 isolates were mixtures of both begomoviruses (Fig. 2b). EACMV was found in samples from all districts under investigation and the major EACMV RFLP pattern (EA1) predominated in every district. In contrast, ACMV appears to occur more sporadically and was found mainly in samples from the western half of the country. Nonetheless, both EACMV and ACMV were found in regions of the country that had experienced the CMD epidemic for different durations (Fig. 2c). Viral DNA was also extracted from *M. glaziovii* plants (white petioles, tree-like growth habit, severe symptoms) as well as from less severely affected plants with red petioles that had a more bushy growth habit, probably a hybrid between *M. glaziovii* and cassava (Fig. 1). PCR-RFLP analysis revealed two polymorphic groups corresponding to EACMV (EA1) and ACMV (A1) (data not shown), suggesting that isolates of these distinct begomovirus species are also present in the wild relative of cassava and their hybrids.

Cassava begomovirus isolates from districts that had experienced the epidemic for more than 10 years (Arua and Nakasongola), for 5–10 years (Kaberamaido, Kapchorwa, Mpigi and Wakiso) and for less than 5 years (Bushenyi, Busia, Kalangala and Iganga) (Fig. 2c) were selected, and full-length copies of the DNA-A and DNA-B components were cloned and characterized by sequence analysis (Table 1). Comparison of the DNA-A components from *M. esculenta* revealed the presence of two clusters showing >98 % (12 sequences) and 97 % identity (two sequences) between isolates within each cluster and 72–74 % identity between clusters. Comparison with published begomovirus sequences showed that the major cluster was most closely related to EACMV-UG isolates (for example, clone 24-ug177-10 shows 99.8 % identity with the Kenyan isolate EACMV-UG[K233]) and the minor cluster to ACMV isolates (for example clone 22-ug56b5 shows 97.2 % identity with the Ugandan isolate ACMV-UGMld). Hence, on the basis of accepted demarcation criteria (Stanley *et al.*, 2005), these begomoviruses should be regarded as isolates of these two species. ACMV and EACMV-UG were also isolated from *M. glaziovii* plants growing in the southern districts of Rakai and Wakiso (Table 1). Phylogenetic analysis of the DNA-A components (Fig. 3a) is consistent with the proposed taxonomic status of the begomoviruses isolated from both cassava and *M. glaziovii*, showing that all EACMV-UG isolates are much more closely related to each other than are the ACMV isolates.

The four sequences of DNA-B components produced in this study are closely related, showing >97 % identity. Phylogenetic analysis of these sequences (Fig. 3b) clustered them with the Group A sequences previously defined by Bull *et al.* (2006). This cluster consists of DNA-B components associated with isolates of EACMV, EACMV-UG and East African cassava mosaic Kenya virus (EACMKV) that are present in Uganda, and western and central Kenya. The latest DNA-B sequences are most closely related to EACMV-UG isolates occurring in western Kenya along the border with Uganda (for example, all four clones show >97 % identity with the Kenyan isolate EACMV-UG[K90]).

Although isolates of both species differed mainly by a series of nucleotide substitutions dispersed throughout the entire genome, the DNA-B component of EACMV-UG[Nak:1] noticeably has a 14 nt deletion located within the intergenic region upstream of the BC1 open reading frame, corresponding to nucleotides 2488–2501 in the other isolates examined. With the exception of EACMV-UG[Bus:289] DNA-A, which has a single nucleotide deletion within the coding sequence of the replication-associated protein (Rep), all DNA-A and DNA-B components have a genetic arrangement typical of Old World geminiviruses (Stanley *et al.*, 2005).

Clones representative of all EACMV-UG RFLP patterns (EA1–EA6) were recovered in addition to a single clone (25-ug128-2) that differed from the most abundant pattern EA1 by the absence of a *Dra*I site resulting from a single nucleotide substitution (Table 1). An ACMV clone corresponding to RFLP pattern A1 was also isolated, although clones 22-ug56b5 and wcass4-3 differed from patterns A1 and A2, respectively, by nucleotide substitutions within either *Mlu*I or *Dra*I sites. This reinforces the contention that, although RFLP analysis can rapidly



(b)

District	EACMV EA1	EA2	EA3	EA4	EA5	EA6	ACMV A1	A2	EACMV +ACMV
Arua	19	–	–	–	1	1	–	–	3
Bushenyi	24	–	1	–	1	–	–	–	–
Busia	6	–	–	–	–	–	1	–	–
Hoima	9	–	1	–	–	–	–	1	1
Iganga	12	–	–	1	1	1	–	–	–
Kabarole	6	–	–	–	1	–	–	7	–
Kaberamaido	6	1	1	–	–	–	–	–	–
Kalangala	16	1	–	1	1	–	2	–	2
Kamuli	5	–	–	–	–	–	–	–	–
Kapchorwa	23	–	–	–	–	–	–	–	–
Kibale	14	–	–	–	–	–	–	1	2
Kiboga	11	–	–	–	–	–	–	1	2
Kumi	13	–	–	–	–	–	–	–	–
Lira	7	–	–	–	–	–	–	–	–
Luweero	15	–	–	–	–	–	–	–	–
Masaka	10	–	–	–	–	–	–	–	–
Masindi	15	–	–	–	–	–	–	–	–
Mbale	24	–	–	–	–	–	–	–	–
Mbarara	14	–	–	–	1	–	–	1	–
Mpigi	6	–	–	–	–	–	1	–	–
Mubende	18	1	–	–	–	1	1	–	1
Mukono	9	–	–	–	–	–	–	–	–
Nakasongola	5	–	–	–	–	–	–	–	4
Nebbi	7	–	–	–	–	–	–	2	4
Pallisa	11	–	–	–	–	–	1	–	–
Rakai	4	1	–	–	2	–	–	–	–
Soroti	8	–	–	–	–	–	–	–	–
Wakiso	10	–	–	–	–	–	–	–	–

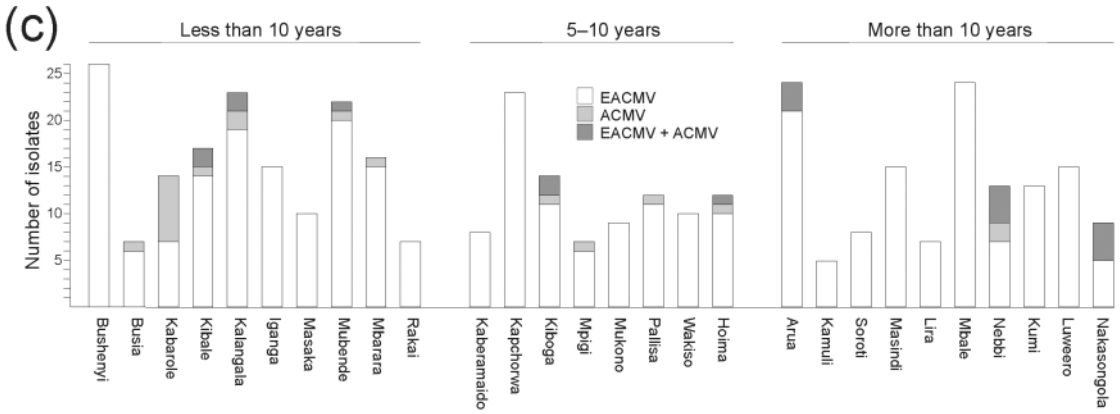


Fig. 2. PCR-RFLP analysis of begomovirus isolates from cassava. (a) DNA-A was PCR amplified using universal primers and digested with *Eco*RV, *Mlu*I and *Dra*I as indicated. RFLP profiles correspond to EACMV isolates EA1–EA6 (lanes 1–6), ACMV isolates A1 and A2 (lanes 7 and 8) and examples of mixtures of EACMV and ACMV isolates EA1 and A1 (lane 9), EA1 and A2 (lanes 10 and 11) and EA1 and EA3 (lane 12). (b) Geographical distribution of EACMV and ACMV isolates according to the PCR-RFLP pattern. (c) Distribution of EACMV and ACMV isolates in regions that had experienced the CMD epidemic for either less than 5 years, between 5 and 10 years or more than 10 years prior to 2001.

provide a general view of the population, establishment of the complete nucleotide sequence of the genomic components is essential for definitive begomovirus identification. While the majority of plants contained a single begomovirus, the nucleotide sequence data confirmed that some cassava plants supported a dual infection of EACMV-UG and ACMV. A map showing the distribution of cassava-infecting begomoviruses in Uganda in 2001–2002, based on the RFLP patterns (Fig. 2) and nucleotide sequence data (Table 1), is presented in Fig. 4.

ACMV and EACMV infections differ in symptom severity

All cloned components isolated during this investigation were full-length and, with the exception of EACMV-UG

Table 1. Geographical origin and characterization of Ugandan cassava begomovirus cloned components

Host	Origin (district)	Virus isolate*	Virus component				
			DNA-A			DNA-B	
			Clone	RFLP pattern†	GenBank accession number	Clone	GenBank accession number
M. esculenta	Arua	EACMV-UG[UG:Aru:177 : 02]	24-ug177-10	EA5	AM502326	24-ug177‡	–
	Arua	EACMV-UG[UG:Aru:175 : 02]	24-ug175-8	EA6	AM502327	24-ug175‡	–
	Bushenyi	EACMV-UG[UG:Bus:289 : 02]	28-ug289-5	EA1	AM502337	28-ug289‡	–
	Busia	EACMV-UG[UG:Bus:108 : 02]	17-ug108-2	EA3	AJ618959	17-ug108‡	–
	Iganga	EACMV-UG[UG:Iga:441 : 02]	32-ug441	EA4	AM502328	–	–
	Kaberamaido	EACMV-UG[UG:Kab:92 : 02]	19-ug92b6	EA4	AJ618956	–	–
	Kalangala	ACMV-[UG:Kal:226 : 02]	27-ug226-2	A1	AM502338	27-ug226-1‡	–
	Kalangala	EACMV-UG[UG:Kal:233 : 02]	27-ug233-2	EA5	AM502329	27-ug233-1	AM502341
	Kalangala	EACMV-UG[UG:Kal:237 : 02]	27-ug237-2	EA4	AM502330	27-ug237-8	AM502342
	Mpigi	EACMV-UG[UG:Mpi:50 : 02]	20-ug50a8	EA2	AJ618958	20-ug50‡	–
	Mpigi	ACMV-[UG:Mpi:56 : 02]	22-ug56b5	–	AM502339	–	–
	Nakasongola	EACMV-UG[UG:Nak:1 : 02]	6-ug1a22	EA3	AJ618957	20-ug1a8	AM502344
	Wakiso	EACMV-UG[UG:Wak:115 : 02]	26-ug115a	EA1	AM502331	–	–
	Wakiso	EACMV-UG[UG:Wak:128 : 02]	25-ug128-2	–	AM502332	–	–
M. glaziovii	Rakai	EACMV-UG[UG:Rak:3 : 03]	wcass3-5	EA1	AM502333	–	–
	Rakai	ACMV-[UG:Rak:4 : 03]	wcass4-3	A1	AM502340	wcass4‡	–
	Rakai	EACMV-UG[UG:Rak:6 : 03]	wcass6-8	EA1	AM502334	wcass6‡	–
	Wakiso	EACMV-UG[UG:Wak:1 : 03]	wcass1-2	EA1	AM502335	wcass1‡	–
	Wakiso	EACMV-UG[UG:Wak:2 : 03]	wcass2-1	EA1	AM502336	wcass2-5	AM502343

*The formal isolate descriptor given here has been abbreviated in the text and Tables 2 and 3.
†Based on established sequence and defined in Fig. 2.
‡Nucleotide sequence data not available.

[Bus:289] which contains a disrupted Rep coding region, are potentially infectious. To confirm this, the DNA-A and DNA-B components of cassava isolates ACMV-[Kal:226], EACMV-UG[Nak:1], EACMV-UG[Kal:233] and EACMV-UG[Kal:237], as well as *M. glaziovii* isolate EACMV-UG[Wak:2], were introduced into plants by biolistic inoculation. All of these cloned components proved to be infectious (Table 2), although marked differences in symptom severity were associated with ACMV and EACMV-UG isolates in *N. benthamiana*. ACMV-[Kal:226] induced mild chlorosis and stunting symptoms in this host while all EACMV-UG isolates were more virulent, causing severe leaf curling and stunting (Fig. 5a). However, the EACMV-UG isolates were distinguishable on the basis of their slightly different latent periods (Table 2). Interestingly, *N. benthamiana* plants inoculated with a mixture of ACMV-[Kal:226] and EACMV-UG[Kal:233] showed more severe symptoms than either virus alone (Fig. 5b). Moreover, the presence of both viruses resulted in a higher infectivity (Table 3). Enhanced symptom severity was also observed when EACMV-UG[Kal:233] was co-inoculated with ACMV-[Kal:226] DNA-A. However, in the reciprocal combination, co-inoculation of ACMV-[Kal:226] with EACMV-UG[Kal:233] DNA-A resulted in only mild symptoms typical of ACMV infection. Co-inoculation of EACMV-UG[Kal:233] DNA-A and ACMV-[Kal:226] DNA-B or the reciprocal combination did not result in infection, consistent with the inability of the DNA-A component of one virus to *trans*-replicate the DNA-B component of the other as predicted from their differing Rep-binding iteron motifs, namely GGGGG (EACMV-UG) and GGAGA (ACMV).

ACMV and EACMV-UG isolates were also infectious in cassava (variety N Mex 55) and induced distinct symptoms that mainly reflected their virulence in *N. benthamiana*. Plants inoculated with EACMV-UG[Kal:233] and EACMV-UG[Nak:1] developed severe leaf curl and mosaic symptoms while ACMV-[Kal:226] induced mild chlorosis and very little leaf distortion in this host (Fig. 5a; Table 2). However, EACMV-UG[Kal:237] produced only a mild phenotype similar to that of ACMV-[Kal:226] and was associated with a long latent period.

Begomovirus isolates from cassava and *M. glaziovii* can infect both hosts

Despite the close relationship between the begomovirus isolates from cassava and *M. glaziovii*, it was important to

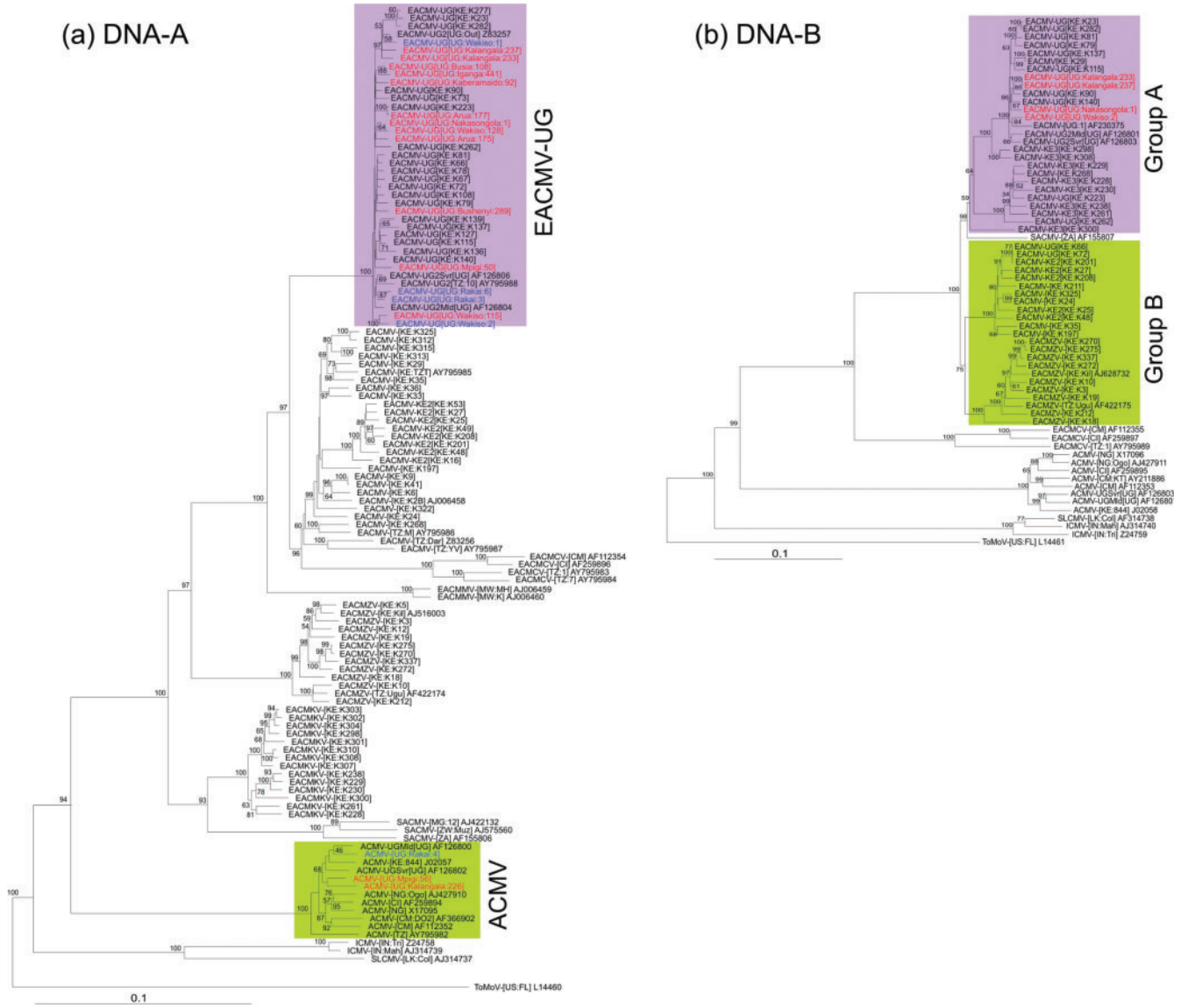


Fig. 3. Phylogenetic dendrograms based upon an alignment of the complete nucleotide sequences of (a) DNA-A and (b) DNA-B components derived in this analysis with those of other selected begomoviruses. Numbers at nodes indicate percentage bootstrap values (1000 replicates). Only values above 50 % are shown. The trees were arbitrarily rooted on the sequence of the genomic components of tomato mottle virus (ToMoV), a distantly related begomovirus originating from the New World. Begomoviruses isolated from cassava and *M. glaziovii* are indicated in red and blue font, respectively. EACMV-UG and ACMV DNA-A groups, and DNA-B groups (defined by Bull *et al.*, 2006) are highlighted. GenBank accession numbers for recently characterized Kenyan begomovirus components have been described by Bull *et al.* (2006), others are indicated in the figure.

confirm that viruses from one host could infect the other, an observation that would have an impact on our understanding of the epidemiology of the disease. To investigate this, plants were inoculated with the *M. glaziovii* isolate EACMV-UG[Wak:2] and the cassava isolate EACMV-UG[Kal:233]. The infectivity of EACMV-UG[Wak:2] was confirmed in *N. benthamiana*, in which it induced severe leaf curl and stunting symptoms similar to those associated with EACMV-UG[Kal:233]. EACMV-UG[Wak:2] also induced mosaic and leaf curl symptoms in its natural host *M. glaziovii*, similar to those observed in the field, by 30 days post-inoculation (Fig. 5c; Table 2). Furthermore, this isolate produced symptoms of severe mosaic and leaf distortion in cassava (variety N Mex 55) that were indistinguishable from those associated with EACMV-UG[Kal:233] infection in this host. In the reciprocal inoculation, EACMV-UG[Kal:233] was able to infect *M. glaziovii*, in which it induced symptoms similar to

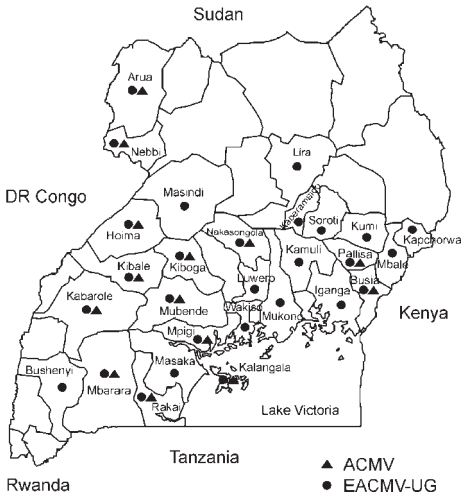


Fig. 4. Map of Uganda showing the distribution of ACMV and EACMV-UG based on PCR-RFLP and sequence analysis data.

those associated with EACMV-UG[Wak:2]. Interestingly, EACMV-UG[Wak:2] induced severe symptoms in the highly susceptible cassava variety Ebwanateraka, although plants subsequently showed signs of recovery. However, EACMV-UG[Wak:2] was less infectious and induced less severe symptoms in cassava variety Nase 4, which contains introgressed *M. glaziovii* genes.

Screening cassava varieties for resistance to CMD

Cassava varieties Ebwanateraka, Njule omumyufu, Njule omweru, Nase 4, TME14 and Nase 12, known to show different responses to begomovirus infection in the field, were screened for susceptibility to CMD. Three plants of each variety were screened for disease development following biolistic inoculation of EACMV-UG[Kal:233] cloned components. Varieties Ebwanateraka and Njule omumyufu developed severe symptoms, Njule omweru and Nase 4 also developed symptoms (Fig. 5d) while TME14 and Nase 12 remained asymptomatic and viral DNA could not be detected in newly developing leaves by blot hybridization (data not shown), indicating that the experimental plants were uninfected.

DISCUSSION

We have undertaken an extensive survey of begomoviruses associated with cassava and its wild relative *M. glaziovii*, sampled throughout the main cassava-growing regions of Uganda between 2001 and 2002. This is the most comprehensive survey to date which complements and extends previous investigations on CMD in the country (Harrison *et al.*, 1997; Zhou *et al.*, 1997; Otim-Nape *et al.*, 2001; Pita *et al.*, 2001; Sseruwagi *et al.*, 2004a). A survey conducted during 1995 and 1996, based on PCR-amplification of DNA fragments using begomovirus-specific primers, suggested that ACMV occurred throughout the

Table 2. Infectivity of begomovirus cloned components isolated from *M. esculenta* and *M. glaziovii*

Inoculum				Experimental host	Infected/ inoculated*		Latent period (days p.i.)	
Host	Isolate	DNA-A	DNA-B		Exp. 1	Exp. 2	Exp. 1	Exp. 2
M. esculenta	EACMV-UG[Nak:1]	6-ug1a22	20-ug1a8	*N. benthamiana*	2/10	3/10	12	13
				M. esculenta (N Mex 55)	2/2	4/4	28	30
	EACMV-UG[Kal:233]	27-ug233-2	27-ug233-1	*N. benthamiana*	7/10	5/10	8	9
				M. esculenta (N Mex 55)	2/2	4/4	22	16
				M. glaziovii	2/2	ND	30	ND
	EACMV-UG[Kal:237]	27-ug237-2	27-ug237-8	*N. benthamiana*	5/10	6/10	10	10
				M. esculenta (N Mex 55)	2/2	4/4	60	66
	ACMV-[Kal:226]	27-ug226-2	27-ug226-1	*N. benthamiana*	4/10	6/10	13	12
				M. esculenta (N Mex 55)	2/2	4/4	30	38
				M. esculenta (Ebwanateraka)	4/4	4/4	30	32
M. glaziovii	EACMV-UG[Wak:2]	wcass2-1	wcass2-5	*N. benthamiana*	3/10	5/10	13	9
				M. esculenta (N Mex 55)	2/2	ND	ND	ND
				M. esculenta (Ebwanateraka)	8/8	8/8	ND	ND
				M. esculenta (Nase 4)	2/8	1/8	ND	ND
				M. glaziovii	2/2	ND	30	ND

*Infected plants showed typical symptoms. The presence of DNA-A and DNA-B components was verified by PCR-RFLP and blot hybridization assays.

ND, Not determined.

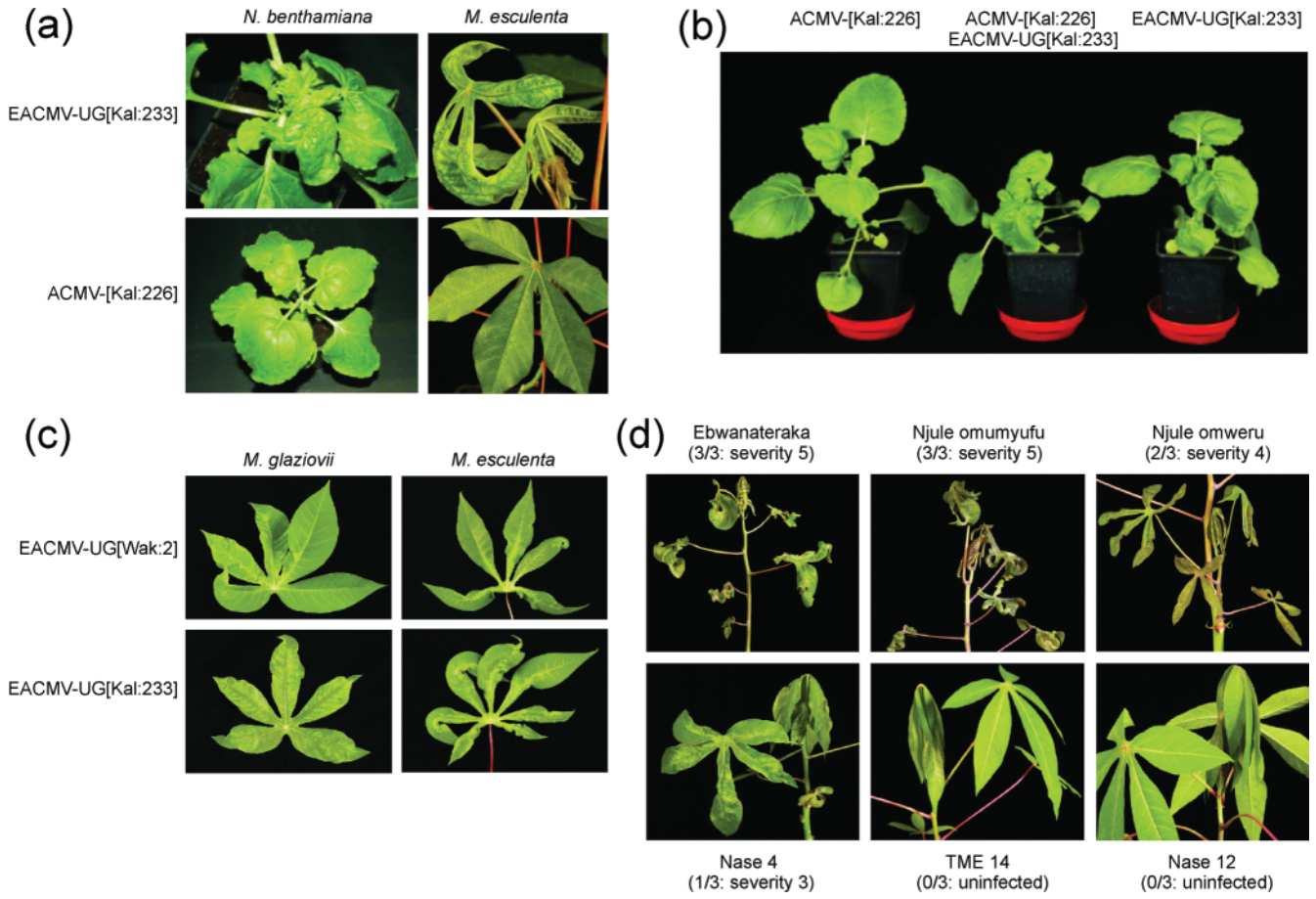


Fig. 5. Infectivity of ACMV and EACMV-UG cloned components. (a) Symptoms induced by cassava isolates EACMV-UG[Kal:233] and ACMV-[Kal:226] in *N. benthamiana* and cassava (variety N Mex 55). (b) Symptoms induced in *N. benthamiana* by ACMV-[Kal:226] (left), ACMV-[Kal:226] and EACMV-UG[Kal:233] (centre) and EACMV-UG[Kal:233] (right). (c) Symptoms induced by cassava isolate EACMV-UG[Wak:2] and *M. glaziovii* isolate EACMV-UG[Kal:233] in *M. glaziovii* and cassava (variety N Mex 55). (d) Screening cassava varieties for resistance to CMD by biolistic inoculation of EACMV-UG[Kal:233]. The number of infected plants and symptom severity are indicated.

sampled areas whereas EACMV-UG occurred only in those regions that had experienced the severe epidemic (Harrison *et al.*, 1997). A subsequent survey in 1997 was based on the use of primers to discriminate begomoviruses as well as the isolation and characterization of full-length genomic components (Pita *et al.*, 2001). It identified ACMV and strains of EACMV, and showed that dual infections of these viruses were common, resulting in a more complex population than previously assumed as a consequence of recombination. An analysis carried out on cassava plants sampled in 2002 again identified both EACMV and ACMV, although in this instance only fragments of the viral DNA components were analysed (Sseruwagi *et al.*, 2004a). Our present findings, based on PCR-RFLP analysis, PCR amplification of full-length genomic components and sequence analysis, are consistent with previous observations that CMD is associated with EACMV-UG and ACMV, and show that EACMV-UG is by far the predominant begomovirus throughout the entire country, consistent with the suggestion that it is displacing ACMV (Legg *et al.*, 2006). As noted previously (Harrison *et al.*, 1997), it is intriguing to find that EACMV-UG occurs throughout Uganda in the absence of its presumed progenitor EACMV. EACMV-UG is a recombinant virus consisting for the most part of sequences derived from EACMV, but with a small fragment of the coat protein gene derived from ACMV (Deng *et al.*, 1997; Zhou *et al.*, 1997) and was prevalent (in association with ACMV) during the severe epidemic of CMD that affected Uganda during the 1990s (Harrison *et al.*, 1997). A concurrent study conducted in Kenya identified EACMV at relatively high incidence east of the Rift Valley but, for the most part, only EACMV-UG in the west of the country at the border with Uganda (Bull *et al.*, 2006). In contrast to the findings of Were *et al.* (2004), who reported ACMV in western Kenya, Bull *et al.* (2006) found no evidence of this

Table 3. Co-infectivity of ACMV and EACMV cloned components in *N. benthamiana*

Virus isolate(s)	Infected/inoculated*		Latent period		Symptoms
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	
EACMV-UG[Kal:233]	4/8	4/6	9	8	Leaf curl, chlorosis, stunting
ACMV-[Kal:226]	4/8	6/6	9	9	Mild chlorosis, stunting
EACMV-UG[Kal:233] + ACMV-[Kal:226]	8/8	6/6	9	7	Severe leaf curl, chlorosis, stunting
EACMV-UG[Kal:233] + ACMV-[Kal:226] DNA-A	8/8	6/6	9	7	Severe leaf curl, chlorosis, stunting
ACMV-[Kal:226] + EACMV-UG[Kal:233] DNA-A	3/8	4/6	9	9	Mild chlorosis, stunting
EACMV-UG[Kal:233] DNA-A + ACMV-[Kal:226] DNA-B	–	0/6	–	–	–
ACMV-[Kal:226] DNA-A + EACMV-UG[Kal:233] DNA-B	–	0/6	–	–	–

*The presence of both begomoviruses in all co-inoculated plants was verified by the identification of A1 and EA5 PCR-RFLP patterns typical of ACMV-[Kal:226] and EACMV-UG[Kal:233], respectively.

begomovirus in Kenya despite using protocols identical to those used in the current study to successfully detect it in Uganda. In spite of this, the overall diversity of CMD-associated begomoviruses in neighbouring Kenya (Bull *et al.*, 2006) and Tanzania (Ndunguru *et al.*, 2005) is significantly higher than in Uganda. This has been attributed to an ongoing repopulation of central and western Kenya by begomovirus isolates and species from coastal Kenya and Tanzania, most probably by human migration and the movement of diseased germplasm (Bull *et al.*, 2006). Certainly, the coastal areas of Kenya have not been affected by the epidemic and neither ACMV nor EACMV-UG has been identified in that region, which otherwise harbours a genetically diverse population of CMD-associated begomoviruses. The lack of spread of the epidemic to coastal areas was likely due to geographical barriers to virus (whitefly) movement between the inland areas and the coast (Bull *et al.*, 2006).

In this survey of 385 cassava plants, the overall frequency of co-infection with ACMV and EACMV-UG was less than 5 %. This value is significantly lower than that previously reported (>50 %) for a more limited plant sample from regions that had experienced the epidemic in 1995 (Harrison *et al.*, 1997), consistent with EACMV-UG emerging as the dominant species in post-epidemic Uganda. Interestingly, the frequency of co-infection is also much lower than the value of 18 % reported during a comprehensive survey of post-epidemic Uganda in 2002 (Sseruwagi *et al.*, 2004a). The reason for this is unclear, although it may simply reflect random sampling at different locations.

M. glaziovii and hybrids between *M. glaziovii* and cassava have been important sources of CMD resistance genes in East African cassava-breeding programmes (Bock *et al.*, 1981; Bock & Woods, 1983). They were used to generate a number of Tropical Manihot Species (TMS) lines that were bred at IITA-Ibadan in West Africa (Legg & Fauquet, 2004; Thresh & Cooter, 2005). These improved varieties, possessing polygenic resistance to CMD derived from *M. glaziovii*, have been distributed widely across Africa and represent a major resource with which to combat CMD. The susceptibility of *M. glaziovii* to viruses associated with severe CMD raises questions about the effectiveness and long-term durability of resistance derived from this species. For example, our infectivity studies demonstrated that the cassava variety Nase 4 (also known as SS4), which derives from parental lines produced from an interspecific cross with *M. glaziovii* and deemed CMD resistant (Sserubombwe *et al.*, 2001; Thresh & Cooter, 2005), is nonetheless susceptible to EACMV-UG infection. Contrary to previous assumptions, therefore, *M. glaziovii* might be a reservoir host for begomoviruses that could contribute to maintenance of the disease. Hence, it would be prudent to remove mosaic-affected *M. glaziovii* plants growing in the vicinity of cassava plants to be used as disease-free planting material.

Harrison *et al.* (1997) showed that cassava plants containing ACMV exhibited mild symptoms while those infected with EACMV-UG or co-infected with ACMV and EACMV-UG produced severe symptoms. They reproduced the symptom phenotype by graft inoculation of healthy cassava plants with scions from plants containing known viruses. Sseruwagi *et al.* (2004b) also found that ACMV occurred more frequently in mildly affected plants. In this study, we have extended these investigations using infectious cloned components and demonstrated that Ugandan isolates of ACMV and EACMV-UG generally produce mild and severe phenotypes, respectively, in both *N. benthamiana* and cassava. Furthermore, we have demonstrated that *N. benthamiana* co-infected with ACMV and EACMV-UG develops symptoms that are more severe than those induced by either virus alone. Using ACMV and East African cassava mosaic Cameroon virus (EACMCV), a distinct CMD-associated begomovirus from Cameroon, Vanitharani *et al.* (2004) demonstrated that synergism is due to the provision by each virus of a

distinct suppressor of post-transcriptional gene silencing (PTGS), an RNA-mediated host defence response against pathogens (Voinnet *et al.*, 1999; Voinnet, 2001). Additionally, despite ACMV and EACMV-UG being functionally incompatible due to their differing iteron sequences, we have shown that the presence of ACMV DNA-A serves to enhance EACMV-UG symptom severity whereas the reciprocal combination, EACMV-UG DNA-A in the presence of ACMV, induces only mild symptoms typical of ACMV. The presence of all viral components in systemically infected tissues in these experiments is consistent with the ability of the movement proteins encoded by DNA-B of one virus to mediate the systemic spread of the autonomously replicating DNA-A component of a second distinct virus, as demonstrated by Frischmuth *et al.* (1993). The symptom phenotype of bipartite begomoviruses is defined largely by the BC1 protein encoded by DNA-B (von Arnim & Stanley, 1992; Pascal *et al.*, 1993; Ingham *et al.*, 1995), consistent with the mild and severe phenotypes observed in these co-inoculation experiments. The enhanced symptom phenotype observed when EACMV-UG is co-inoculated with ACMV DNA-A suggests that the latter provides a PTGS suppressor, possibly AC4 as demonstrated for a Cameroon isolate of ACMV (Vanitharani *et al.*, 2004). It is unclear why co-inoculation of ACMV with EACMV-UG DNA-A failed to enhance the symptoms of ACMV infection, particularly as AC2 (of EACMCV) has also been shown to function as a silencing suppressor (Vanitharani *et al.*, 2004). However, this might simply reflect differences in DNA-A gene functions between isolates from different geographical locations and indicate that the ACMV DNA-B genes determine the phenotype for this particular combination.

One of the main objectives of our concurrent studies in Uganda (reported here) and Kenya (Bull *et al.*, 2006, 2007) has been to provide clones representative of all CMD-associated begomovirus species and strains present in East Africa for use in the resistance breeding programme. Undoubtedly, the use of biolistic inoculation of cloned components has advantages over conventional field screening procedures; it allows results to be obtained more rapidly and a wider range of defined virus species and strains can be screened, since it does not rely on chance infections by locally occurring begomoviruses in the field. New cassava varieties often have to be screened at numerous locations to challenge the resistance with a representative range of species and strains, an approach that is limited by natural variation in the incidence and severity of the disease as well as its genetic makeup. Here, we have demonstrated the feasibility of a clone-based approach for screening germplasm for resistance to the disease. We have shown that several so-called resistant lines, including Nase 4 derived from TMS germplasm, are in fact susceptible to EACMV-UG, while TME14 and Nase 12 lines remained uninfected, although it should be emphasized that only a limited number of test plants were challenged on this occasion. This small-scale experiment shows that biolistic inoculation could provide a useful tool for preliminary screening for resistance, requiring relatively little material and thus overcoming time-consuming propagation required to produce sufficient material for a standard field screen. In addition, a new line can be screened against a wide range of defined species and strains to assess resistance at a single location, providing a distinct advantage over conventional screening procedures. The clones produced during this study and the concomitant study in Kenya (Bull *et al.*, 2006, 2007) will be made available for screening purposes.

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