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Efficient whitefly transmission of African cassava mosaic geminivirus requires sequences from both genomic components

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Clones of two subgroup III geminiviruses, the common strain of tomato golden mosaic virus (csTGMV) and African cassava mosaic virus originating from Kenya (ACMV-K), were shown to be non-transmissible by whiteflies. Lack of transmissibility of cloned ACMV-K was investigated by exchanging genomic components with a whitefly-transmissible ACMV isolate from Nigeria (ACMV-NOg). Neither pseudorecombinant was transmissible, indicating that defects in both genomic components contributed to the lack of transmissibility. Analysis of the acquisition of the pseudorecombinants by *Bemisia tabaci* indicated that accumulation of virus within the insect was DNA B dependent. Return of virus to plants was determined by DNA A, although the coat protein was essential for acquisition. Repeated passaging of both the wild strain of ACMV-NOg and the cloned virus led to loss of insect transmissibility of the wild isolate but not the cloned virus. Products encoded on both genomic components are required for transmission of bipartite geminiviruses by *B. tabaci*.

The geminiviruses are a group of plant-infecting viruses with a unique twinned icosahedral particle morphology and encapsidated genomes of circular, single-stranded DNA. Three genera of geminiviruses are recognized based upon such factors as host range, insect vector and genome arrangement (Briddon & Markham, 1995). The species of transmitting insect forms a major basis for the taxonomic division of geminiviruses. The viruses of genera Subgroup I and Subgroup II are each transmitted by different species of leafhopper, or in a single case a treehopper (Briddon *et al.*, 1996). Viruses belonging to the genus Subgroup III are transmitted in field situations exclusively by a single species of whitefly, *Bemisia tabaci*, although a few may also be transmitted by mechanical means. Subgroup III viruses fall into two classes in having either one or two genomic components (termed DNA A and DNA B).

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Of the large number of Subgroup I and II geminiviruses for which infectious clones have been obtained, all of the cloned progeny tested have been shown to be transmissible from plant-to-plant by their respective insect vectors, including the type members of each of these two genera, respectively maize streak virus and beet curly top virus. However, for the cloned bipartite whitefly-transmitted geminiviruses (WTG) for which insect transmission has been investigated, some important members have proven non-transmissible by *B. tabaci*, including ACMV-K (Stanley & Gay, 1983; Table 1) and the common strain of tomato golden mosaic virus (csTGMV; von Arnim & Stanley, 1992*a*). The lack of transmissibility of the cloned ACMV-K was investigated by exchange of genomic components (termed pseudorecombination) with a cloned ACMV isolate originating from Nigeria (ACMV-N*Og*). Clones of ACMV-N were produced from purified viral supercoiled DNA isolated from *Nicotiana benthamiana* infected by mechanical inoculation from *N. tabacum* cv. Samsun plants in which the virus isolate had been maintained by whitefly transmission (the genomic components of ACMV-N*Og* will be referred to as NA and NB, whilst those of the Kenyan isolate will be referred to as KA and KB respectively). Insect transmissions were carried out from infected *N. tabacum* cv. Samsun plants essentially as described previously (Bedford *et al.*, 1994).

The transmission of geminiviruses by insects occurs by a circulative, non-propagative mechanism (Harrison, 1985) which is similar in many respects to the transmission of luteoviruses by aphids. This type of transmission mechanism involves the passage of the virus through the body of the insect and consists of two distinct phases; firstly acquisition during which the virus passes into the insect's blood (probably via the gut wall) and secondly the inoculation of the virus back into the plant, which involves the passage of the virus from the blood into the salivary secretions. The work we present here provides evidence of the distinction between these two processes. For discussion purposes we shall describe these two stages as acquisition and inoculation respectively.

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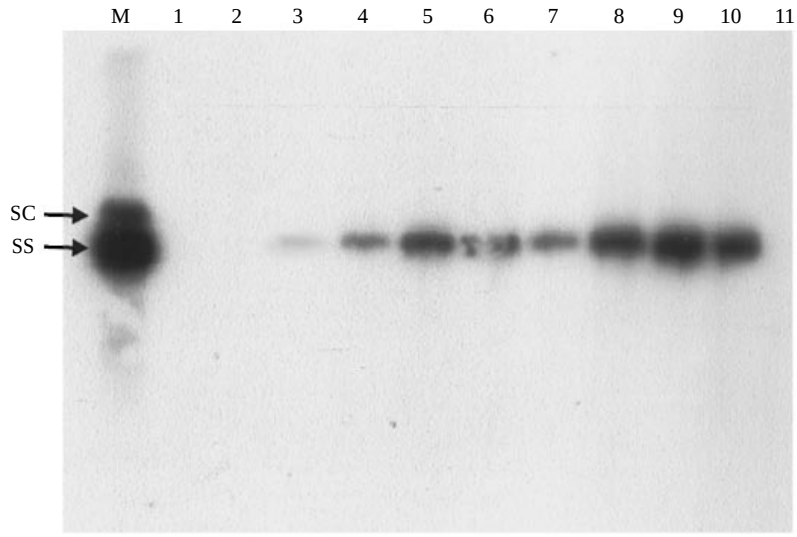

Fig. 1. Southern hybridization of nucleic acids extracted from *B. tabaci* insects which had been fed on healthy (lane 11) or infected *N. tabacum* plants. Samples originate from insects fed on plants infected with NA + KB (lane 1), KA + KB (lane 2), KA + NB (lane 3), NA + NB (lanes 4–10). A total nucleic acid sample extracted from an ACMV-infected *N. benthamiana* plant was run as a marker to show the positions of single-stranded (ss) and supercoiled (sc) DNA forms (lane M). The blot was probed with the full-length insert of the DNA A clone of ACMV-K.

Table 1. Infectivity and transmission of pseudorecombinant ACMV

Inoculum	Infectivity by mechanical inoculation of *N. benthamiana* (plants infected/ inoculated)	Infectivity by sap inoculation of *N. tabacum* (plants infected/ inoculated)	Symptom type*	Insect transmission† (plants infected/ inoculated			Detection in *B. tabaci*‡ (positive samples/total samples)
				I	II	III	
KA + KB	9/10	3/5	K	0/11	1/10	0·8	7/8
NA + NB	2/10	7/10	N	7/11	4/4	4/6	0/15
NA + NB	8/10	7/10	K	0/11	0·7	0/4	0/13
KA + NB	6/10	7/10	N	0·11	0/4	0/7	3/5
Wild-type N	15/15	2/4	N	3/8	6/13	3/6	8/10

* Symptoms either typical of the Nigerian (N) or Kenyan (K) clones.
† Transmissions assays using 100 whiteflies per *N. tabacum* test seedling.
‡ Detection of ACMV by Southern hybridization.

The symptoms of infection by the clones of ACMV-N*Og* differed from those of ACMV-K mainly on mature leaves of both *N. benthamiana* and *N. tabacum*. These species showed a patchy yellow mosaic upon infection by ACM-N*Og*, rather than the distinct chlorotic lesions typical of ACMV-K. Symptoms produced by the pseudorecombinants in plants were a function of the DNA B component used (Table 1). The importance of the DNA B component in pathogenicity and symptom production has been noted previously (von Arnim & Stanley, 1992*a*, *b*; Frischmuth *et al*., 1993). In fact, ACMV DNA A has been shown to be able to infect *N. benthamiana*, in the absence of DNA B, under certain conditions (Klinkenberg & Stanley, 1990). Such infections produce no symptoms. However, very similar pseudorecombinant work conducted by Stanley *et al*. (1985) concluded that some part of symptom determination for ACMV resides on DNA A, a finding that we were unable to confirm.

Neither of the pseudorecombinant virus infections proved transmissible by *B. tabaci* from plants inoculated with linearized clone inserts (Stanley, 1983; Table 1) to healthy seedlings, even when over 1000 insects were used per assay (results not shown). This contrasts with the wild ACMV-N*Og* isolate, which was transmitted at near 50% efficiency under these conditions. Southern blot analysis of nucleic acids extracted from groups of *B. tabaci* given 48 h acquisition access to plants infected with cloned ACMV-K, ACMV-N*Og* and their pseudorecombinants, probed for the presence of DNA A, showed that in addition to ACMV-N*Og* and wild-type Nigerian virus, the pseudorecombinant between KA and NB could be acquired (Fig. 1). This indicated that some defect of KB

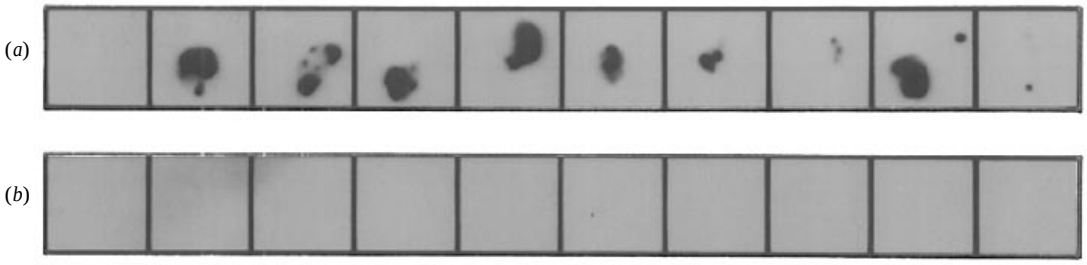

Fig. 2. Squash blot analysis of single *T. vaporariorum* insects which had been fed for 48 h on an *N. benthamiana* seedling infected with the clones of ACMV-K (*a*) or a seedling infected with the coat protein mutant pCHI001 (*b*). The blot was probed with the full-length insert of the DNA A clone of ACMV-K.

was preventing acquisition of the virus by whiteflies. The two genes encoded on the DNA B components of WTGs have been shown to be involved in spread of the virus through plant tissues (Etessami *et al*., 1988; Noueiry *et al*., 1994). It would seem likely, therefore, that the lack of acquisition of cloned ACMV infections involving KB is due to anomalous tissue distribution caused by a defect in one of the two movement genes. This defect must preclude movement to, and display of, the virus in the correct plant tissues for acquisition by *B. tabaci*.

Although the pseudorecombinant involving KA and NB could be acquired by *B. tabaci* from plants (Fig. 1), it remained non-transmissible. It has previously been shown that the coat protein (CP) is essential for acquisition/transmission (Azzam *et al*., 1994) and is the determining factor in interactions with insects that allows inoculation (for a leafhopper-transmitted virus at least; Briddon *et al*., 1990). A defect in the CP of KA is the most likely explanation for inability to transmit infections involving this clone. Analysis of further WTG clones has shown that defects in DNA A leading to lack of transmission are not limited to ACMV. Like ACMV-K, clones of csTGMV (von Arnim & Stanley, 1992*a*) have proven non-transmissible by whiteflies (results not shown). Again, progeny virions from plants infected with these cloned genomic components could be acquired by whiteflies from plants, as determined by Southern blot analysis of insect extracts (results not shown), but were not transmissible.

A number of other reports in the literature indicate that lack of transmissibility of WTGs is not uncommon. A number of 'wild' geminiviruses have been identified which are not transmissible by whiteflies, including Abutilon mosaic and honeysuckle yellow vein viruses (Wu *et al*., 1996; Bedford *et al*., 1992). Lack of insect transmissibility for these viruses is usually attributed to prolonged vegetative propagation in the absence of the vector. Our attempts to mimic this process by repeated passaging of the wild-type ACMV-N*Og* virus in *N. benthamiana*, by mechanical inoculation, indicated that insect-transmissibility can be lost quite rapidly. In two experiments, insect transmissibility was lost after the 20th and 25th passage. For identical experiments carried out with the clones of ACMV-N*Og*, transmissibility was maintained past 40 passages. This finding suggests that ACMV field isolates may be mixtures of insect-transmissible and non-transmissible forms of the virus, with the non-transmissible form being maintained either by vegetative propagation of cassava or by *trans*-complementation. Alternatively, there may be hyper-susceptible regions of the genome which are rapidly mutated upon introduction of the virus to alternative hosts such as *N. benthamiana*. Once in the permissive host *N. benthamiana*, the whitefly-non-transmissible form must have some selective advantage, during repeated passage, allowing it to become the dominant population. For mutations affecting cell-to-cell movement such a course of events seems plausible. A virus with an altered tissue specificity (altered DNA B-encoded product) may be able to spread more quickly through *N. benthamiana*, thereby out-competing the insect-transmissible virus, as has previously been suggested by McGrath & Harrison (1995). For mutations within the CP, which abolish transmission, a selective advantage is more difficult to imagine. Possibly the CP of bipartite WTGs play a part in cell-to-cell movement (Jeffrey *et al*., 1996), as is the case for monopartite geminiviruses (Briddon *et al*., 1989; Boulton *et al*., 1989). Certainly these findings highlight the pitfalls of using 'laboratory-adapted' virus strains for cloning and analysis, as these may not be a true representation of the field isolate.

It has been reported that acquisition of geminiviruses by *B. tabaci*, and some other whitefly species, is non-specific but that the inoculation stage is virus-vector specific (Cohen *et al*., 1989).

In squash-blot experiments (Boulton & Markham, 1986) the acquisition of cloned ACMV-K by *Trialeurodes vaporariorum* (Fig. 2) and *Myzus persicae* (results not shown), two non-vector species, can be demonstrated. However, the CP is shown to be essential for the acquisition process since viruses which are not encapsidated (clone pCHI001; Stanley & Townsend, 1986) cannot be acquired, as previously concluded by Azzam *et al*. (1994) using *B. tabaci*. This non-specific acquisition by whitefly species could be limited to geminiviruses or might also occur for other viruses. The ability of *M. persicae* to acquire WTGs may indicate that this insect feeds in the same tissues as whiteflies and therefore comes into contact with the virus. Whether there is any form of specificity for the acquisition of geminiviruses by this and other aphid species will require

further investigation. This finding also indicates that care should be taken, when searching for vector insect species using methods that detect viruses in insects, to avoid false positives.

From the results presented here it is clear that geminivirus movement proteins play an important role in insect transmission. Although not directly interacting with the vector, they must determine the correct location of the virus in plant tissues for acquisition by the insect. Sequence analysis of ACMV-N*Og* clones should allow us to identify the defects in both components which affect insect transmissibility, giving us a better understanding of both the transmission and intercellular movement of this group of viruses.

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