Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus

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Nicotiana benthamiana was transformed with three different constructs (pCRA1, pCRA2 and pJC1) containing the coat protein coding sequence of African cassava mosaic virus (ACMV). Transformed plants were inoculated with a coat protein deletion mutant of ACMV that induces mild systemic symptoms in control plants. Several inoculated plants of transgenic lines CRA1/3, CRA1/4, CRA2/1 and CRA2/2 developed severe systemic symptoms typical of ACMV. DNA analysis revealed that, in these plants, recombination had occurred between the mutant viral DNA and the integrated construct DNA, resulting in the production of recombinant virus progeny with ‘wild-type’ characteristics. No reversion of mutant to ‘wild-type’ virus was observed in pJC1-transformed plants. Recombinant virus from several transgenic plants was analysed by PCR and parts of DNA A of virus progeny were cloned. Sequence analysis revealed that only a few nucleotides were changed from the published sequence.

Introduction

Non-conventional resistance to plant virus diseases has been explored for several years. Coat protein-mediated resistance is, so far, the best studied method of protecting plants against virus infection (Hull & Davies, 1992; Wilson 1993). Two possible risks associated with viral coat protein-expressing plants are conceivable: heterologous encapsidation and recombination. Heterologous encapsidation has been demonstrated in transgenic plants that express the coat protein of plum pox virus and are infected by beet necrotic yellow vein virus (Maiss et al., 1994). However, in the event of heterologous encapsidation, the changed feature (i.e. the different coat protein) of the virus progeny is limited to one generation because the virus genetic information remains intact. In contrast, recombination between viral genomes and a coat protein transgene results in a permanent change in genetic constitution of the virus progeny. This has been demonstrated for the RNA plant virus cowpea chlorotic mottle virus; recombination between transgenic coat protein transcripts and mutant viral RNA resulted in infectious recombinant virus progeny (Greene & Allison, 1994).

In this paper, we examine recombination between viral DNA and the transgenic coat protein gene of a geminivirus, African cassava mosaic virus (ACMV). Geminiviruses are small plant viruses with a circular single-stranded (ss) DNA genome that is encapsidated in twinned (geminate) particles. Members of this virus family have been divided into three subgroups on the basis of their genome organization and host range (Murphy et al., 1995). Members of the genus Begomovirus (previously known as subgroup III), typified by ACMV (Stanley & Gay, 1983), infect dicotyledonous plants. The majority of the members of this subgroup have bipartite genomes (DNAs A and B) and are transmitted by the whitefly Bemisia tabaci Gennadius. To date, seven reports of transgenic plants conferring resistance to geminivirus infection have been published (Stanley et al., 1990; Day et al., 1990; Frischmuth & Stanley, 1994; Stenger, 1994; Kunik et al., 1994; Hong et al., 1996; Noris et al., 1996).

Methods

Construction of clones. Recombinant DNA techniques were performed as described by Sambrook et al. (1989) and restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

For construction of pCRA1, an EcoRI(2732)–BamHI(291) fragment (clockwise numbering via nucleotide 1; Fig. 1a) of DNA A was cloned into pBluescript KS(−) and an EcoRI(2732–1868; clockwise) fragment of DNA A was introduced into the unique EcoRI site. The construct DNA
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Fig. 1. (a) The organization of ACMV DNAs A and B showing the location of virion-sense (AV1, AV2, BV1) and complementary-sense (AC1–AC4, BC1) genes. The common region between DNA A and B is indicated (CR). The positions of selected restriction sites are shown and the position numbers given are according to the published sequence of ACMV (Stanley & Gay, 1983). The deletion of the mutant ∆11 of ACMV is indicated by the arrow between the DraI and ScaI sites. The position and orientation of primers AA3 and AA4 are indicated by arrows. (b) Diagrammatic representation of pBin19 constructs pCRA1, pCRA2 and pJC1 used for transformation of *N. benthamiana*. Open reading frames are indicated by arrows.

was transferred as a XbaI–KpnI fragment into pBin19 (pCRA1; Fig. 1b). For construction of pCRA2, the EcoRI(2732–1868; clockwise) fragment of DNA A was cloned into pBin19 (Fig. 1b). In a third construct, the coat protein of ACMV was expressed under the control of the 35S promoter of cauliflower mosaic virus. The β-glucuronidase (*GUS*) gene of plasmid pSLJ4D4 (kindly supplied by Jonathan Jones, Sainsbury Laboratory, John Innes Centre, Norwich, UK), containing a 35S promoter, the *GUS* gene and an octopine synthetase terminator (ocs3'), was removed by digestion with NcoI, followed by treatment with Klenow enzyme (to produce a blunt end) and subsequent digestion with Xhol. The coat protein gene of ACMV was amplified by PCR with primers AACP1 (5' TCGAAGCGACGAGGAGAT 3'; position on the ACMV genomic sequence 449–466) and AACP2 (5' CGTCTAGATTAATTGCCAATACTGTC 3'; position 1223–1205) from pCLV1.3A (Klinkenberg et al., 1989). The resulting fragment was cleaved with XbaI and introduced into the prepared pSL4d4 plasmid. An *EcoRI–HindIII* fragment, comprising the 35S promoter, the coat protein gene and the ocs3' terminator, was transferred to pBin19 giving construct pJC1 (Fig. 1b). Constructs were transferred to *Agrobacterium tumefaciens* LBA4404 by triparental mating (Frischmuth & Stanley, 1991).

The construction of infectious clones of ACMV DNA A (pCLV1.3A) and DNA B (pCLV2B) as well as *A. tumefaciens* LBA4404 bearing a partial repeat of ACMV DNA A (AgA) and a dimer of DNA B (AgB) have been described (Klinkenberg et al., 1989; Frischmuth & Stanley, 1991). The coat protein deletion mutant (pJS170), which lacks the DNA A genomic part from nucleotide 221–941 (Fig. 1a), has been described by Etessami et al. (1989) and the construction of a tandem repeat of pJS170 (∆11) by Klinkenberg et al. (1989).

■ Plant transformation, leaf disk assay and characterization of viral DNA forms. *N. benthamiana* was transformed using a leaf disk method described by Stanley et al. (1990). The numbers of integrated construct copies were determined by cleavage of 10 µg total plant genomic DNA, extracted essentially as described by Frischmuth & Stanley (1991), with endonuclease *EcoRV* or *HindIII*. Samples were run on agarose gels in 0.5 x TBE and the DNA was transferred to nylon membranes by Southern blotting. Blots were hybridized with the digoxigenin-labelled *EcoRI* fragment of construct pCRA2. The probes were labelled with the Boehringer DIG DNA Labelling kit according to the manufacturer's instructions. Following self-fertilization, F₁ and F₂ progeny were tested for antibiotic sensitivity by germinating seeds on 0.5 mg/ml kanamycin.

The ability of the pCRA1 construct to be trans-replicated by virus DNA A and to be amplified, after integration, from the plant genome was tested in leaf disks as described by Frischmuth & Stanley (1991). The source for the leaf disks were either control or transformed *N. benthamiana* of generations F₁ (original transformants), F₂ and F₃. Leaf disks were harvested 9 days post-agroinoculation. Agroinoculation and mechanical inoculation of plants have been described previously (Stanley et al., 1990).
For characterization of viral DNA forms, total cellular nucleic acids were extracted as described by Frischmuth & Stanley (1991). Samples containing 5 or 10 µg nucleic acid were either run directly, or after treatment with restriction endonucleases, on agarose gels in 0±5 TBE and viral DNA forms were detected using probes specific to each genomic component (Stanley et al., 1990). Samples were further analysed by PCR with primers AA3 (5’ GTGACCGGTGGGCTTGGTTGAA-CACAGG 3’; position 731–760) and AA4 (5’ CCCACGCTCAC-ATCCTA 3’; position 742–723), which contain the MluI site (underlined) at position 734 in the ACMV genome (see Fig. 1; Stanley & Gay, 1983).

To analyse likely recombination sites of ∆11 recombinant virus progeny, full-length PCR amplified recombinant virus was digested with EcoRI and resulting fragments were cloned into pBluescript KS(−) double digested with HindII and EcoRI. The sequence of resulting clones was determined by automatic sequence analysis with the Li-Cor system according to the manufacturer’s instructions.

Characterization of coat protein production. Proteins from plants and leaf disks were isolated as described (von Arnim et al., 1993). Essentially 0±5–2 g leaf tissue was homogenized in liquid nitrogen and proteins were extracted in grinding buffer (10 mM KCl, 5 mM MgCl₂, 400 mM sucrose, 10 mM mercaptoethanol, 100 mM Tris–HCl, pH 8±1, 10% glycerol) using approximately 2 ml/g tissue. The homogenate was centrifuged for 5 min at 12000 g. The supernatant was boiled for 5 min in SDS–PAGE loading buffer (2% SDS, 10% glycerol, 5% mercaptoethanol, 62±5 mM Tris–HCl, pH 6±8, 0±001% bromophenol blue) and loaded onto 12±5% polyacrylamide gels (Laemmli, 1970). Proteins were transferred to nitrocellulose (Schleicher and Schuell) using a Bio-Rad semi-dry trans-blot assembly. The presence of coat protein was detected using polyclonal antiserum raised against purified ACMV (Townsend et al., 1985). Blots were developed using alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma).

Results

Plant transformation

N. benthamiana leaf disks were transformed with each construct and, for each construct, several transformed plants were analysed. For further investigation, two plant lines transformed with construct pCRA1 (CRA1/3 and CRA1/4),

![Fig. 3. (a) Southern blot analysis of viral DNA forms produced in leaf disks derived from CRA1/4 (lanes 1 and 2) and CRA2/2 (lane 3 and 4) plants. Leaf disks were inoculated with ACMV DNA A (lanes 1 and 3) and ∆11 (lanes 2 and 4). Single-stranded (ss) and supercoiled (sc) forms of genomic DNA, construct DNA (CRA1) and ∆11 DNA are indicated. (b) Southern blot analysis of viral DNA forms produced in systemically infected leaves of ACMV- and ∆11-inoculated control and transformed plants. Control plants were inoculated with ACMV (lane 1) and ∆11 (lane 7). CRA1/4- (lanes 2–6), CRA2/1- (lane 8) and CRA2/2- (lanes 9 and 10) transformed plants were inoculated with ∆11. Plants 1–5 and 9 exhibited severe infection symptoms and plants 6–8 and 10 had mild symptoms. Viral single-stranded (ss) and supercoiled (sc) DNA forms are indicated. Both blots were hybridized with an ACMV DNA A-specific probe.](image-url)

![Fig. 2. (a) Western blot analysis of coat protein production in pJC1/S transformed plant. Proteins analysed were from an ACMV-infected control plant (lane 1), uninfected control plant (lane 2) and transformed line JC1/5 (lane 3). The position of ACMV coat protein is indicated by an arrowhead. (b) Western blot analysis of coat protein production in inoculated control and transformed plants. Proteins were extracted from ACMV (lane 1) and ∆11 (lane 2), inoculated control plants and ∆11-inoculated CRA2/2 plants (lanes 3 and 4, respectively), CRA1/4 (lanes 5–8 and 12) and CRA1/3 (lanes 9–11) plants. Plants 1, 3 and 5–11 exhibited severe symptoms of systemic infection and plants 2, 4 and 12 had mild symptoms. The molecular sizes of standard proteins are indicated (lane M).](image-url)
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Table 1. Infection of CRA1, CRA2 and JC1 F₂-transgenic N. benthamiana plants

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Plant line†</th>
<th>Infectivity (infected/ inoculated)</th>
<th>Symptom type‡</th>
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<tbody>
<tr>
<td>pJS170 + pCLV2B</td>
<td>CRA2/1</td>
<td>24/104</td>
<td>Mild</td>
</tr>
<tr>
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<td>CRA2/2</td>
<td>17/43</td>
<td>4</td>
</tr>
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<td>8/00</td>
<td>0</td>
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<td>pJS170 + pCLV2B</td>
<td>CRA1/4</td>
<td>19/28</td>
<td>6</td>
</tr>
<tr>
<td>pJS170 + pCLV2B</td>
<td>JC1/4</td>
<td>22/54</td>
<td>22</td>
</tr>
<tr>
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<td>19</td>
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<td>24</td>
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<td>Control</td>
<td>14/15</td>
<td>0</td>
</tr>
<tr>
<td>∆11 + AgB</td>
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<td>48/54</td>
<td>44</td>
</tr>
<tr>
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<td>Control</td>
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<td>0</td>
</tr>
</tbody>
</table>

* Clones pJS170, pCLV2B, pCLV1.3A, ∆11, AgA and AgB are described in the text. Clones pJS170, pCLV2B and pCLV1.3A were inoculated as DNA. Clones ∆11, AgA and AgB were used for agroinoculation.
† Transformed plant lines are described in the text.
‡ Symptom types are described in the text. Figures represent the number of infected plants displaying each symptom.

two transformed with pCRA2 (CRA2/1 and CRA2/2) and five transformed with pJC1 (JC1/1–5) were selected arbitrarily. Transformed plant lines CRA1/3 and CRA2/1 contained a single copy of constructs pCRA1 and pCRA2, respectively. Transgenic plants were self-fertilized, and homozygous F₂ plants were used for infection experiments. One plant (F₀) transformed with pCRA1 initially contained seven copies of the construct. After self-fertilization, plant line CRA1/4 (F₁), containing four copies, was selected. This genetic status was preserved in the F₂ generation (25 F₂ plants out of 25 tested by endonuclease cleavage with HindIII). One F₂ plant transformed with construct pCRA2 initially contained five copies. After self-fertilization, plant line CRA2/2, containing three copies in the F₂ as well as the F₃ generation (32 plants out of 32 tested), was selected. All five pJC1-transformed plant lines contained only a single copy of the construct DNA (data not shown). Transgenic plants were self-fertilized and homozygous F₂ plants were used for infection experiments.

Production of ACMV coat protein was not detected in any of the pCRA1- or pCRA2-transformed plants (data not shown). Coat protein was detected in plant line JC1/5 (Fig. 2a, lane 3), but it was not found in the other four JC1 lines (data not shown).

Inoculation of N. benthamiana

Inoculation of leaf disks. After co-agroinoculation of non-transgenic leaf disks with ACMV DNA A and construct pCRA1, replication of viral construct DNA was observed. In contrast, no such amplification products were detected from construct pCRA2 after co-agroinoculation with ACMV DNA A (data not shown). In leaf disks derived from the original transformed CRA1 plants (F₀), viral construct DNA was amplified from the transgene by both wild-type virus and the coat protein deletion mutant ∆11 (Fig. 3a, lanes 1 and 2). No amplification of viral construct DNA was observed in leaf disks derived from the original transformed CRA2 plants (Fig. 3a, lanes 3 and 4). After inoculation of leaf disks derived from CRA1/3 and CRA1/4 F₂ plants with ∆11, production of coat protein was detected (data not shown).

Inoculation of plants. After self-fertilization, F₂ plants were challenged with wild-type ACMV or the coat protein deletion mutant either mechanically with cloned virus DNA or plant...
nor inoculation. In contrast to the leaf disk results, neither ACMV-blotting (Fig. 3) levels of construct DNA that were detectable by Southern analysis of nucleic acid extracts of severely infected transgenic plants exhibited a level of accumulation of ssDNA comparable to wild-type ACMV infections (data not shown). In contrast, viral progeny of mild ∆11-infected CRA1/3 and CRA1/4 plants was not sap-transmissible (none of the 25 inoculated plants developed symptoms). Also, ∆11 progeny failed to be sap-transmissible from control plants (none of the 10 inoculated plants developed symptoms).

To examine recombinant virus progeny, nucleic acid samples were analysed either by digestion with the endonucleases SphI and EcoRV or by PCR. The endonuclease SphI cleaves wild-type ACMV dsDNA, as well as that of the coat protein mutant ∆11, but no SphI cleavage site is present in the constructs pCRA1, pCRA2 or pJC1 (Fig. 1). The second endonuclease, EcoRV, cleaves within the coat protein region that has been deleted from ∆11 (Fig. 1). The restriction pattern of such treated nucleic acid samples from severely ∆11-infected transformed plants was similar to the restriction pattern of samples from wild-type ACMV-infected plants (Fig. 5, compare lane 17 with lanes 6 and 8–13). In contrast, the restriction pattern of virus from plants with mild symptoms varied and some examples are shown in Fig. 5 (lanes 4 and 14).

Recombinant virus progeny of severely infected transgenic plants was analysed by PCR (Fig. 4). The primers used for PCR are located within a coat protein region that is deleted in ∆11 (Fig. 1); this allows amplification of replicative forms of wild-
type ACMV and construct pCRA1, but not of Δ11. In all transgenic plants infected with Δ11 that showed severe symptoms, full-length recombinant virus DNA, containing the coat protein gene, was detected and some examples are shown in Fig. 4 (lanes 7–12). Amplified DNA samples of recombinant virus progeny from fourteen plants were arbitrarily chosen for cloning and sequence analysis. In 12 out of 14 plants, the sequences of the EcoRI(2732)–MluI(734) (clockwise) and EcoRI(1868)–MluI(734) (counter-clockwise) fragments were identical to that of wild-type ACMV. In two samples, we found a sequence variation, i.e. substitution of a thymine for a cytosine at nucleotide position 91 in the published ACMV sequence (Stanley & Gay, 1983).

Samples of Δ11 agroinoculated leaf disks derived from transgenic CRA1 and CRA2 plants were also analysed by PCR; production of full-length recombinant virus DNA, containing the coat protein gene, was detected at a low frequency (3 out of 17 samples) in leaf disks derived from CRA1/4 plants indicating that recombination had taken place between incoming Δ11 DNA and the transgene (e.g. Fig. 4, lane 4).

Discussion

Coat protein-mediated virus resistance has been successfully applied for a number of RNA plant viruses (Hull & Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993). For the geminivirus tomato yellow leaf curl virus (TYLCV) Israel strain, Kunik Davies, 1992; Wilson, 1993).

We transformed N. benthamiana with various constructs, outlined in Fig. 1, containing the coat protein of ACMV. Transformed plants were inoculated with the ACMV coat protein deletion mutant Δ11. Several CRA1 and CRA2 plants developed symptoms that were similar to those observed in normal ACMV infections (Table 1). In these plants, ssDNA accumulated to levels comparable to those in ACMV-infected control plants, and immunoblots revealed coat protein accumulation. Recombinant Δ11 progeny from infected transformed plants with mild symptoms showed various restriction patterns, whereas the restriction pattern of all the analysed Δ11 progeny from infected plants was comparable to that of wild-type ACMV (Fig. 5). These wild-type characteristics of Δ11 progeny were confirmed by sap-transmission to control plants. PCR and sequence analysis confirmed that the recombinant Δ11 virus progeny was, in most cases, identical with wild-type ACMV. Therefore, our analysis does not enable us to pinpoint the recombination sites between Δ11 and the transgenic construct DNA.

The occurrence and frequency of recombination between the coat protein transgene and incoming Δ11 DNA seems to be dependent on the construct configuration. Recombination was observed in CRA1 and CRA2 plants, but not in JC1 plants (Table 1). In contrast to JC1, both pCRA1 and pCRA2 contain the entire common region of DNA A (Fig. 1). The common region seems to be a prime target for recombination. Homologous recombination between the common regions of ACMV DNA A and B was suggested as a repair mechanism to correct lethal substitutions and deletions in the DNA B stem–loop (Roberts & Stanley, 1994). Furthermore, constructs pCRA1 and pCRA2 contain extensive homologous sequences on both sides of the deletion in Δ11 while pJC1 contains homologous sequences only at the 3' end of the deletion (Fig. 1). Both the length and the multiplicity of locations of homologous sequences probably enhance the possibility of homologous recombination.

The recombination frequency is increased in CRA1 plants compared to CRA2 plants. This might be due to amplification of pCRA1 construct DNA either to a substantial level in a few infected cells or to a very low level in every cell. Such amplification of the construct DNA may increase the possibility of recombination with Δ11.

Geminiviruses are plant DNA viruses that replicate in the nuclei of infected cells (Abouzid et al., 1988; Pilartz & Jeske, 1992). The nucleus is generally considered as the cell compartment in which DNA recombination occurs. Recombination is a typical event in meiosis and, more recently, recombination has been described in mitotically dividing somatic cells (Swoboda et al., 1994). Thus, cellular proteins involved in recombination were probably available in every cell supporting recombination of viral mutant DNA and the integrated construct DNA.

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References


Recombination between viral DNA and transgenes


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