Pseudorecombination and complementation between potato yellow mosaic geminivirus and tomato golden mosaic geminivirus

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Pseudorecombinants made by exchanging the cloned, infectious genome components (DNAs A and B) of potato yellow mosaic geminivirus (PYMV) and the common strain (cs) of tomato golden mosaic geminivirus (csTGMV) are not infectious in their common host Nicotiana benthamiana. In an N. benthamiana leaf disc assay neither PYMV DNA A nor TGMV DNA A trans-replicated each other's DNA B component. The ability of PYMV and TGMV to mediate the systemic movement of each other's DNA A was investigated following co-inoculation of N. benthamiana with both genome components of one virus (the helper virus) and DNA A of the other virus (the dependent virus). Movement of the dependent virus DNA A in both cases illustrates interchangeability between the DNA B-encoded movement proteins of New World geminiviruses which infect solanaceous hosts. We have studied this genetic interchangeability further in separate co-agroinoculation experiments with N. benthamiana plants using TGMV DNA A to complement mutations in PYMV open reading frame (ORF) AC2, which encodes a protein that trans-activates the expression of virion sense promoters, and in PYMV ORF AC3, which specifies a protein that enhances viral DNA replication. TGMV DNA A complemented a PYMV AC2 mutant and restored its infectivity and it also complemented a PYMV AC3 mutant and restored the reduced DNA phenotype.

Introduction

Geminiviruses are single-stranded (ss) DNA viruses with a characteristic geminate capsid structure that replicate via double-stranded (ds) DNA intermediates in infected cell nuclei (Stanley, 1991; Lazarowitz, 1992). Potato yellow mosaic geminivirus (PYMV), which originates from Venezuela, is a member of the bipartite, geminivirus subgroup III (Mayo & Martelli, 1993) and is the only whitefly-transmitted geminivirus known to infect potato naturally (Roberts et al., 1986, 1988). The PYMV genome consists of two DNA molecules designated A and B (Coutts et al., 1991), both of which are required for infectivity (Roberts et al., 1986; Buragohain et al., 1994). The PYMV genome contains six or seven (Stanley & Latham, 1992) open reading frames (ORFs) that are arranged in a similar way to the genomes of other subgroup III geminiviruses such as tomato golden mosaic virus (TGMV) and abutilon mosaic virus (AbMV) (Hamilton et al., 1984; Frischmuth et al., 1990). Recent genetic analysis has shown that the organization and function of the ORFs in PYMV and TGMV are similar (Sung & Coutts, 1995) and places the two viruses in the same phylogenetic cluster (Arguello-Astorga et al., 1994; Faria et al., 1994; Fontes et al., 1994a, b; Padidam et al., 1995). The DNA A components of both viruses encode all of the viral information necessary for replication [the AC1, replication-associated (rep) protein] and encapsidation (the AV1, coat protein) of both DNAs (Rogers et al., 1986; Sunter et al., 1987; Sung & Coutts, 1995). The protein encoded by the AC3 ORF, while not absolutely required for viral replication, is necessary for normal accumulation of viral DNA in infected plants (Elmer et al., 1988; Sung & Coutts, 1995) and protoplasts (Sunter et al., 1990). The AC2 gene product transactivates transcription of the TGMV AV1 and BV1 genes (Sunter & Bisaro, 1991, 1992). The B components (encoding the BV1 and BC1 proteins) provide functions necessary for efficient systemic spread in infected plants (von Arnim & Stanley, 1992; Sung & Coutts, 1995).

The viability of pseudorecombinants produced by reassortment of the genome components of strains of the subgroup III geminiviruses, African cassava mosaic virus (ACMV), squash leaf curl virus (SqLCV), TGMV and bean golden mosaic virus (Stanley et al., 1985; Morris et al., 1990; Lazarowitz, 1991; von Arnim & Stanley, 1992; Faria et al., 1994) or of tomato mottle virus (ToMoV) and bean dwarf mosaic virus (BDMV) (Gilbertson et al.,...
1993) implies that trans-acting functions are interchangeable between different strains of the same virus and between very closely related viruses. In contrast, attempts to produce infectious pseudorecombinants between other closely related subgroup III geminiviruses such as ACMV and TGMV, TGMV and AbMV, ACMV and Indian cassava mosaic virus (ICMV) (Frischmuth et al., 1993) and TGMV and SqLCV (Lazarowitz, 1991) were unsuccessful, even though they share common hosts. Minimal differences between these viruses in iterated elements upstream of the putative TATA box sequences for the transcription of the respective rep genes (Arguello-Astorga et al., 1994; Fontes et al., 1994a), which in turn have high-affinity for and bind rep proteins (Fontes et al., 1994b) may determine the ability of DNA A to replicate heterogenic DNA B. However, it has been shown that ACMV can mediate the systemic movement of ICMV, TGMV and AbMV DNA A but, in reciprocal experiments, neither TGMV nor AbMV mediated systemic movement of ACMV DNA A although the former can support the movement of each other’s DNA A (Frischmuth et al., 1993).

Recently, it has been shown that both the AC2 and AC3 proteins of subgroup III geminiviruses can complement mutations in a heterologous viral genome (Sunter et al., 1994). The AC3 protein was (to some extent) interchangeable between different dicot-infecting geminivirus (subgroup II and III), whereas AC2 protein was more specific in its function but did not distinguish between Western and Eastern hemisphere subgroup III viruses (Sunter et al., 1994).

In this study we have produced pseudorecombinants of TGMV and PYMV by exchanging DNAs A and B and investigated their ability to infect whole plants and replicate in leaf discs of Nicotiana benthamiana Domin. We have also studied the ability of PYMV and TGMV to mediate the systemic movement of each other’s DNA A, and the complementation of mutations in TGMV ORFs AC2 and AC3 by TGMV AC2 and AC3 gene products in planta.

**Methods**

**Clone construction.** Recombinant DNA techniques were essentially as described (Sambrook et al., 1989). Restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers.

The construction of the wild-type monomeric clones of PYMV DNA A in pEMBL18 (pMAH2, referred to here as PA) and DNA B in pBR328 (pMBN1, referred to here as PB) have been described previously (Roberts et al., 1988). The construction of dimers of PYMV DNA A (referred to here as P2A) and DNA B (referred to here as P2B) in the Agrobacterium tumefaciens binary vector pBin19 (Bevan, 1984) has been described by Buragohain et al. (1994). The construction of monomeric clones of the common strain (cs) of TGMV DNA A and DNA B (referred to here as csTA and csTB) and partial dimers of TGMV DNA A and DNA B in pBin19 (referred to here as csTA1.6 and csTB1.4) has been described by von Arnim & Stanley (1992).

Two PYMV DNA A constructs, which had mutations in either the AC2 or AC3 ORF were made. The construction of these mutant clones, called respectively pAL2-1 (referred to here as PA:AC2-) and pAL3-1 (referred to here as PA:AC3-), and the production of pBin19 clones containing tandem repeats of each mutant respectively P2A:AC2- and P2A:AC3- in Agrobacterium tumefaciens strain LBA 4404 has been described before (Sung & Coutts, 1995).

**Maintenance and inoculation of N. benthamiana leaf disc assay for viral DNA replication.** N. benthamiana plants were maintained in accordance with the requirements of the Advisory Committee on Genetic Manipulation in an insect-free facility as previously described (Roberts et al., 1988). Clone combinations were introduced into the plants either by biolistic inoculation (Sung & Coutts, 1995) or by stem agroinoculation (Buragohain et al., 1994). Following inoculation, all plants were returned to the greenhouse to allow virus replication and symptom development.

The ability of viral DNAs to replicate in N. benthamiana leaf discs was assayed as described previously (Elmer et al., 1988; Buragohain et al., 1994) in accordance with established procedures (Horsch & Klee, 1986). Individual leaf discs or batches of five discs were harvested 5–8 days after inoculation and screened for the presence of viral DNAs by dot blot analysis (Maule et al., 1983).

**Isolation and characterization of viral DNA forms.** Total cellular nucleic acids were extracted from the leaves of all plants (symptomless or otherwise) and leaf discs as described by Frischmuth & Stanley (1991) and assayed for viral DNA replication by dot blot and Southern blot analysis. Samples containing 5-10 µg total nucleic acids were analysed by agarose gel electrophoresis and Southern transfer to Hybond N+ membranes (Amersham) which was performed under alkaline conditions according to the manufacturer’s recommendations. Viral DNAs were detected by hybridization with random-primed, radiolabelled probes (Feinberg & Vogelstein, 1983). PYMV genome components were specifically detected using a HindIII (1636)–Xhol (2232) fragment of DNA A and a BamHI (1038)–NcoI (1898) fragment of DNA B (Sung & Coutts, 1995). TGMV genome components were specifically detected using a BamHI (1356)–EcoRI (2251) fragment of DNA A and an EcoRI (650)–PstI (2092) fragment of DNA B (Frischmuth et al., 1993). Reciprocal hybridization with TGMV-specific and PYMV-specific DNA probes was performed using stringent washing conditions to avoid cross-hybridization between the respective DNA components (Frischmuth et al., 1993).

**Sequencing and PCR.** Maintenance of the PYMV AC2 and AC3 mutants was assessed by PCR sequencing of PCR-amplified products and by restriction digestion of PCR-amplified products using total nucleic acid extracts of infected plants (Sung & Coutts, 1995). The oligonucleotide primers used for PCR were: viral strand primer 5’TATGATTGCATTTAAATT3’ (nucleotide 1057–1074) and complementary strand primer 5’AAGGACCTACATATGCAGTC-3’ (nucleotide 1623–1639) (Coutts et al., 1991). The complementary strand primer was also used as the PCR sequencing primer and BglII and CofI were used for restriction digestion.

**Results**

**Infectivity of pseudo-recombinants in N. benthamiana**

Both csTGMV and PYMV induce similar but distinguishable symptoms in N. benthamiana following agroinoculation with mixtures of the cloned partial dimers of csTGMV or dimeric PYMV DNA components or biolistic inoculation with the excised, monomeric
PYMV and TGMV pseudorecombinants

Table 1. Infectivity of cloned PYMV and TGMV DNA combinations in N. benthamiana*

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Inoculum clones</th>
<th>Plants infected/ inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biolistic†</td>
<td>csTA + csTB</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>PA + PB</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>PA + csTB</td>
<td>0/7</td>
</tr>
<tr>
<td></td>
<td>csTA + PB</td>
<td>0/7</td>
</tr>
<tr>
<td>Agroinoculation‡</td>
<td>P2A + P2B</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>csTA1.6 + csTB1.4</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>P2A + csTB1.4</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>csTA1.6 + P2B</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Plants were inoculated with combinations of the cloned genome components of TGMV and PYMV.
† Inoculum consisted of 5 µg of each plasmid as described in Methods and by Sung & Coutts (1995). The recombinant plasmids were digested with the appropriate restriction endonuclease before inoculation of the excised monomeric DNAs to N. benthamiana.
‡ Agroinoculation was performed as described in Methods and by Buragohain et al. (1994).

DNAs of either TGMV or PYMV (Table 1). In this host, csTGMV induces severe stunting of the stem, extensive chlorosis, and severe leaf curling of systemically infected leaves (von Arnim & Stanley, 1992). In contrast, PYMV induces less marked stunting of the stem with restricted chlorosis and severe leaf curling of systemically infected leaves (Roberts et al., 1988). However, neither of the pseudorecombinants produced by mixing the cloned components of csTGMV and PYMV induced a systemic infection in N. benthamiana (Table 1), implying genome component incompatibility. Dot blot analysis of the DNA from upper leaves that emerged after inoculation confirmed the above results (results not shown).

Trans-replication in agroinoculated N. benthamiana leaf discs

To investigate the reason for component incompatibility csTGMV/PYMV clone combinations were agroinoculated into N. benthamiana leaf discs. The results for the combined inoculations are shown in Table 2. The independent replication of DNA A of PYMV and TGMV and the trans-replication of their cognate DNA B component were readily detected but trans-replication between each other’s DNA A and B components was not detected.

DNA B-mediated virus movement in N. benthamiana

To investigate the ability of TGMV (the helper virus) to spread PYMV DNA A (the dependent component) and PYMV to spread TGMV DNA A, combinations of cloned genome components were co-inoculated into N. benthamiana using biolistics.

Analyses of the viral DNAs present in the systemically infected leaves of randomly selected plants (three are shown in Fig. 1) consistently showed that plants inoculated with TGMV DNA A and B together with PYMV DNA A contained the ss and dsDNA forms of all three genome components (Fig. 1a shows a blot

Table 2. Trans-replication of PYMV and TGMV B DNA genome components in N. benthamiana leaf discs*

<table>
<thead>
<tr>
<th>Probe</th>
<th>P2A</th>
<th>P2A + P2B</th>
<th>P2A + csTB1.4</th>
<th>csTA1.6</th>
<th>csTA1.6 + csTB1.4</th>
<th>csTA1.6 + PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA-specific</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PB-specific</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>csTA-specific</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>csTB-specific</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</tr>
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</table>

* N. benthamiana leaf discs were agroinoculated with combinations of genomic components of PYMV (P2A and P2B) and csTGMV (csTA1.6 and csTB1.4) as described in Methods and by Buragohain et al. (1994). Replica dot blots of total nucleic acids (1 µg samples, harvested 7 days after inoculation) were probed with specific probes for each component of each virus as described in Methods. +, positive signal; -, negative signal.
Fig. 2. Complementation of a PYMV AC2 ORF mutant (a) and a PYMV AC3 ORF mutant (b) by TGMV. Total nucleic acids were extracted from leaves of representative, systemically infected N. benthamiana plants which were harvested 21 days after agroinoculation. Samples (5 μg) were fractionated, probed and blots washed as in Fig. 1. In (a), plants were inoculated with P2A + P2B and csTA1.6 (lane 1), P2A:AC2- + P2B and csTA1.6 (lanes 2–4) and P2A:AC2- and P2B (lane 5). In (b), plants were inoculated with P2A + P2B and csTA1.6 (lane 1), P2A:AC3- + P2B and csTA1.6 [two plants with severe symptoms (lanes 2 and 3) and one plant with mild symptoms (lane 4)] and P2A:AC3- and P2B (lane 5). Replica membranes were hybridized with probes specific for PYMV DNA A [panels (i)], PYMV DNA B [panels (ii)] or TGMV DNA A [panels (iii)], as described. Lane 6 in both (a) and (b) contains an extract from healthy N. benthamiana leaves. Lane 7 in (b) contains a HindIII digest of PA. Abbreviations for the DNA forms are as indicated in Fig. 1.

hybridized with a PYMV DNA A probe; TGMV probe results not shown). Probing replica blots with a PYMV DNA B-specific probe gave negative results and eliminated the possibility of chance contamination with PYMV DNA B mediating PYMV DNA A movement (results not shown). The amounts of PYMV DNA A found in these extracts were similar to those found in leaves systemically infected with a mixture of the wild-type PYMV DNA A and B components (results not shown), illustrating that TGMV DNA B can efficiently
mediate the systemic movement of the PYMV component.

In the reciprocal experiment, plants were co-inoculated with PYMV DNA A and B together with TGMV DNA A using biolistics or agroinoculation (see below), which showed that PYMV DNA B can also mediate the systemic movement of the TGMV component (Fig. 1b shows a blot hybridized with a TGMV DNA A probe; PYMV probe results not shown). Replicate results are shown in Fig. 2(a, b) [lane 1 in panels (iii)].

The symptoms on all systemically infected plants above were typical of the helper virus throughout and the dependent component did not obviously influence symptom type.

**Complementation of PYMV AC2 and AC3 mutants by TGMV**

To determine if the TGMV AC2 and AC3 proteins were capable of complementing mutants in the respective, equivalent genes of PYMV, *N. benthamiana* plants were agroinoculated with mixtures of P2A:AC2-, P2B and csTA1.6 or P2A:AC3-, P2B and csTA1.6 and compared with plants agroinoculated with either P2A:AC2- and P2B or P2A:AC3- and P2B. All plants agroinoculated with P2A:AC2-, P2B and csTA1.6 showed severe symptoms. Two-thirds of the plants agroinoculated with P2A:AC3-, P2B and csTA1.6 showed severe symptoms and the rest showed mild symptoms. In both cases the symptoms elicited were of an intermediate phenotype. All plants agroinoculated with a combination of P2A:AC2- and P2B were uninfected, while plants agroinoculated with a mixture of P2A:AC3- and P2B showed a considerable delay in the production of attenuated symptoms as compared to wild-type PYMV infections.

Viral DNA accumulation in extracts of systemically infected leaves from representative plants in the different experiments were examined by Southern blot analysis using PYMV DNA- and TGMV DNA-specific probes. Extracts from all plants inoculated with P2A:AC2-, P2B and csTA1.6 contained slightly reduced levels of PYMV DNA as compared to those found in wild-type infections [Fig. 2a, panels (i) and (ii); compare lane 1 and lanes 2-4]. Also, while the extracts from plants inoculated with P2A:AC2-, P2B and csTA1.6 showed differentially increased levels of PYMV DNA A as compared to those found in extracts of plants inoculated with P2A:AC3- and P2B [Fig. 2b, panels (i) and (ii); compare lanes 2-4 and lane 5] the amounts were lower than those found in wild-type infections [Fig. 2b, panels (i) and (ii); compare lane 1 and lanes 2-4]. Both sets of plants contained similar levels of the TGMV DNA A component which were comparable to the amounts found in plants inoculated with a mixture of P2A, P2B and csTA1.6 [Fig. 2a, b; panels (iii); compare lane 1 and lanes 2-4 in both blots].

PCR amplification of a PYMV DNA A 0.56 kb fragment (which included both the AC2 and AC3 ORFs) from nucleic acid extracts of representative plants and PCR sequencing of the product (for the AC2 mutant) or restriction analysis (loss of a BglII site and acquisition of a ClaI site) of the product (for the AC3 mutant) revealed that both mutants had been maintained (results not shown).

**Discussion**

Viable geminivirus pseudorecombinants have only been observed between virus strains or between very closely related viruses (see Introduction) and in all but one combination these viruses have highly homologous common regions and identical AC1 binding motifs (Fontes et al., 1994a). The only combination capable of forming pseudorecombinants with different AC1 recognition sequences is BDMV and ToMoV (Gilbertson et al., 1993) whose predicted AC1 binding motifs diverge at one nucleotide position via a conservative G/A replacement. The BDMV/ToMoV pseudorecombinants did not replicate as efficiently as the homologous combinations which suggests that even a single nucleotide difference significantly interferes with the interaction between AC1 and its recognition sequence.

While PYMV and TGMV are closely related to one another it was not surprising that combinations of their genomic components were not infectious for the common host, *N. benthamiana* following either agroinoculation or highly efficient biolistic inoculation (Table 1).

An examination of the nucleotide sequence of the PYMV DNA B common region (which is presumably concerned with TGMV rep protein binding and DNA replication) did not reveal significant identity with motifs crucial as cis-acting elements for TGMV replication (Fontes et al., 1994a, b) and vice versa.

In addition to a defect in a trans-replication function (Table 2) the non-viability of the heterologenous pseudorecombinants of PYMV and TGMV DNAs (Table 1) may be the result of an inability of the DNA B-encoded movement proteins to interact functionally with the heterologous DNA A component and cause a systemic infection. To investigate this, *N. benthamiana* plants were biolistically inoculated with mixtures of monomeric DNAs consisting of the A and B components of one virus and the A component of the other virus. We found that the DNA A components of both PYMV and TGMV do autonomously co-replicate, that DNA B is trans-replicated, and that DNA B gene expression is trans-activated by the cognate DNA A component (Fig. 1).
These results illustrate that one or possibly both of the DNA B gene products of PYMV and TGMV can fully recognize and functionally interact with each other's DNA and are consistent with the results found with plants co-agroinoculated with comparable combinations of TGMV and AbMV DNAs (Frischmuth et al., 1993). We also found that in mixed infections the symptoms induced were usually those of the helper virus even though the dependent DNA A was amplified in the systemically infected tissue. This observation confirms the importance of the DNA B-encoded movement proteins in symptom phenotype, previously noted using pseudorecombinants of phenotypically distinct TGMV strains (von Arnim & Stanley, 1992) and TGMV and AbMV (Frischmuth et al., 1993).

Agroinoculation of N. benthamiana plants with a combination of dimers of PYMV AC2 mutant DNA and PYMV DNA B did not cause infection and no replicating DNA was detected [Fig. 2a, panels (i) and (ii), lane 5]. Also, inoculation of N. benthamiana with combinations of dimers of the PYMV AC3 mutant DNA and PYMV DNA B resulted in plants with attenuated and delayed symptoms of infection and a pronounced reduction in viral DNA as compared to wild-type infections [Fig. 2b, panels (i) and (ii); compare lane 1 and lane 5]. These results are consistent with previous findings following biolistic inoculation of N. benthamiana with the same clone combinations (Sung & Coutts, 1995). However co-agroinoculation of N. benthamiana plants with partial dimers of TGMV DNA A and dimers of PYMV DNA B, together with either dimers of the PYMV AC2 or the AC3 mutant DNAs resulted in systemic symptoms of infection which were as severe as wild-type symptoms of an intermediate phenotype in nearly all cases. These results suggest that either or both of the two PYMV mutants had reverted and produced wild-type AC2 and AC3 proteins, or that the respective TGMV proteins had complemented the mutations in planta. Restriction and sequence analysis of the viral DNA confirmed that both mutants had been retained in the progeny and it is concluded that in separate inoculations both the TGMV AC2 and AC3 proteins can complement mutations to their respective equivalent ORFs in PYMV.

These results confirm that both the AC2 and AC3 gene products of subgroup III geminiviruses can function in heterologous viral systems and that mutations in both the AC2 and AC3 ORFs can be complemented in planta as well as in protoplasts (Sunter et al., 1994). However, the levels of PYMV DNA found in either case, whilst variable, were generally lower than those found in plants inoculated with wild-type genomic components [Fig. 2a, b, panels (i) and (ii); compare lane 1 and lanes 2-4]. We believe that this observation and the slight variability in symptom severity may be due to an inherent variability of the N. benthamiana host which has been noted previously (Evans & Jeske, 1993; Sung & Coutts, 1995). Alternatively, such variability reflects a randomness in the onset of infection by three components, as well as sampling systemically infected leaves at different stages of development and infection. We plan to repeat the experiments in potato, the natural host, using biolistic inoculation.

In conclusion, our results show that at least for TGMV and PYMV, although the AC1 rep protein is virus specific, movement proteins encoded by the DNA B components, and replication modulation and transcription activation functions, endowed by AC3 and AC2 respectively, are not the primary determinants of the ability of bipartite geminiviruses to form pseudorecombinants. We have recently expressed the PYMV AC2 ORF in Escherichia coli and Saccharomyces cerevisiae cells (unpublished results) and are now extending our studies to a functional analysis of this protein.

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replication protein and origin DNA is essential for viral replication. *Journal of Biological Chemistry* 269, 8459-8465.


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