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Mutational Analysis of the Putative Nicking Motif in the Replication-Associated Protein (AC1) of Bean Golden Mosaic Geminivirus

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Geminiviruses are circular single-stranded DNA viruses that replicate by a rolling circle mechanism involving the viral-encoded AC1 protein. DNA nicking is necessary both for initiating replication of the covalently closed double-stranded DNA templates and for releasing unit-length monomers. The effects of mutations in a putative nicking motif (K^{101} A Y I D K^{106}; E. V. Koonin and T. V. Ilyina, J. Gen. Virol. 73:2763-2766, 1992) of the AC1-derived protein for bean golden mosaic geminivirus isolate GA (BGMV-GA) were studied. The amino acids equivalent to Y^{103} and K^{106} of BGMV-GA are invariant in all whitefly-transmitted geminiviruses. *Phaseolus vulgaris* plant infectivity assays showed that the mutants $K^{101}\rightarrow H$, $K^{101}\rightarrow A$, and $D^{105}\rightarrow T$ produced symptoms, but mutants $Y^{103}\rightarrow A$, $Y^{103}\rightarrow F$, $K^{106}\rightarrow R$, and $K^{106}\rightarrow H$ did not. A mutant with a stop codon in the N terminus of the AC4 open reading frame (ORF) produced the same symptoms as the wild-type BGMV-GA. Only those that were infectious replicated in NT-1 tobacco suspension cells. These results indicate that the Y^{103} and K^{106} residues are essential for replication, and that this putative DNA-nicking motif of the AC1 ORF may be functional in the rolling circle mechanism of replication for geminiviruses. The potential role of these mutants in the design of antiviral strategies is discussed.

Bean golden mosaic geminivirus (BGMV) causes a serious disease of common bean (*Phaseolus vulgaris* L.) in the tropical and subtropical Americas and the Caribbean Basin. Characteristic symptoms of bean golden mosaic are a yellow-green mosaic on leaves, and stunted or distorted growth. BGMV is a member of the geminivirus subgroup III, whose characteristics include dicot hosts, whitefly transmission, and a bipartite or monopartite, single-stranded DNA genome with ≈2,600 nucleotides (nt) in each component. In BGMV and most other bipartite geminiviruses, both DNA components (DNA-A and DNA-B) are required for plant infectivity (Gilbertson et al. 1991a; see review by Timmermans et al. 1994). Both components have a 200-nt segment of homologous sequence (Faria et al. 1994), referred to as the common region, that contains the viral origin of replication (Lazarowitz et al. 1992; Orozco and Hanley-Bowdoin 1996); and together they have seven open reading frames (ORFs) potentially coding for proteins greater than 9 kDa. The products and functions of the ORFs have been studied primarily in tomato golden mosaic geminivirus (TGMV) and African cassava mosaic geminivirus (ACMV) (see review by Timmermans et al. 1994). The AC1 ORF is the only ORF essential for replication of geminiviruses (Hanley-Bowdoin et al. 1989), and DNA cleavage and joining activities of the C1 (AC1) protein are associated with the 211 amino acids of the N terminus of the Sardinia tomato yellow leaf curl geminivirus (STYLCV; Heyraud-Nitschke et al. 1995).

Geminiviruses replicate by a rolling circle mechanism (see review by Timmermans et al. 1994; Laufs et al. 1995a, 1995b; Stanley 1995), and the role of the AC1 protein in replication is analogous to that of the gene A protein of bacteriophage ϕX174 (Rogers et al. 1986), which causes cleavage of the viral polarity strand at a specific site and then binds covalently to the 5′ end of viral sense DNA through one of a pair of tyrosine residues (van Mansfeld et al. 1986). Recently, Laufs et al. (1995b) showed that the C1 protein of STYLCV cleaved and joined DNA of the conserved nonanucleotide sequence in the stem loop in the origin of replication. The ability of replication-associated proteins, such as the gene A protein and C1 (AC1) proteins, to cleave DNA is central to their function in initiating and terminating rolling circle replication. Amino acid sequence comparisons among replication-associated proteins from bacteriophages and prokaryotic plasmids known to nick DNA have revealed a putative nicking domain with three conserved motifs (Ilyina and Koonin 1992). Geminivirus consensus protein sequences for these motifs are FLTYpxC (motif I), HlHxUUQ (motif II), and xxYxxK (motif III) (Faria et al. 1994; Ilyina and Koonin 1992). Capital letters represent residues conserved in all 14 geminivirus sequences considered (9 monopartite, 5 bipartite); lowercase letters represent residues that are conserved in all but one aligned sequence; and U represents a bulky hydrophobic residue.

Certain amino acids in motif III are conserved among diverse geminiviruses and prokaryotic plasmids (Koonin and Ilyina 1992). In particular, the tyrosine residue corresponding to Y^{103} of the BGMV AC1 protein is conserved (Faria et al. 1994). When this residue was mutated to phenylalanine in the *rep*A ORF of plasmid pC194 of *Escherichia coli*, the plasmid

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replicated at 5% of the wild-type (wt) plasmid (Noirot-Gros et al. 1994). The similar mutant (Y to F) in the NS-1 gene of a parvovirus (minute virus of mice) is replication deficient (Skiadopoulos and Faust 1993).

Motif III occurs in the BGMV AC1-derived protein of DNA-A at amino acid positions 101 to 106: lysine (K^{101}) alanine (A) tyrosine (Y) isoleucine (I) asparagine (D) lysine (K^{106}) (Koonin and Ilyina 1992). Putative nicking motifs in 25 geminiviruses include both the Y^{103} and K^{106} residues (Faria et al. 1994; Koonin and Ilyina 1992), and this N terminus region is known to have DNA cleavage activity for the C1 proteins of STYLCV and wheat dwarf geminivirus (Heyraud-Nitschke et al. 1995). Site-directed mutagenesis was performed on both conserved and unconserved amino acid codons in this putative nicking motif in BGMV to assess its role in geminivirus replication. Certain amino acid codons were changed to codons for amino acids from a different family (with different side-chain properties) and some were changed to codons for amino acids from the same family (similar side-chain properties). Mutant clones were analyzed for their ability to infect beans and to replicate in tobacco suspension cells. These results are discussed with regard to their potential use in a trans-dominant interference strategy for viral resistance.

RESULTS

Inoculation of bean plants with AC1 mutants.

To evaluate infectivity and symptom development, *P. vulgaris* radicles were inoculated with mutant BGMV-GA DNA by particle acceleration (Gilbertson et al. 1991a). Bean radicles were coinoculated with either a mutant DNA-A or wt DNA-A, plus wt DNA-B. Nine radicles were inoculated with each DNA combination in each of two to four experiments. After 16 days, plants inoculated with wt DNA-A and DNA-B showed typical golden mosaic symptoms on the second trifoliolate leaf (Table 1). Plants inoculated with either wt DNA-A or one of the DNA-A mutants, $K^{101}{\rightarrow}H$, $K^{101}{\rightarrow}A$, or $D^{105}{\rightarrow}T$ (excised full-length DNA-A inserts from mutant plasmids $pK^{101}{\rightarrow}H$, $pK^{101}{\rightarrow}A$, and $pD^{105}{\rightarrow}T$), plus wt DNA-B had similar symptoms and rates of development. These three mutants are classified as symptom-positive.

One plant from each group of 27 inoculated with DNA-A mutant $Y^{103}{\rightarrow}A$ or $K^{106}{\rightarrow}R$ showed symptoms. The data from restriction analysis of polymerase chain reaction (PCR)–amplified virus fragments from these two plants (Table 1) indicated that the viruses infecting these plants could be contaminating wt viruses, and that the plants were not infected with the expected mutant. None of the 36 or 18 plants inoculated with DNA-A mutants $Y^{103}{\rightarrow}F$ or $K^{106}{\rightarrow}H$, respectively, showed symptoms. These four mutants are classified as symptom-negative.

The presence of BGMV-GA nucleic acids in upper noninoculated leaves was determined by squash blot hybridization of plant tissue collected 16 days after inoculation (Gilbertson et al. 1991b). All symptomatic beans contained nucleic acids that hybridized under high stringency conditions to the ^{32}P-labeled probe, pGAA2 (Table 1). Nucleic acids from BGMV were not detected in symptomless plants. The association of symptom development with the presence of BGMV-GA nucleic acids indicated that the symptoms were caused by BGMV infection.

Restriction site analysis confirmed the presence of the engineered codon changes in viral DNA from all mutant-infected beans. In every case, PCR with primers PCRc2 and PAC1v1978 generated the expected 671-bp fragment, and these amplified fragments, generated from DNA extracted from beans inoculated with DNA-A mutants $K^{101}{\rightarrow}H$, $K^{101}{\rightarrow}A$, and $D^{105}{\rightarrow}T$, gave the expected fragment sizes when digested with *Nsi*I, *Bbv*I, and *Hph*I, respectively. Thus, these mutants are infectious and produce typical golden mosaic symptoms in bean plants.

Replication of nicking motif mutants in NT-1 tobacco suspension cells.

The ability of these mutants to replicate was further evaluated through transfection of NT-1 tobacco suspension cells by electroporation with wt or mutant DNA-A plus wt DNA-B, and subsequent analysis of DNA extracted from these cells at 0 h and 72 h. This analysis used a PCR system that selectively amplifies circular DNA-A replicated in the plant cells, not linear inoculum or recircularized, nonreplicated inoculum (Hanson et al. 1995). In no treatments were PCR products generated from DNA extracted from tobacco suspension cells for the time zero samples. The expected 1.1-kb fragments were obtained by PCR with DNA extracted from the tobacco suspension cells 72 h after transfection with either wt DNA-A or

Table 1. Biological activity of DNA-A with mutations in the putative DNA-nicking motif III of AC1 open reading frame (ORF) and in the AC4 ORF of bean golden mosaic geminivirus (BGMV)

Inoculum[a]	Symptoms[b]	Squash blot hybridization[c]	Replication[d]	Mutation confirmation[e]
wt	33/36	33/36	9/9	NA
Mock	0/24	0/24	0/8	NA
$K^{101}{\rightarrow}H$	12/18	12/18	4/4	*Nsi*I+
$K^{101}{\rightarrow}A$	11/18	11/18	5/8	*Bbv*I+
$Y^{103}{\rightarrow}A$	1/27[f]	1/27	0/6	*Bbv*I–
$Y^{103}{\rightarrow}F$	0/36	0/36	0/6	NA
$D^{105}{\rightarrow}T$	11/15	11/15	6/6	*Hph*I+
$K^{106}{\rightarrow}R$	1/27[f]	1/27	0/6	*Bsa*OI–
$K^{106}{\rightarrow}H$	0/18	0/18	0/6	NA
$AC4^-$	21/24	21/24	3/3	NA

[a] Linearized monomers of viral DNAs. wt = wild-type BGMV-GA DNA-A. Mock = water (bean plants) or carrier DNA (NT-1 tobacco cells). $K^{101}{\rightarrow}H$ = codon change of K to H at nucleotide position 101 in AC1 ORF. $K^{101}{\rightarrow}A$ = codon change of K to A. $Y^{103}{\rightarrow}F$ = codon change of Y to F. $Y^{103}{\rightarrow}A$ = codon change of Y to A. $D^{105}{\rightarrow}T$ = codon change of D to T. $K^{106}{\rightarrow}R$ = codon change of K to R. $K^{106}{\rightarrow}H$ = codon change of K to H. $AC4^-$ = AC4 ORF mutant with stop codon after three codons in the N terminus end.

[b] Bean plants inoculated with DNA-As and wt DNA-B. Number of bean plants with viral symptoms/total number of plants 16 days after inoculation of radicles by particle acceleration.

[c] Squash blot hybridization at high stringency with pGAA2 cleaved with *Spe*I and radiolabeled with ^{32}P as a probe. Number of plants with positive hybridization signal/total number of plants. All plants with viral symptoms had positive hybridization signals with the pGAA2 probe.

[d] Replication in NT-1 tobacco suspension cells, detected by polymerase chain reaction (PCR). Number of replication positive tests/total number of replication tests. Only DNA-As used in electroporation.

[e] Confirmation of site-directed codon changes by restriction analysis of PCR fragments obtained from all plants with symptoms. + = restriction site added during site-directed mutagenesis is present; – = restriction site added during site-directed mutagenesis is not present, therefore infection could have resulted from contaminating wild-type molecules. NA = Not applicable.

[f] The single infected plant was from the second of three inoculations.

mutants $K^{101}\rightarrow H$, $K^{101}\rightarrow A$, or $D^{105}\rightarrow T$ (Fig. 1). Digestion of these fragments with the appropriate restriction enzymes gave restriction fragment patterns that indicated the preservation of the mutant codon changes. Thus, these three mutant AC1 ORFs do support replication. Replication was not detected in tobacco suspension cells inoculated with mutants $Y^{103}\rightarrow A$, $Y^{103}\rightarrow F$, and $K^{106}\rightarrow R$ (Fig. 1), or $K^{106}\rightarrow H$ (data not shown).

Plant infectivity and replication of AC4 mutant.

Since the mutations in the AC1 ORF also caused codon changes in the overlapping AC4 ORF, the replication and infectivity of a truncated AC4 mutant was determined. This AC4 mutation did not change the derived amino acid sequence of the AC1 ORF. The AC4 mutant and wt DNA-B combination infected bean plants with the same frequency and symptomatology as wt infections. All plants with symptoms gave positive squash blot hybridization signals with the pGAA2 probe, and symptomless plants gave none. Southern blot analysis showed that viral DNA accumulated to similar levels in plants infected with the AC4 mutant and with wt viral DNA. PCR fragments, which included the mutated region of the AC4 ORF, were obtained from four plants inoculated with the AC4 mutant, and the sequence of these fragments showed that the stop codon mutation had been retained in PCR fragments from these plants. Furthermore, the AC4 mutant did replicate in the NT-1 tobacco suspension cells (Fig. 1).

DISCUSSION

Seven site-directed mutations in the codons for the putative nicking motif III (K^{101} A Y I D K^{106}) of the BGMV-GA AC1 ORF were analyzed for their effect on the ability of the viral DNA to infect beans and to support viral replication in tobacco suspension cells (Table 1). Some of these codon changes caused the loss of both of these abilities. When the codon for Y^{103} was mutated to that for A or F, or the codon for K^{106} to R or H, neither infectivity nor replication was observed, so these conserved residues are essential for BGMV-GA replication. The loss of activity in Y^{103} mutants is consistent with the hypothesis that this tyrosine functions in DNA nicking and covalent binding of the AC1 protein to the 5′ end of the cleaved strand in the origin of replication (Laufs et al. 1995b). During the course of this research, a substantiating study by Laufs et al. (1995a) reported the binding of the Y^{103} of the STYLCV Rep protein, which is equivalent to the Y^{103} of the BGMV-GA AC1 protein, to the 5′ end of the cleaved DNA strand; and they also found that a $Y^{103}\rightarrow F$ mutant did not replicate in tobacco suspension culture cells. The codon changes, $K^{101}\rightarrow H$ or $K^{101}\rightarrow A$, and $D^{105}\rightarrow T$, in less conserved areas of the putative DNA-nicking motif, did not lead to a loss of infectivity or the ability to replicate in tobacco suspension cells. It is not surprising that the $D^{105}\rightarrow T$ mutant had no effect, since the threonine codon is present at this position in the AC1 ORF of squash leaf curl geminivirus.

Interpretation of the effects of AC1 mutations is based on the assumption that the proteins with presumed amino acid substitutions retain the overall conformational structure of the wt AC1 protein. In the absence of structural or biochemical studies, the possibility cannot be discarded that Y^{103} and K^{106} of AC1 protein are not directly involved in rolling circle replication, but rather are important for maintaining the native conformation of the AC1 product. This seems unlikely because $Y^{103}\rightarrow F$ is such a conservative change compared with more drastic ones ($K^{101}\rightarrow H$, $K^{101}\rightarrow A$, and $D^{105}\rightarrow T$), which have no effect, and because changes in another K residue nearby (K^{101}) have no effect. Therefore, we suggest that Y^{103} and K^{106} are involved in DNA nicking.

Ilyina and Koonin (1992) analyzed the conservation of the DNA-nicking motif in rolling circle replication-associated proteins across a wide variety of bacteriophages and plasmids, as well as plant and animal viruses. The tyrosine corresponding to Y^{103} of the BGMV-GA AC1 ORF is conserved across all 59 proteins studied and its function has been extensively studied in bacteriophage ϕX174 (van Mansfeld et al. 1986; Kornberg and Baker 1992). Specific biochemical analysis of the protein-DNA complex has strongly implicated this tyrosine residue in covalent DNA binding (van Mansfeld et al. 1986); and studies by others (Noirot-Gros et al. 1994; Skiadopoulos and Faust 1993; Yasukawa et al. 1991), using site-directed mutagenesis of the Y codon to F, have shown that it is essential for replication in the bacterial plasmids, pKYM and pC194, and in the minute virus of mice. Our work shows that this tyrosine (Y103) is essential for replication and infectivity of BGMV-GA, and the homologous tyrosine is highly likely to be essential for other geminiviruses as well (Faria et al. 1994, Laufs et al. 1995a). Our results and those of Laufs et al. (1995a, 1995b) support the hypothesis that geminiviruses replicate by a rolling circle mechanism similar to that of ϕX174 (van Mansfeld et al. 1986) or other DNA molecules containing the DNA-nicking motifs identified by Ilyina and Koonin (1992), such as pC194 (Noirot-Gros et al. 1994). The lysine corresponding to BGMV-GA K^{106} is conserved among the replication-associated proteins from 22 plant and animal vi-

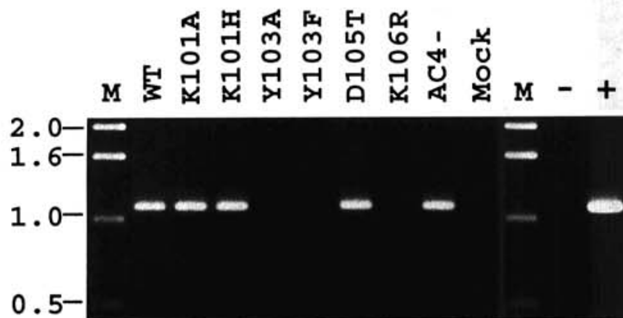

Fig. 1. Replication in NT-1 tobacco suspension cells of the bean golden mosaic geminivirus isolate GA (BGMV-GA) mutants variant in the DNA-nicking motif III of AC1 open reading frame (ORF) or of a mutant with an introduced stop codon in the N terminus of AC4 ORF. Wild-type (wt) or mutant DNA-A was electroporated into NT-1 tobacco suspension cells; cells were incubated for 72 h and total DNA was extracted and digested with *Dpn*I. Polymerase chain reaction (PCR) fragments were produced from viral replicative form DNA with primers PAv1903 and PAc342. Lane 1: 1-kb ladder (sizes of individual marker bands indicated at left of gel. Gibco BRL, Gaithersburg, MD); lane 2: wt BGMV-GA DNA-A; lane 3: $K^{101}\rightarrow A$ mutant; lane 4: $K^{101}\rightarrow H$ mutant; lane 5: $Y^{103}\rightarrow A$ mutant; lane 6: $Y^{103}\rightarrow F$ mutant; lane 7: $D^{105}\rightarrow T$ mutant; lane 8: $K^{106}\rightarrow R$ mutant; lane 9: AC4-truncation mutant; lane 10: mock = carrier DNA; lane 11: 1-kb ladder as marker DNA; lane 12: PCR negative control (no added DNA); lane 13: PCR positive control (BGMV-GA dimer of DNA-A as PCR template). DNA isolated from cells inoculated with $K^{106}\rightarrow H$ mutant did not produce a fragment (data not shown).

ruses described in Koonin and Ilyina (1992) with the exception of adeno-associated virus, which contains a proline at this position. This K^{106} residue also appears to be essential for replication of BGMV-GA, since conservative amino acid codon changes to arginine or histidine abolish replication and infectivity. As in the assumed ϕX174 mechanism, however, this residue is not thought to be directly involved in covalent binding to DNA.

Because of the overlap between the AC1 and AC4 ORFs, the effects of mutations in the AC1 ORF could result from concomitant codon changes in the AC4 ORF rather than the AC1 ORF. Since introduction of a stop codon in the N terminus of AC4 ORF of BGMV-GA did not affect plant infectivity or replication, as is also the case for three other bipartite geminiviruses (Elmer et al. 1988; Etessami et al. 1991; Sung and Coutts 1995), interpretation of the effects of AC1 ORF mutations is based on changes in the AC1, rather than the AC4, protein.

Competition between a genetically engineered, nonfunctional version of a viral protein and the wt viral protein is a possible antiviral strategy (Li and Rhode 1990; Smith and Deluca 1992; Stow et al. 1993). Lethal mutants could yield trans-dominant AC1 proteins that interfere with viral replication. This possibility was investigated by means of a binary vector system in tobacco suspension cells, and the mutants $Y^{103} \rightarrow F$ and $K^{106} \rightarrow R$ both reduced replication of wt BGMV-GA DNA-A (S. F. Hanson and D. P. Maxwell, unpublished data). Thus, transgenic plants with these altered AC1 ORFs could be produced and tested for resistance to geminiviruses.

MATERIALS AND METHODS

Recombinant plasmids.

An infectious, full-length *Spe*I monomer of BGMV-GA DNA-A was cloned from a previously constructed *Eco*RI dimer of DNA-A (Gilbertson et al. 1991a) into pBluescript II KS(+) (pBSII, Stratagene, La Jolla, CA) to create clone pGAA2 (Hanson et al. 1995). Clone pGAB1 had a full-length, infectious insert of BGMV-GA DNA-B cloned at the *Bam*HI^{2377} site (Gilbertson et al. 1991a).

Molecular biology techniques were performed according to Sambrook et al. (1989), except as noted. Restriction endonucleases were obtained from Promega Corp. (Madison, WI) and from New England Biolabs, Inc. (Beverly, MA).

Site-directed mutagenesis.

The sequence of BGMV-GA DNA-A (GenBank no. M91604) was used to derive the amino acid sequences for the AC1 and AC4 proteins and to design oligonucleotides to be used with site-directed mutagenesis to create recombinant plasmids with the codon changes $K^{101} \rightarrow A$ (pK^{101}A), $K^{101} \rightarrow H$ (pK^{101}H), $Y^{103} \rightarrow A$ (pY^{103}A), $Y^{103} \rightarrow F$ (pY^{103}F), $D^{105} \rightarrow T$ (pD^{105}T), $K^{106} \rightarrow R$ (pK^{106}R), and $K^{106} \rightarrow H$ (pK^{106}H) in AC1 and with a stop codon at nucleotide position 2472, which is four codons from the N terminus of AC4 ORF. Codon changes in the AC1 ORF were designed to create additional restriction sites (Table 2) to facilitate identification of DNA containing the desired nucleotide changes. The oligonucleotide CTTCATGCGCTAATTCAATTCG, in which the underlined letter is the mismatch with the wt viral DNA, was used to introduce the stop codon in the AC4 ORF. Plasmid pGAA2 was transformed into *E. coli* strain CJ236 (*dut*$^-$, *ung*$^-$), and the resulting colonies were screened for the presence of the transformed plasmid. Site-directed mutagenesis was performed as described by Kunkel et al. (1989) except that T7 DNA polymerase (United States Biochemical, Cleveland, OH) was used. Recombinant plasmids containing an added site were sequenced, using the dideoxy chain termination method and Sequenase version 2.0 enzyme (United States Biochemical), from the *Bgl*II^{2288} site through the *Spe*I^{107} cloning site and to the *Not*I site in the pBSII multiple cloning region to determine the presence of the desired codon changes and to detect other changes in this region.

Following confirmation that the sequence from *Bgl*II^{2288} to *Spe*I^{107} contained only the expected codon changes, a 500-bp fragment was excised with *Bgl*II and *Not*I enzymes, gel purified with the GeneClean kit (Bio 101, La Jolla, Ca), and cloned into *Bgl*II and *Not*I-digested pGAA2 (also gel purified as above). Plasmid DNA preparations from the resulting colonies were screened by digestion with the appropriate restriction endonuclease. Clones exhibiting the expected restriction digest pattern were used to analyze mutant phenotypes.

Plant inoculation by particle acceleration.

Plasmid DNA was prepared by a scaled-up version of the alkaline lysis method described in Sambrook et al. (1989). Full-length linear monomers of DNA-A and DNA-B were inoculated into *Phaseolus vulgaris* cv. Topcrop radicles 0.5 to 1.0 cm long by particle acceleration (Gilbertson et al. 1991a). Inoculated seeds were planted in Jiffy Mix, and held at 26°C

Table 2. Oligonucleotides used in site-directed mutagenesis to produce AC1 mutants

Name[a]	Sequence[b]	Location[c]	Enzyme[d]
wt	...........AAGGCATACATCGACAAA.........	2336 to 2353	. . .
$K^{101} \rightarrow H$	.....GACGTCCATGCATACATCGACAAAGATG.....	2332 to 2359	*Nsi*I^{2351}
$K^{101} \rightarrow A$	GTTCAGACGTCGCAGCATACATCGACAAAG........	2335 to 2364	*Bbv*I^{2344}
$Y^{103} \rightarrow A$	........GTCAAGGCAGCCATCGACAAAG........	2335 to 2356	*Bbv*I^{2333}
$Y^{103} \rightarrow F$	.......CGTCAAGGCATTCATCGACAAAG........	2335 to 2357	*Bsm*I^{2351}
$D^{105} \rightarrow T$	............GGCATACATCACCAAAGATGGAG..	2329 to 2351	*Hph*I^{2351}
$K^{106} \rightarrow R$	.............GCATACATCGACCGAGATGGAGTC	2327 to 2350	*Bsa*OI^{2340}
$K^{106} \rightarrow H$	.............GCATACATCGATCATGATGGAGTC	2327 to 2350	*Cla*I^{2340}

[a] Change in codon for one of the six amino acids in the putative DNA-nicking motif of the AC1 protein of bean golden mosaic geminivirus. wt = wild type.

[b] Sequence (5′→3′) of oligonucleotide used in site-directed mutagenesis. Nucleotides different from those in wt (complementary strand) are underlined.

[c] Nucleotide position on the viral sense ssDNA to which the oligonucleotide anneals.

[d] Restriction endonuclease site created by the codon changes underlined in the oligonucleotides. The superscript indicates the restriction endonuclease cleavage site.

for 14-h light periods alternating with 10-h dark periods at 21°C.

Detection of viral nucleic acids by squash blot hybridization and Southern blot analysis.

Plants from inoculated seeds were tested for the presence of BGMV-GA nucleic acids by squash blot hybridization under high stringency conditions as described by Gilbertson et al. (1991b). Random priming (kit from United States Biochemical) was used to produce a ^{32}P-labeled probe from pGAA2 cleaved with *Spe*I. For Southern analysis, DNA was extracted from fresh leaf tissue as described by Dellaporta et al. (1983), and its concentration determined with a fluorometer (Hoefer Scientific Instruments, San Francisco, CA). One microgram of each DNA was run in a 1% agarose gel with 0.5× Tris-borate-EDTA (TBE) buffer and then capillary-blotted onto nylon membrane (Zetabind, CUNO, Inc., Meriden, CT) as described by Sambrook et al. (1989).

Analysis of BGMV DNA in inoculated plants.

BGMV-GA DNA was detected in infected plants by PCR amplification of viral DNA (Rojas et al. 1993) from DNA extracted from 8-mm leaf disks (Dellaporta et al. 1983). PCR primers PCRc2 and PAC1v1978 (Rojas et al. 1993) amplify a 671-bp viral DNA fragment that includes the DNA-nicking motif III. PCR fragments generated from DNA from plants inoculated with mutant DNA-As were digested with appropriate restriction enzymes to detect the expected site-directed mutations. PCR fragments from plants inoculated with the AC4 mutant were sequenced at the Biotechnology Center, University of Wisconsin–Madison with an ABI 373 automated sequencer and fluorescent dye terminators.

Replication of mutant BGMV-GA DNA-A in tobacco suspension cells.

NT-1 suspension cells (originated from *N. tabacum* cv. bright yellow 2) (An 1985) were a gift from R. Amasino, Department of Biochemistry, University of Wisconsin–Madison. NT-1 suspension cells were subcultured weekly in NT-1 medium by transferring 15-ml aliquots from stationary phase culture to 50 ml of NT-1 medium (per liter: [pH 5.5-5.7] 4.3 g of Murashige/Skoog basal salts [Sigma Chemical Co., St. Louis, MO], 10 ml of B1 inositol [per liter: 10 g of inositol, 0.1 g of thiamine ·HCl], 3 ml of Miller's I [per liter: 60 g of KH_2PO_4, 30 g of sucrose, 0.2 mg of 2,4-dichlorophenoxyacetic acid]) in sterilized 125-ml Erlenmeyer flasks with silicone stoppers. Cell cultures were grown on a rotary shaker (Model G33, New Brunswick Scientific, Edison, NJ) at 180 rpm at room temperature (20 to 25°C).

Isolation and transfection of partially digested NT-1 suspension cells.

Partially digested NT-1 tobacco suspension cells (An 1985) were prepared according to the protocol of Fontes et al. (1994), except that the enzymes used were cellulysin cellulase and macerase pectinase (Calbiochem, San Diego, CA). The number of viable cells, estimated after staining with fluorescein diacetate (Widholm 1972), was adjusted to 5×10^6 cells/ml in NT-1 electroporation buffer and held on ice prior to electroporation (Fontes et al. 1994).

BGMV-GA DNA (20 µg/treatment) was added to electroporation cuvettes (Gene Pulser Cuvettes, BIORAD, Hercules, CA), mixed with salmon testes carrier DNA (80 µg/treatment; Sigma), and the volume adjusted to 400 µl with electroporation buffer (Fontes et al. 1994). Four hundred microliters of NT-1 suspension cells was added to each cuvette and the mixture electroporated and incubated as in Fontes et al. (1994). The inoculated cells were incubated in petri dishes in a box with moist paper towels at 23°C. Aliquots were removed at 0 h and at 72 h, and the cells pelleted and stored at –80°C. Total DNA was extracted as described in Dellaporta et al. (1983), except for the addition of an extraction with phenol/chloroform before DNA precipitation. DNA was quantified by means of a Hoefer Model TKO 100 fluorometer.

Detection of replication in NT-1 suspension cells with PCR.

The PCR method developed by Hanson et al. (1995) and used to detect viral replication in these cells specifically amplifies replicated viral DNA, not inoculum DNA. The primers PAC1v1903 and PAV1c342 anneal to opposite sides of the cloning site (*Spe*I^{107}) of BGMV-GA DNA-A, so the linear inoculum DNA-A cannot give rise to a PCR product. A PCR fragment of the expected size (1.1 kb), therefore, unambiguously demonstrates viral replication in the tobacco cells. This PCR product is then digested with the appropriate restriction enzyme to confirm the presence of the site-directed mutation.

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