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Identification of amino acid residues of the coat protein of Sri Lankan cassava mosaic virus affecting symptom production and viral titer in *Nicotiana benthamiana*

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ABSTRACT

Sri Lankan cassava mosaic virus (SLCMV) is bipartite begomovirus infecting cassava in India and Sri Lanka. Interestingly, the DNA-A component of the SLCMV alone is able to infect *Nicotiana benthamiana* causing symptoms of upward leaf rolling and stunting. One of the differences between monopartite and bipartite begomoviruses is the requirement of Coat Protein (CP) for infectivity; CP being essential for the former, but dispensable in the latter. This investigation was aimed to determine the importance of CP in the infectivity of the bipartite SLCMV, behaving as a monopartite virus in *N. benthamiana*. We tested CP-null mutants, single amino acid replacement mutants and double, triple and quadruple combinations of the above in SLCMV DNA-A, for infectivity, symptom development and viral DNA accumulation in *N. benthamiana*. While CP-null mutants were non-infectious, a majority of the single amino acid replacement mutants and their combinations retained infectivity, some with attenuated symptoms and reduced viral titers. Some of the combined mutations restored the attenuated symptoms to wild type levels. Some of the mutations were predicted to cause changes in the secondary structure of the CP, which roughly correlated with the attenuation of symptoms and the reduction in viral titers.

1. Introduction

Geminiviruses are a large group of plant-infecting viruses, which are composed of either one (monopartite) or two (bipartite) single-stranded circular DNA components and have a characteristic twinned geminate shaped particles. Based on their genome organization, host range and vector specificity, seven genera of geminiviruses have been recognized by the International Committee on Taxonomy of Viruses, namely, *Becurtovirus*, *Begomovirus*, *Curtovirus*, *Eragrovirus*, *Topocuvirus*, *Mastrevirus* and *Turncurtovirus* (Varsani et al., 2014). Of the above, genus *Begomovirus* contains the most members and are transmitted by whiteflies belonging to the species complex *Bemisia tabaci*. In the circular viral DNA, open reading frames (ORFs) are present both in the viral (rightward) as well as complementary (leftward) directions and hence the ORFs are termed V(R) or C(L) based on their direction. In bipartite begomoviruses, DNA-A encodes for at least six viral proteins AC1-4, AV1 and AV2. The protein encoded by ORF AV1 is known as coat protein (CP). DNA-B, on the other hand encodes two movement-related proteins; BC1 or the movement protein (MP) and BV1 or the nuclear shuttle protein (Hanley-Bowdoin et al., 2013). CP is essential for the infectivity in monopartite geminiviruses such as maize streak virus (MSV, Boulton et al., 1989), beet curly top virus (BCTV, Briddon et al., 1989) and tomato yellow leaf curl virus (TYLCV, Wartig et al., 1997). Certain bipartite begomoviruses, on the other hand, show much less dependency on CP for infectivity and can retain infectivity even with mutations or deletions in the CP, provided the DNA-B encoded movement functions remain intact (Pooma et al., 1996; Guevara-González et al., 1999). For example, viral DNA constructs with deletion across the CP gene of the bipartite tomato golden mosaic virus (TGMV, Brough et al., 1988) were reported infectious through agroinoculation in *Nicotiana benthamiana* plants, albeit with attenuated symptoms. Similar observations were reported also in the bipartite squash leaf curl virus (SqLCV, Qin et al., 1998), bean golden mosaic virus (BGMV, Azzam et al., 1994), tomato leaf curl virus (ToLCV, Padidam et al., 1995) and pepper huasteco virus (Guevara-González et al., 1999), illustrating the fact that CP shares functions of movement with genes encoded by DNA-B (Qin et al., 1998).

The importance of CP to the infectivity of monopartite geminivirus was further revealed when it was shown that single amino acid changes in the monopartite tomato yellow leaf curl virus

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(TYLCV) CP completely abolished the infectivity (Noris et al., 1998), the proper assembly of virion particles being a deciding factor.

Sri Lankan cassava mosaic virus (SLCMV) is a bipartite begomovirus infecting the root crop cassava (*Manihot esculenta*) in India and Sri Lanka (Saunders et al., 2002; Dutt et al., 2005; Patil et al., 2005). Interestingly, in *N. benthamiana*, SLCMV DNA-A alone induces upward leaf roll and vein swelling symptoms (Saunders et al., 2002; Mittal et al., 2008), unlike any other bipartite begomovirus, the symptoms being similar to those produced by the curtovirus BCTV (Stanley et al., 1986) and the monopartite begomovirus Ageratum yellow vein virus (AYVV, Tan et al., 1995). Hence, it would be interesting to study the effect of various mutations in the CP on the infectivity of SLCMV DNA-A in *N. benthamiana*.

To determine the effects of mutations in the CP of SLCMV DNA-A, we generated CP non-expressing mutants, several single amino acid replacements and combinations of double, triple and quadruple amino acid replacements in the CP of SLCMV DNA-A. We then studied the symptoms produced in *N. benthamiana* following inoculations using those mutants and determined the viral titers. This allowed us to identify amino acid residues that are important for determining symptom production and viral titers and show that some of them work in a combinatorial fashion.

2. Materials and methods

2.1. Site directed mutagenesis of the specific amino acid residues in the SLCMV CP

SLCMV-[Ker20] DNA-A (accession no. AJ579307, Dutt et al., 2005; Mittal et al., 2008) fragment cloned into TA vector was digested using restriction enzymes *Eco*RV and *Eag*I (Thermo Scientific, USA) and the released fragment of 1039 bp was cloned in *Eco*RV and *Eag*I restriction sites of pBSK vector generating Amut-pBSK construct carrying nucleotide residues 317nt to 1355nt of SLCMV DNA-A. Mutations were introduced using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene Agilent Technologies, Inc., La Jolla, CA) by following the manufacturer's instructions. Primers used for introducing the mutations are mentioned in Supplementary Table 1. After transformation, ten colonies were randomly selected and DNA was sequenced using M13 forward and reverse primers to confirm introduced mutations. The Amut-pBSK with the introduced single mutation was digested by *Xcm*I restriction enzyme and the 400 bp fragment was cloned in A1.06-pCAMBIA construct that carried a partial dimer of SLCMV DNA-A along with the full length SLCMV DNA-A fragments-A11 cloned in the same orientation into the binary vector pCAMBIA2300 in *Sal*I-*Sac*I sites (Mittal et al., 2008). The CP gene was amplified from A1.06-pCAMBIA* construct carrying single mutation using SLCMVCP Eco-FP/Bam-RP primers (Supplementary Table 1). The amplified DNA fragment was cloned into TA vector (InsTA vector-Thermo Scientific, USA) and the generated SLCMV CP-TA construct was used for the introduction of the second mutation at the specified positions. Similarly, double mutants were used for introducing a third mutation and finally fourth mutation. Each SLCMV CP-TA construct was digested with by *Xcm*I restriction enzyme and released 400 bp fragment was cloned in digested SLCMV A1.06-pUC19 construct carrying the SLCMV DNA A 3.7 kb partial dimer to generate clones of single, double, triple and quadruple mutants. The SLCMV DNA A 3.7 kb partial dimer was excised from pUC19 backbone using *Sal*I-*Sac*I and cloned in pCAMBIA binary vector to obtain mutated A1.06/pCAMBIA2300 construct.

SLCMV CP-null mutant STCP was constructed by inserting a stop codon ahead of AV1 ORF in A1.06-pUC19 construct, using ST-CP forward and reverse primers (Supplementary Table 1) and later excising the A1.06 3.7 kb partial dimer with stop codon and cloning it in pCAMBIA binary vector. Another CP null mutant JSdCPgfp was used in which *gfp* gene replaced the *cp* gene in A1.06 partial SLCMV DNA, cloned in pCAMBIA2300 backbone in between *Sac*I-*Kpn*I restriction sites. The authenticity of the clones were checked by DNA sequencing.

2.2. Agroinoculation and infectivity of the wild-type and CP mutant SLCMV clones in N. benthamiana plants

Agrobacterium EHA105 competent cells were transformed with wild type, mutated A1.06/pCAMBIA2300 binary construct of SLCMV and CP null mutants. A total of 400 *N. benthamiana* plants were agroinoculated in the petiole region of the 3rd leaf from the top (5 plants per construct, repeated 5 times) using a method reported earlier (Mittal et al., 2008). Mock-inoculations were performed with empty vector and control plants were kept un-inoculated. After agroinoculation, the plants were kept at 30 °C with 16/8 h light/dark cycle. The inoculated plants were observed for symptom development every week. Symptoms or other morphological changes were recorded.

2.3. DNA extraction and PCR amplification of the SLCMV CP gene

The genomic DNA was isolated, using standard protocols (Dellaporta et al., 1983), from 100 mg of 4th or 5th systemic leaf from top of agroinoculated *N. benthamiana* plants at 25 dpi. The prepared dilutions of 50 ng/μl of the isolated genomic DNA for each mutant were used as a template in 15 μl reaction volume for the PCR amplification reaction of SLCMV CP gene using CP specific primers. To the reaction 200 μM of dNTP mix, 10 μM of each CP FP/RP pair, 1X reaction buffer, 1 mM $MgCl_2$ and 1U of *Taq* DNA polymerase enzyme (New England Biolabs) were added. The reactions were performed at 95 °C for 3 min of initial denaturation followed by 30 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s, 72 °C extension for 40 s, followed by 72 °C for 5 min of final extension.

2.4. Quantitative real-time PCR assay

Real time PCR-based quantification of SLCMV DNA-A was performed using a method described earlier (Sharma and Dasgupta, 2012). The primers for SLCMV CP were designed by using Primer Express™ software (Applied Biosystems). The amplification efficiency of SLCMV primers was determined through the calibration curve representing the C_t values on *Y*-axis vs log of input DNA on *X*-axis, prepared by using the tenfold dilution series of linearized SLCMV plasmid DNA, starting with 1 μg/μl of concentration. The percentage efficiency was calculated using the formula, efficiency (%) = [10 (−1/slope) − 1] × 100. The Real time PCR amplification was performed using 96-well MicroAmp optical plates (Applied Biosystems) in 25 μl of the reaction mix volume consisting of 1X SYBR Green I PCR master mix (Applied Biosystems), 1 μg to 1 pg template DNA and 200 nM of specific primer pair RT-SLCMV FR/RP (Supplementary Table 1). For quantification of SLCMV DNA in the inoculated plant tissue sample, 1 ng of prepared dilution of genomic DNA isolated from the inoculated *N. benthamiana* plants systemic leaf at 25 dpi and used as a template with the above mentioned primer pair in reaction. Cycling parameters for the amplification were the following: 1 cycle at 95 °C for 10 min (DNA polymerase activation) and 40 cycles, each consisting of 30 s at 95 °C (denaturation) and 1 min at 60 °C (annealing and extension). One cycle of melting curve with 95 °C for 1 min, 55 °C for 30 s and again 95 °C for 30 s was added at the end of the amplification reaction. Three technical replicates and no-template control (negative control) were used for each sample and the experiment was repeated thrice.