Journal of Virological Methods 170 (2010) 57–66

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocol

# A new strategy for generating geminivirus resistant plants using a DNA betasatellite/split barnase construct

Ali Pakniat-Jahromy[a], S.A. Akbar Behjatnia[a,*], Ian Barry Dry[b], Keramatollah Izadpanah[a], Mohammad Ali Rezaian[b,1]

[a] Plant Virology Research Center, College of Agriculture, Shiraz University, Shiraz, Iran
[b] CSIRO Plant Industry, PO Box 350, Glen Osmond, SA 5064, Australia

*Article history:*
Received 30 May 2010
Received in revised form 16 August 2010
Accepted 23 August 2010
Available online 8 September 2010

*Keywords:*
Geminivirus
Betasatellite
Tomato leaf curl virus
Cotton leaf curl Multan virus
Barnase
Resistant

A B S T R A C T

The betasatellite DNA associated with cotton leaf curl disease contains a single ORF, βC1, which is a pathogenicity determinant. Deletion of the βC1 ORF showed that it was not required for betasatellite replication in the presence of Tomato leaf curl virus-Australia (TLCV-Au). A series of betasatellite/split mutant barnase gene constructs, in which a direct repeat of the *Bacillus amyloliquefaciens* barnase gene flanked the betasatellite, were shown to replicate in tobacco in the presence of TLCV-Au. A betasatellite/split intact barnase gene construct, with the optimal direct repeat unit of the barnase gene, was introduced into *Nicotiana tabacum* plants. Approximately one third of the transgenic lines containing the betasatellite/split barnase gene constructs were shown to be completely resistant to the TLCV-Au infection. The betasatellite/split intact barnase gene cassette ensures that there is no expression of the barnase in the absence of TLCV-Au, but upon infection of the cell with the virus, release of the betasatellite/split barnase cassette as a replicating molecule resulting in the reconstitution and expression of an active barnase gene and the destruction of the infected cell. This system offers the potential to provide resistance in a variety of plant species against geminiviruses that support the replication of betasatellite.



## 1. Introduction

*Geminiviridae* is a large family of plant viruses that infect a broad range of plants and cause significant crop losses worldwide. They are characterized by twin icosahedral capsids and circular ssDNA genomes that replicate through dsDNA intermediates in infected cells (Hanley-Bowdoin et al., 1999), and utilize three replication modes: complementary-strand replication, rolling-circle replication and recombination-dependent replication (Erdmann et al., 2010). The *Geminiviridae* exhibit considerable diversity in terms of their genome structure, sequence, host range, tissue tropism and insect vectors. Based on these properties, geminiviruses have been classified into four genera, *Begomovirus*, *Curtovirus*, *Mastrevirus* and *Topocuvirus* (Rojas et al., 2005).

Begomoviruses are responsible for numerous diseases of economically important crops (Brown and Bird, 1992; Moriones and Navas-Castillo, 2000; Polston et al., 1994) including a severe disease of tomato in the northern parts of Australia caused by Tomato leaf curl virus-Australia (TLCV-Au; Dry et al., 1993). The genome of TLCV-Au contains a single DNA species of 2766 nucleotides that contains six open reading frames (ORFs), two on the virion-sense strand and four on the complementary-sense strand, interspersed by an intergenic region.

Circular ssDNA satellites have been isolated from plants infected with certain monopartite begomoviruses including Cotton leaf curl Multan virus (CLCuMV). These satellites, referred to as betasatellite (DNAβ), are approximately 1350 nt in size and depend on the replication-associated protein (Rep) of the helper virus for their replication. They encode only a single complementary-sense ORF, βC1, which is a pathogenicity determinant (Briddon et al., 2003; Stanley, 2004). It has been shown previously that deletion of the βC1 ORF of a betasatellite associated with Tomato yellow leaf curl China virus (TYLCCV) has little effect on betasatellite replication. Furthermore, a betasatellite molecule carrying foreign DNA sequences in place of the βC1 ORF were capable of inducing gene silencing (Xiaorong and Xueping, 2004) indicating that betasatellites can be used as gene delivery vectors.

Eini et al. (2009) have described sequence domains which regulate βC1 promoter activity of the CLCuMV betasatellite. They

* Corresponding author. Tel.: +98 711 2286154; fax: +98 711 2287165.
*E-mail addresses:* behjatni@shirazu.ac.ir, akbar_behjatnia@hotmail.com (S.A.A. Behjatnia).
[1] Present address: School of Agriculture, Food and Wine, The University of Adelaide, Waite Campus, SA 5064, Australia.

0166-0934/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2010.08.019

**Table 1**
List and sequences of primers used in this study.

| Primers | Size | Nucleotide position [a] | Sequences from 5' to 3' [b] | Underlined Restriction site |
|---|---|---|---|---|
| *Bar*1[f]- *Pst*I | 31 | 1-22 | CTGCAG**ATG**GCACAGGTTATCAACACGTTTG | Pst I |
| *Bar*1[f]-m | 31 | 1-22 | CTGCAG***TAA***GCACAGGTTATCAACACGTTTG | Pst I |
| *Bar*39[f] | 26 | 39-58 | TCTAGATCTTCAGACATATCATAAGC | Xba I |
| *Bar*138[r] | 27 | 118-138 | AAGCTTAGCGACGTCTGCAAGGTTCCC | Hind III |
| *Bar*187[r] | 29 | 165-187 | AAGCTTGTTTGCCTTCCCTGTTTGAGAAG | Hind III |
| *Bar*304[r] | 30 | 281-304 | AAGCTTGGTCCGTTGTTTTGTAAATCAGCC | Hind III |
| *Bar*333[r] | 28 | 312-333 | GGATCCTTATCTGATTTTTGTAAAGGTC | Bam HI |
| DNAβ218[c] | 25 | 200-218 | GGATCCTTCAATAAAAAGTTCACCG | Bam HI |
| DNAβ236[c] | 37 | 549-564 and 222-236 | CAAATAAGCAGAAATGCCATGGGTAAACGAAC CGTAC | |
| DNAβ549[v] | 37 | 222-236 and 549-564 | GTACGGTTCGTTTACCCATGGCATTTCTGCTT ATTTG | |
| DNAβ552[v] | 28 | 552-573 | CTGCAGTTCTGCTTATTTGATGGAAATG | Pst I |
| DNAβ1285[v] (β02T) | 25 | 1285-1309 | GGTACCACTACGCTACGCAGCAGCC | Kpn I |
| DNAβ1290[c] (β01T) | 25 | 1266-1290 | GGTACCTACCCTCCCAGGGGTACAC | Kpn I |
| pBin19-NPTII[f] | 26 | 7969-7994 | AGATCCCGTGGGCGAAGAACTCCAGC | |
| pBin19-NPTII[r] | 29 | 8924-8952 | GGATCGTTTCGCATGATTGAACAAGATGG | |
| TLCV-Au748[c] | 21 | 728-748 | GGACCAAGAAGAACATAACTG | |
| TLCV-Au2025[v] | 24 | 2025-2048 | CTCGAGTTCTTCTGGAACTCGATC | |

[a] Nucleotide position of TLCV-Au, CLCuMV betasatellite DNA, pBin19 and *Bacillus amyloliquefaciens* gene for barnase as in the GenBank database under accession numbers S53251, AJ298903, XXU09365 and X12871, respectively.
[b] Letters in the bold indicate extra nucleotide residues inserted into the primer sequence which did not exist in the original sequence in the GenBank database. X12871 excludes the initiation codon (ATG) of the barnase gene. Letters in italics indicate the mutated residues. Underlined residues are natural occurring restriction sites and double underlined residues are artificially insertion restriction sites in primers.
[c] Complementary-sense strand primers.
[v] Virion-sense strand primers.
[r] Non-viral negative-sense (reverse) strand primers.
[f] Non-viral positive-sense (forward) strand primers.

identified a 68 nt region upstream of the start codon of the βC1 ORF which is important for βC1 transcription. This region contains a G box (CACGTG) which regulates βC1 promoter activity (Eini et al., 2009). The promoter activity of CLCuMV betasatellite indicated that this DNA could be used as a vector for the expression of foreign genes in plants.

To date, the control of infection caused by begomoviruses has relied heavily on control of the whitefly insect vector through the application of insecticides. The excessive use of agrochemicals over the past decades, has contributed to an exponential increase in whitefly populations and incidence of begomoviruses transmitted by this vector (Morales, 2001). Thus, the generation of begomovirus resistant germplasm either by conventional plant breeding or transgenic strategies is of major importance. Conventional plant breeding has led to some improvements in productivity of a limited number of crops including cassava, bean and tomato but in the main this, at least in the case of tomato, has been the result of increased tolerance rather than improved resistance (Morales, 2001). For the last 10 years a range of genetic engineering strategies have been evaluated for the development of crops resistant to geminiviruses which have focused mainly on pathogen-derived resistance strategies including the expression of mutant or truncated viral proteins that interfere with virus infection, or transcription of viral RNA sequences that silence the expression of virus genes. Recently, however, alternatives to pathogen-derived resistance have been investigated. These include the use of geminivirus-inducible toxic proteins to kill infected cells, and the expression of DNA binding proteins, peptide aptamers or GroEL homologues that either disrupt geminivirus infections or lessen their harmful effects (Shepherd et al., 2009).

In this study, a resistance strategy is presented which is based on geminivirus-induced host cell death that is activated by expression of an integrated barnase gene. The basis of this strategy is a betasatellite/split barnase expression cassette, which is constructed in such a way that there is no expression of the barnase in the host cells until infection by the geminivirus, at which time the betasatellite/split barnase construct is mobilized as a replicating circular molecule resulting in the reconstitution and expression of an active barnase gene and the destruction of the infected cell.

## 2. Materials and methods

### 2.1. TLCV-Au and betasatellite clones

A full-length KpnI monomeric CLCuMV betasatellite clone in pBS SK+ (pBS-β), a KpnI dimeric betasatellite clone in the same vector (pBS-2β) and the infectious constructs TLCV-Au XbaI head-to-tail dimer in the pBin19 (pBin-2TLCV) and betasatellite KpnI/SnaBI 1.2 mer in the pBin20 (pBin-1.2β) were described (Dry et al., 1993; Rigden et al., 1996; Saeed et al., 2005). A mutant version of the latter construct in which the βC1 gene was deleted (pBin-1.2βΔC1) was generated using thermal cycled fusion mutagenesis (Kahn et al., 1990). Two fragments flanking the 312-bp βC1 region to be deleted were first amplified from pBS-β by PCR in separate reactions using primer pairs, DNAβ1290[c]/DNAβ549[v] and DNAβ236[c]/DNAβ1285[v] (Table 1), respectively. Aliquots of the two PCR reactions were used