An iterated sequence in the genome of *Banana bunchy top virus* is essential for efficient replication

Virginia A. Herrera-Valencia,† Benjamin Dugdale, Robert M. Harding and James L. Dale

Science Research Centre, Queensland University of Technology, GPO Box 2434, Brisbane, QLD 4001, Australia

*Banana bunchy top virus* (BBTV) has a multi-component genome of circular, single-stranded DNA. BBTV replicates via a rolling-circle mechanism, probably involving sequence-specific interaction of the replication initiation protein (Rep) with iterated sequences (iterons) within the viral genome. Three putative iterons (designated F1, F2 and R), with the sequence GGGAC, have been identified in the intergenic region of each BBTV component. To investigate their role in replication, each of the iterons was mutated, singularly and in tandem, in a BBTV DNA-N 1·1 mer and the ability of these molecules to be replicated by the BBTV ‘master’ Rep was evaluated in banana cells using transient biolistic assays. All iteron mutants were replicated less efficiently than the native DNA-N. Mutation of the F1 and R iterons caused a 42 and 62 % reduction in DNA-N replication, respectively, whereas mutation of the F2 and combined F1F2 iteron virtually abolished DNA-N replication.

*Banana bunchy top virus* (BBTV) is the type member of the genus *Babuvirus* in the family *Nanoviridae* and has a genome comprising at least six circular, single-stranded DNA components (referred to as DNA-R, -S, -C, -M, -N and -U3; Vetten et al., 2005), each of approximately 1 kb (Burns et al., 1995). Based on similarities among nanovirus DNAs and those of the geminiviruses, it has generally been accepted that BBTV replication occurs by a rolling-circle-type mechanism (Stenger et al., 1991). In geminiviruses, iterated DNA sequences (iterons) play an important role in the replication process by acting as recognition sites for sequence-specific binding of their cognate replication initiation proteins (Reps). Mutation of these sites can affect Rep binding negatively *in vitro* and replication *in vivo* (Chatterji et al., 2000; Choi & Stenger, 1996; Fontes et al., 1994a, b; Orozco et al., 1998). Although putative iteron sequences have been identified in the non-coding regions of some nanovirus DNAs, including *Faba bean necrotic yellows virus* (FBNYV), *Milk vetch dwarf virus* (MDV) and *Subterranean clover stunt virus* (SCSV) (Timchenko et al., 2000), their exact role in replication has not been demonstrated experimentally.

Analysis of the intergenic regions of BBTV DNA-R, -S, -C, -M, -N and -U3 (Horser, 2000) has identified a putative iteron sequence (GGGAC) occurring as a tandem repeat (designated iterons F1 and F2, respectively) on the virion-sense strand, 5’ of the stem–loop, and as a single iteron (designated iteron R) on the complementary strand, 5’ of the stem–loop. The direct repeat iterons, F1 and F2, were located 2 nt 3’ of the stem–loop in DNA-R, -S, -C, -M and -N, whereas in DNA-U3, they commenced 1 nt 3’ of the stem–loop. However, the location of iteron R varied, being 10 (DNA-N), 19 (DNA-R, -S, -C and -M) and 90 (DNA-U3) nt upstream of the 5’ base of the stem–loop.

We investigated the role of the F1, F2 and R iterons in BBTV replication by assessing the ability of the BBTV ‘master’ Rep (M-Rep) (encoded by DNA-R) to replicate native and iteron mutants of DNA-N in banana embryogenic cells. DNA-N was selected as a representative genome component because it encodes a putative nuclear-shuttle protein that is not intrinsic to the replication process. In all cases, the native iteron sequence in the BBTV DNA-N backbone was mutated to that of the unique restriction site *XbaI* (TCTAGA) (Fig. 1). The system used to assess replication involved the use of greater-than-unit-length BBTV clones (1·1mers), which incorporated two stem–loops. In the presence of BBTV M-Rep, the 1·1 mer acts as a template for rolling-circle replication, is excised at the conserved nonanucleotide loop sequence and is subsequently recircularized by the nucleotidyl-transfer activity of Rep and converted into a double-stranded DNA transcriptionally active molecule. In addition to DNA-R, a 1·1 mer of BBTV DNA-C (encoding the cell-cycle link protein Clink) (Aronson et al., 2000) was also co-delivered, as this protein has been shown to enhance replication (Horser et al., 2001). The construction of 1·1mers of BBTV DNA-R, -C and -N has been described previously (Horser et al., 2001).
DNA-N 1·1mers containing mutations in F1 (pMutF1), F2 (pMutF2), R (pMutR) or the tandem F1F2 iterons (pMutF1F2) were generated using either an overlapping PCR-based approach or a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturers’ instructions. The integrity of all constructs was confirmed by sequencing. Microprojectile bombardment of banana (Musa spp. cv. ‘Lady finger’) embryogenic cell suspensions was carried out as described previously (Dugdale et al., 1998). Plasmids pMutR, pMutF1, pMutF2 and pMutF1F2 were independently co-bombarded (in equimolar amounts) in combination with 1·1mers of both DNA-R and DNA-C. The native DNA-N 1·1mer (pWtN) was similarly co-bombarded as a positive control for replication. In total, ten replicate bombardments were performed per plasmid combination in order to account for variation between independent transformation events and individual treatments. At 4 days post-bombardment, 60 μg total nucleic acid was extracted (Stewart & Via, 1993), electrophoresed through agarose, blotted onto nylon (Sambrook et al., 1989) and hybridized with a digoxigenin (DIG)-labelled probe specific for the DNA-N ORF (nt 281–742). Pre-hybridization, hybridization (42°C) and chemiluminescent-detection assays were done according to the DIG (Roche) protocol. In some cases, nylon membranes were stripped (according to the manufacturer’s instructions) and rehybridized with a DIG-labelled probe specific for the DNA-R ORF (nt 129–989) to ensure even loading of the DNA-R 1·1mer. To assess replication, densitometry readings were based on the supercoiled, replicative episomal forms of DNA-N and were analysed using TotalLab v1.11 and Phoretix software (Nonlinear Dynamics). On each autoradiograph, densitometry readings were initially made relative to a lane containing a DNA extract from a BBTV-infected plant. The same DNA extract was used for each blot. Differences in the mean densitometry readings between the mutated and native DNA-N 1·1mers were subsequently analysed using one-way analysis of variance (ANOVA; spss 13.0 for Windows) and significant differences between means were identified with a least significant difference post-hoc test using a significance level of 0·05.

The effect of mutating putative iterons F1 and R on the replication of BBTV DNA-N is shown in Fig. 2(a). In comparison with the native DNA-N control plasmid (pWtN), mutation of the F1 and R iterons reduced progeny replication. This was confirmed by Southern blot analysis using a DNA-N-specific probe. The different BBTV replicative intermediates [open circular (oc), supercoiled (sc) and single-stranded (ss)] are indicated, with densitometry readings based only on the sc replicative intermediate. Four representative nucleic acid extracts of mutations pMutF1 and pMutR (a) and five of pMutF2 and pMutF1F2 (b) are shown in comparison with pWtN. The lower panels are loading controls and show the ethidium bromide-stained DNA extracts in agarose gels prior to blotting. pN, BBTV DNA-N plasmid control; I, nucleic acid extracted from a BBTV-infected plant; U, nucleic acid extracted from non-bombarded banana embryogenic cells.
of the 5\textsuperscript{th} interactions between Asn10 in Rep and the third base pair family replication was mapped to mutation of F2 virtually abolished replication. Differential F1 decreased replication by approximately 42\%, whereas although the F1 and F2 iterons were identical, a mutation in most important for replication. However, the arrange-
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These results indicate that sequence-specific recognition of the iterons by BBTV M-Rep is required for optimal replication of DNA-N, as mutagenesis of these sequences, individually and in tandem, reduced replication significantly in transient biolistic assays. However, the degree to which replication is affected appears to depend on the individual iteron, with the F2 iteron appearing to be the most important for replication.

Although the F1 and F2 iterons were identical, a mutation in F1 decreased replication by approximately 42\%, whereas mutation of F2 virtually abolished replication. Differential contributions of the 5′- and 3′-proximal iterons in tandem repeats have been reported in geminiviruses. For example, with the begomovirus Tomato golden mosaic virus (TGMV), the 3′ repeat contributes more to replication specificity and probably functions as an essential cis-acting element for replication, whilst the 5′ repeat possibly enhances replication (Fontes et al., 1994a). In contrast, specificity in Tomato leaf curl New Delhi virus replication was mapped to interactions between Asn10 in Rep and the third base pair of the 5′ iteron repeat, GGTGTG (Chatterji et al., 1999). Generally, there appears to be no strict set of guidelines governing the number, position or sequence of iterons in the family Geminiviridae. The geminivirus iterative sequences do not seem to follow any phylogenetic relationship, with similar iterons found in viruses from the New and Old Worlds (Arguëllo-Astorga & Ruiz-Medrano, 2001). Furthermore, the DNA β satellite molecules associated with some geminiviruses are replicated efficiently in the absence of any obvious iterons (Saunders et al., 2000). In relation to the geminiviruses, BBTV appears to be more similar to TGMV in that the 3′ direct repeat, here the F2 iteron, is essential for replication. However, the arrangement of the tandem iterons in TGMV differs greatly from that of BBTV in that they are located 5′ of the origin of replication (Fontes et al., 1994a). Like the F1 iteron, mutation in the BBTV R iteron caused a significant reduction in the accumulation of DNA-N replicative forms. These results suggest that both the F1 and R sequences, whilst not essential to the replication process, play an important role in Rep recognition and may function as enhancers of the BBTV replication process.

BBTV (the sole member of genus Babuvirus) differs from members of the genus Nanovirus in several ways, including host range, number of genomic components, aphid vectors and serology (Vetten et al., 2005). These differences are also reflected at the iteron level: BBTV has fewer iterons than the nanoviruses (three compared with six for FBNYV and MDV and seven for SCSV) and their sequence and arrangement also differ (Timchenko et al., 1999, 2000). In contrast, all three nanoviruses have iteron-like sequences that are very similar in sequence and arrangement, and their M-Reps are able to support interspecies cross-replication of heterologous non-Rep-encoding DNAs, although efficiency of cross-replication appears to correlate with the relatedness of the two species being tested (Timchenko et al., 2000). To date, the exact role of iterons in nanovirus replication has yet to be investigated. Interestingly, iterons in the non-essential BBTV Rep-encoding components S1, S2, S3 and Y1 (Bell et al., 2002; Horser et al., 2001), and the analogous components associated with FBNYV, MDV and SCSV, share a similar structural arrangement to the mastreviruses, suggesting that they may have a common origin.

In Porcine circovirus type 1 (PCV1), a member of the family Circoviridae and reportedly the smallest autonomously replicating mammalian virus, an iterative sequence (CGGGAG) is repeated four times immediately 3′ of the stem–loop (Mankertz et al., 1997; Steinfeldt et al., 2001). PCV1 encodes two replication proteins, the full-length Rep and a spliced isoform (Rep′). Interestingly, the minimal binding-site requirements of the two proteins differ and their binding action results in complexes of different stoichiometry.

In summary, we have shown that the three BBTV iterons are involved in virus replication, but their roles in this process differ, as an alteration in their sequence negatively affects replication to varying degrees. Taken together, in vivo results suggest that interaction of Rep with the F2 iteron is an essential step in DNA replication, whilst the F1 and R iterons may function in synergy to enhance virus replication.

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References


