Virusdisease. 2014 Dec; 25(4): 460–466.
Published online 2014 Nov 28. doi: 10.1007/s13337-014-0241-9
PMCID: PMC4262304

Population structure of *Banana bract mosaic virus* reveals recombination and negative selection in the helper component protease (HC-Pro) gene

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Introduction

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Banana bract mosaic virus (BBrMV) is a serious constraint in the production of banana and plantain in India [18] and the Philippines [11]. BBrMV, a member of the genus *Potyvirus*, family *Potyviridae*, is the causal agent of the bract mosaic disease (BBrMD) and have flexuous filamentous particles of approximately 725-nm long which encapsidate a monopartite ssRNA genome of 9,711 bp in length. The virus contains a major open reading frame (ORF) encoding a large polyprotein of 3,125 amino acids (aa) which is subsequently cleaved into at least ten mature proteins by three viral proteinases [2, 7]. Furthermore, an overlapping PIPO exists in the +2 reading frame within the protein 3 (P3 protein) encoding region [4]. BBrMV is transmitted non-persistently by several aphid species viz. *Pentalonia nigronervosa*, *Rhopalosiphum maidis*, and *Aphis gossypii* [11]. The main host of BBrMV is *Musa* and recently, BBrMV is reported in small cardamom (*Elettaria cardamomum*) in India [20] and also infects flowering ginger, *Alpinia purpurata* (Vieill.) K. Schum in Hawaii [25].

The recent increasing incidence of BBrMD in banana fields in south India suggested possible accumulation of genetic variations in BBrMV. Understanding the genetic variability and recombination of BBrMV populations is an important prerequisite for designing efficient diagnosis, management and long-term control of the disease. Recently, based on coat protein (CP) gene sequence analysis, we reported that BBrMV isolates of India had a divergence of 0–21 % at nucleotide (nt) and 0–20 % at aa level, respectively. There would be a risk of loss-of resistance if CP mediated virus resistant transgenic banana plants due to the presence of new variants [3]. Potyviral helper component-proteinase (HC-Pro) is a multifunctional protein essential in the viral infection cycle, such as aphid transmission, genome amplification, cell-to-cell and long distance movement, suppression of RNA silencing defense responses, synergism between co-infecting viruses and symptom development [15]. There are several reports on genetic structure of potyvirus population and these reports showed that virus population have been shaped by selection, founder effects and genetic recombination [14, 22, 26]. To our knowledge there are no reports on diversity and recombination analysis of the HC-Pro gene of BBrMV population. Therefore, the present investigation was undertaken to characterize the genetic structure of BBrMV isolates occurring in India.

Materials and methods

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Plant materials

Banana leaves or bracts showing symptoms of bract mosaic disease were collected from different variety and regions (Tamil Nadu, Kerala, Karnataka and Andhra Pradesh states) of India during 2008–2014 (Table 1 in Supplementary material). A total of 22 isolates were collected from different locations were used for analysis. Host plants of some of the isolates were maintained in insect free glass house at National Research Centre for Banana (NRCB), Trichy, Tamil Nadu.

RNA extraction, RT-PCR,cloning and sequencing

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. Viral RNA was reverse transcribed in a PCR machine (Mastercycler gradient; Eppendorf, Germany) using a Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) using oligo (dT) as primer by following the manufacturer’s protocol. The HC-Pro gene from 22 Indian BBrMV isolates (Table 1 in

Supplementary material) was amplified by PCR with forward primer (RSR10FP: 5′ATAGGATCCTCTGGAACGGAGTCAACC3′) and reverse primer (RSR10RP: 5′TTCATGTTTCATCCCAAGCAGAG 3′). RT-PCR products were separated by electrophoresis in 1.0 % agarose gels and purified using the Genelute Gel Extraction Kit (Sigma, USA). Purified PCR products were cloned onto the pTZ57R/T vector (Thermo Fisher Scientific, USA), and transformed into competent *E. coli* DH5α cells as per manufacturer's instructions. Selected recombinant plasmids were sequenced by Eurofins Genomic India Pvt Ltd., Bangalore using universal M13 forward and reverse primers. A minimum of three independent clones were sequenced in both directions to obtain the consensus sequence for each region studied. Sequence data for HC-Pro gene sequences obtained in this study were deposited into GenBank with accession numbers (Table 1 in Supplementary material).

Sequence analysis

Two complete genome sequences of BBrMV isolates from India and Philippines were retrieved from NCBI and used for analysis. Alignments of 24 nucleotide sequences were done using CLUSTALW [23]. Sequence identity matrix and sequence difference count matrix were calculated using Bioedit sequence alignment editor version 5.09.04 [8]. Phylogenetic analysis was performed using Maximum-likelihood phylogenetic tree constructed in the MEGA 5.0 software [21]. DnaSP 5.10 [10] was used to estimate Tajima's D, Fu and Li's D* and F* statistical tests for each BBrMV protein coding sequence. The pairwise nt and aa sequence identity scores were represented as color-coded blocks using SDT v.1 software [13]. Recombination sites in the aligned sequences were identified by implementing the RDP4 Beta 4.24 software [12]. To determine the site-specific selection pressures acting on HC-Pro gene, four different codon-based maximum-likelihood methods implemented on Datamonkey server (http://www.datamonkey.org) [5] were used.

Results

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Sequence identity, sequence difference count matrix and phylogeny analysis of HC-Pro gene

From the present investigation, 23 HC-Pro gene sequences of BBrMV isolates originating from India and one sequence from the Philippines (PHI) were analyzed. We found that the sequence length of the HC-Pro gene of all the BBrMV isolates was 1,371 nt and encoding a protein of 457 aa having a deduced MW of 51.88 kDa and p*I* of 8.39. The pairwise sequence identity of HC-Pro genes among all 24 BBrMV isolates ranged from 92 to 100 % both at the nt and aa level (Fig. 1). When the Indian isolates compared with the PHI isolate, the nt and aa sequence identities ranged from 92 to 95 and 96 to 98 % respectively. Among Indian isolates, TN32 shared 94–96 and 92–95 % identity in the nt and aa sequences between them. Sequence difference count matrix varied from 4 to 106 nt among the BBrMV isolates. A maximum of 106 nt difference was noticed in the TN32 and PHI isolate of BBrMV while only 4 nt differences was observed with the isolates of TN7 and TN8. The aa sequence difference count matrix ranged from 1 to 37 aa. A maximum of 37 aa difference was noticed in isolates TN24 and TN32 while only one aa difference was observed in five isolates namely TN8, TN22, TN31, KAR3 and AP7 (Table 2 in Supplementary material).

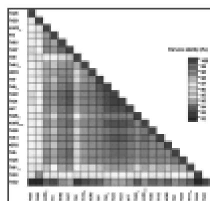

Fig. 1
Graphical representation of pair wise and amino acid identity (with percentage identity scale) of 24 BBrMV isolates. For the detailed of isolates, refer Table 1 in Supplementary material

Phylogenetic trees were constructed excluding four potential recombinants (TN16, TN28, TN32 and PHI) by the maximum likelihood method based on nt sequence alignments are shown (Fig. 2). The isolates TN9, TN15 and TN24 grouped in one cluster (Group I) and the remaining other isolates formed another cluster (Group II). The phylogenetic tree generated based on alignment of deduced aa sequences showed no significant clustering into groups (data not shown). Schematically, the HC-Pro protein can be divided into three regions: an N- terminal

region associated with aphid transmission, a C-terminal region associated with proteinase and RNA silencing suppressors (RSS) activity [24], and a central region implicated in many functions, including RSS activity [15]. All BBrMV isolates except isolate TN27 have a unique RISC motif which, corresponds to aa 51–54 and a PSA motif corresponding to aa 309–311 in their HC-Pro gene instead of KITC and PTK motifs that have been reported for the aphid transmissibility of other potyviruses [2, 7] and the significance of these changes in BBrMV is not known. However, in the central region of HC-Pro, several motifs are highly conserved in BBrMV isolates which are also present in all other potyviruses, including the FRNK box at aa positions 180–183 in HC-Pro, which is associated with symptom severity and suppression of gene silencing. In addition, IGR motif (aa 249–251) and CCC motif (aa 291–293) in the central part of HC-Pro reported to be involved in virus movement and amplification also found conserved in all BBrMV isolates used in this study (Fig. 3).

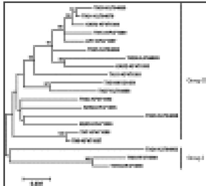

Fig. 2
Phylogenetic analysis of the HC-Pro gene of nucleotide sequences of non recombinant BBrMV isolates from India using Maximum Likelihood method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used **...**

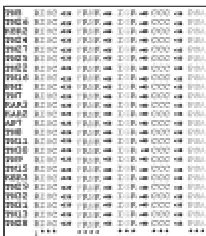

Fig. 3
Alignment showing conserved motif's of HC-Pro gene of BBrMV sequences. * indicates identical sequence

In silico recombination and selection pressure analysis

Recombination has been reported as one of the main forces driving plant virus evolution [6, 17], and this phenomenon may have also contributed to the evolution of HC-Pro proteins and helped in adaptation of members of the family of *Potyviridae* with a wide host range. In silico recombination analysis was done for HC-Pro gene sequences of 24 BBrMV isolates using eight recombination detection methods. Four potential recombinants were detected from 24 analyzed isolates (Table 1). Six recombination break points were detected in the isolate TN32 followed by five in TN16, three in TN28 and one in the PHI isolate. All the six recombination breakpoints of recombinant TN32 were detected from N-terminal region of HC-Pro gene whereas, five recombination breakpoints detected in the recombinant isolate TN16 were located in C-terminal region. Of the four recombinants identified, two (TN32 and TN16) were detected by five to eight programs with significant P values always lower than 10^{-5}, adding to the validity of the results. Indian isolate TN11 appeared as parental isolate for a recombinant PHI isolate and PHI isolate as parental for recombinant TN16. This suggests a possible derivation of PHI isolate by genetic exchange involving Indian isolates.

Table 1
Recombination events detected in the HC-Pro gene of *Banana bract mosaic virus* (BBrMV) isolates using RDP methods showing the parental and recombinant sequences

To determine the gene and site-specific selection pressures acting on BBrMV HC-Pro, four different codon-based maximum-likelihood algorithms SLAC, FEL, IFEL, and REL within the HyPhy software package as

implemented in Datamonkey server with significance levels set at P = 0.01 for the first three programs and Bayes factor = 50 for REL, respectively, were used to estimate the value of dN/dS at each codon site (Table 2). Total number of codons found under negative selection in HC-Pro gene was 21, 91, 04, 136 and 140 detected by the methods SLAC, FEL, IFEL, REL, and FUBAR respectively. Although, most of the codons were under negative or neutral selection, the codon at position 74 in the N-terminal region of HC-Pro was found under positive selection which was detected by FEL, IFEL, REL and FUBAR methods. Additionally, codon 79 was identified under positive selection only by FEL method. To evaluate the importance of natural selection to shape the population structure of BBrMV, the Tajima's D and Fu and Li's D*and F* statistics were used to test the mutation neutrality hypothesis using the DnaSP 5.10 program. These three statistics were significantly negative ($P > 0.05$), suggesting a strong negative or purifying selection indicating the occurrence of recent BBrMV population expansions.

Table 2
Codon positions of HC-Pro coding region of BBrMV isolates significantly affected by selection by five methods implemented by Data Monkey Software

Discussion

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Understanding the driving forces acting on virus evolution and the evolutionary mechanisms is a central aspect of evolutionary biology and would enable the development of efficient strategies for managing viral diseases. Studies of the genetic variations at regional or global scales have been reported in several potyviruses [14, 22, 26]. These analyses allow a higher level of detail that may be particularly informative. In this study, we have collected the BBrMV isolates from India and analyzed their genetic variability using the HC-Pro gene sequences. The pair wise sequence identity of HC-Pro gene of all 24 BBrMV isolates ranged from 92 to 100 % both at the nt and aa level. Sequence similarity at nt and aa of HC-Pro gene suggests that BBrMV population in India represent a similar molecular architecture and the virus might be under host adaptive selection. Our previous study showed that CP gene sequence analysis grouped the isolate TN16 as a distinct variant whereas in this study the HC-Pro gene of the same isolate showed 94–97 % similarity with all the other isolates. Bagyalakshmi et al. [1] reported a high level of diversity of 3–28 % at nt and 1–17 % at aa level in the HC-Pro gene of *Sugarcane streak mosaic virus* (SCSMV) and this wider variation was due to the use of diverse virus isolates from the world sugarcane germplasm collection. The HC-Pro gene sequences of 25 *Potato virus Y* (PVY) isolates characterized were 81.6–99.7 and 89.4–100 % identical at nt and aa levels, respectively [22]. Zhang et al. [26] showed that the nt and aa identities of *Tobacco vein banding mosaic virus* (TVBMV) HC-Pro genes were 88.9–99.9 and 96.4–100 % respectively.

The phylogenetic tree generated based on alignment of deduced aa sequences showed no significant clustering into groups. Further, it was observed that the sequences derived from isolates of different regions and banana genotypes were clustered as together. Phylogenetic analysis did not indicate a clear relationship between genetic variability of BBrMV isolates and their geographical origin. Seo et al. [19] reported that the phylogenetic trees were inconsistent and did not support geographic clustering of the *Soybean mosaic virus* (SMV) isolates. The phylogenetic inconsistency and clustering pattern in BBrMV isolates might be due to the movement of infected suckers across the districts of Tamil Nadu and other states in south India. Since BBrMV is transmitted efficiently through suckers and also by aphid transmission, the possibility of spread of same strains to other varieties cannot be ruled out. The significance of grouping of BBrMV isolates would become clear when complete genome of more number of isolates are examined.

In our study four recombinants were detected from 24 analyzed isolates. Similar to our study, potential recombinants were detected in HC-Pro gene of PVY [22], SCSMV [1] and *Turnip mosaic virus* (TuMV) [14]. Zhang et al. [26] showed that the hot spot of recombination sites were located at 5′ and 3′ termini of HC-Pro gene of TVBMV. Interestingly we found that the involvement of potential recombinant as major parent for one or the other recombination events detected in this study. Since, banana is a clonally propagated plant; we presume that these parental isolates would have co-existed in the same banana clone through aphid transmission leading to the

occurrence of recombinants. To our knowledge, this study is the first to report evidence of recombination in HC-Pro gene of BBrMV isolates. Based on the results of recombination analysis, we speculate that the BBrMV would have occurred first in India and later it spread to the Philippines. Rodoni et al. [16] have also proposed that BBrMV persisted in India for a longer period of time and the movement of BBrMV into Philippines occurred as one event. Observations made while collecting samples suggested that locally cultivated traditional banana cultivars namely Poovan, Nendran, Neypoovan were more severely affected than a widely adopted tissue culture friendly banana cv. Grand Naine (data not shown). This may be due to the fact that the traditional cultivars have been propagated vegetatively for many years and the virus has accumulated to a higher concentration whereas, cv. Grand Naine is mainly propagated mainly through virus free certified mother plants in India and new infection with BBrMV would have arose from aphid transmission from infected traditional cultivars.

Different functional motifs of the HC-Pro gene of BBrMV isolates were found conserved. The codon based selection analysis revealed that most of the codons were under negative or neutral selection, the codon at position 74 in the N-terminal region of HC-Pro was found under positive selection. The HC-Pro encoding regions in PVY isolates analyzed were mainly under purifying selection but seven aa were found to be under positive selection [22]. The aa of HC-Pro that were under positive selection were located in domain 1, which is responsible for aphid transmission, RNA binding, and genome amplification [16]. Though SCSMV isolates had high nt diversity, none of codons of HC-Pro gene of the isolates were reported to be under positive selection [1]. The significance of this positive selection in BBrMV isolates on the codon at position 74 and 79 needs further investigation. The three neutrality test statistics were significantly negative ($P > 0.05$), suggesting a strong negative or purifying selection indicating the occurrence of recent BBrMV population expansion. The recombination, together with strong negative selection, enhances the speed of elimination of deleterious mutations in the HC-Pro gene of potyviruses [1, 9]. Finally, this study showed the level of genetic variation, phylogenetic relationship, recombination and operation of selection among the different isolates of BBrMV with respect to HC-Pro gene. HC-Pro genes are subjected to purifying selection, which may play an important role in the host vector interaction and in shaping their evolution.

Electronic supplementary material

Go to:

Supplementary material 1 (DOCX 22 kb)(23K, docx)

Acknowledgments

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This is a part of the Ph.D work of the first author and authors are grateful to the Director, NRCB, Trichy, for encouraging and providing necessary facilities to carry out the work.

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