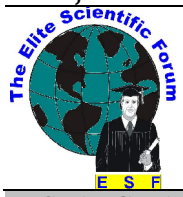
Pak. j. life soc. Sci. (2012), 10(2): 91-97 E-ISSN: 2221-7630;P-ISSN: 1727-4915



Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Use of Rolling Circle Amplification for the Identification of Unknown Components of Banana Bunchy top Virus from Pakistan

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ARTICLE INFO
Received: May 08, 2012
Accepted: Jul 16, 2012
Online: Aug 11, 2012

Keywords
Babuvirus
Banana
Nanoviruses
Rolling circle amplification
Restriction fragment length polymorphism

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ABSTRACT

In the present study rolling circle amplification (RCA) was used to characterize the unknown components of *Banana bunchy top virus* (BBTV) from the samples collected from Tando Jam Sindh, Pakistan. The RCA product was treated with several types of restriction endonuleases (*ClaI*, *KpnI*, *EcoRV*, *BglII*, *MboI*, *TruI*, *HpaII* and *HaeIII*) for Restriction Fragment Length Polymorphism (*RFLP*) analysis. This restriction pattern was further confirmed by Southern blot analysis and hybridized to all respective fragments. On the basis of results, it has been suggested that five components of BBTV are prevalent in Pakistani isolate of BBTV. Furthermore, it was found that the cloned components were highly homologous to South Pacific group of BBTV reported earlier in Pakistan. So, the results of present study confirmed that similar to geminiviruses, the RCA technology can also be used for characterization of nanoviruses. It can also be suggested that the technique is of great value to nanovirus research since the components that make up this group are still being discovered.

INTRODUCTION

Banana bunchy top disease (BBTD) is a major threat to banana cultivation throughout the world (Dale et al., 1998). This disease was first reported in Fiji in 1889 and then introduced to Australia, South Pacific and Africa while Central and South America and Caribbean are free from this disease (Dale, 1987). In 1989, this disease was first reported from Pakistan and caused a huge loss to banana production (Khalid et al., 1993). The initial symptoms of BBTD include dark green streaks on the lower portion of midrib, vein clearing and the dwarf bushy appearance. This disease is caused by *Banana bunchy top virus* (BBTV); belong to the family *Nanoviridae* that is transmitted by aphid *Pentalonia nigronervosa* (Allen, 1978). The family *Nanoviridae* is divided into two genera; Nanovirus and Babuvirus. The genus Nanovirus include *Faba bean necrotic yellows virus* (FBNYV) (Katul et al., 1998), *Subterranean clover stunt virus* (SCSV) (Chu and Helms, 1988) and *Milk vetch dwarf virus* (MDV) (Sano et al., 1998). Recently, another nanovirus called *Faba bean necrotic stunt virus* (FBNSV) is reported from Ethiopia (Grigoras et al., 2009), while the genus *Cocunut foliar decay virus* (CFDV) is a possible member of this genus (Rohde et al., 1990). The genus Babuvirus includes *Banana bunchy top virus* (BBTV) (Harding et al., 1991) and *Abaca bunchy top virus* (ABTV) (Sharman et al., 2008).

Nanoviruses are ssDNA viruses with a multi-component genome, consisting of 6 to 8 circular ssDNA molecules of approximately 1100bp. BBTV also have multi-component genome, consisting of 6 to 8 circular ssDNA molecules of approximately 1000 to 1100bp each. The components of BBTV are known as DNA-R (MRep), DNA-C (Clink), DNA-S (Coat protein), DNA-M (MP) and DNA-N (NSP). DNA-R component is called master Rep (MRep) protein which helps in the trans-replication of all other components of BBTV but not in replication of satellite Rep encoding components (Horser et al., 2001). These Rep encoding components are believed to be parasitic, satellite

molecules which are not required by the virus (Horser et al., 2001) In addition to this, DNA U1, U2, U3 encode the proteins of unknown functions (Timchenko et al., 2006; Vetten et al., 2005). Each DNA component contains the stem loop common region (CR-SL) consists of 69 nucleotides which is 62% identical between all components of BBTV and the major common region (M-CR) (Burns et al., 1995). The major common region incorporates 66-92 nucleotides which is 76% identical between components (Burns et al., 1995). Each of six components also contains one open reading frame (ORF) in the virion sense (Burns et al., 1995; Xie and Hu, 1995) with a potential TATA box.

Rolling circle amplification (RCA) is a novel technique used for the amplification of circular DNA such as plasmids and viruses having circular genome including geminiviruses and nanoviruses (Johne et al., 2009). Though this technique has been widely used for the characterization of geminiviruses but its use for the characterization of nanoviruses has not been yet reported. In RCA, Phi 29 DNA polymerase show high affinity to viral molecules. This technique is useful for microorganisms for which no sequence information is availableIt can be applied directly to sequencing, restriction analysis and cloning of whole genome. Therefore, this technique can be used for differentiation and detection of known geminivirus species by restriction analysis (Haible et al., 2006). Until now, this technique has been applied to six different virus families but this technique is not used for ssDNA viruses such as nanoviruses uptil now. We have already cloned five components of BBTV (Amin et al., 2008). In the present study, we used this technique to identify the unknwon components in BBTV.

MATERIALS AND METHODS

Plant Material

Seven infected banana plants were located from various areas of Tando Jam Sindh, Pakistan. Young leaves of infected plants were collected and kept in plastic bags labeled with permanent marker and stored at -80^{o}C until utilized. Total nucleic acids were extracted from the frozen leaves by CTAB method (Doyle and Doyle, 1990).

Rolling circle amplification (RCA)

Reaction mixture (20µl) comprised of 100 to 200ng of template DNA, 50µl of random hexamer primers, 1mM dNTPs, 2µl of 10X Φ29 DNA polymerase reaction buffer and enzyme mixture comprised of Φ 29 polymerase and pyrophosphatase in 1X reaction buffer was prepared and incubated at 94^{o}C for 3min to denature dsDNA. The mixture was allowed to cool down to room temperature and mixed with 5-7units of Φ 29 DNA polymerase and 0.02 units of pyrophosphatase and incubated at 30°C for 20 hrs. After this, the reaction was heated at 65°C for 10min to stop the activity of enzyme. 2µl of RCA product was resolved on 1% agarose gel.

Cloning of RCA products

For the cloning of RCA products, the concatameric DNA was digested with restriction endonucleases to yield monomeric copies. A cloning vector pTZ57R was also restricted with the same enzyme. Restricted RCA product and vector were extracted with phenol-chloroform to remove protein and quantified. Vector and RCA product was then ligated. The ligation mixture was kept at 16^{o}C overnight and the following day transformed into competent *E. coli* cells.

Sequence analysis

Sequence information was assembled and analyzed using the Lasergene sequence analysis package (DNAStar Inc., Madison, WI, USA). Sequence alignments were produced using CLUSTAL W (Thompson et al., 1994). Phylogenetic analysis was conducted using the neighbour-joining and bootstrap options of Phylip (ver 3.5c). Phylogenetic dendrograms were viewed using treeview (Page, 1996)

RFLP analysis

Phi product was digested wit unique hexacutter enzymes like *ClaI, KpnI, EcoRV, BglII* and tetracutter enzymes like *MboI, TruI, HpaII* and *HaeIII*.

Southern hybridization

RCA product was digested with the restriction enzymes as mentioned above and resolved on 1.3% agarose gel and transferred to nylon membrane (Amersham Biosciences). Probes of components DNA-S, DNA-N and DNA-M were prepared by dioxigin (DIG) PCR kit (Roche). Hybridization and detection was done by the same method ad described by the manufacturers.

RESULTS

Phylogenetic relationship of BBTV components from Pakistan cloned from RCA product

Seven BBTV infected samples were collected from Tando Jam Sindh, Pakistan. These samples were amplified by Rolling circle amplification (RCA) method. Out of seven samples, only three samples gave amplification (Fig. 1A). These phi products were then restricted with hexacutters like *Hind*III and *Bgl*II. These samples gave band of 1.1kb with restriction enzyme *Hind*III, while these samples remain unrestricted with *Bgl*II. So, these samples were then again restricted randomly with hexacutters like *Eco*RI, *BamH*I, *Xho*I and *Not*I. But these samples remain unrestricted with all these restriction enzymes. The restricted product with *Hind*III was then cloned into circular vector pTZ57R. On the basis of restriction analysis 3 clones of 1.1kb sizes were selected for sequencing (Fig. 1B). The sequences obtained were compared with already available sequences in the NCBI database using

BLAST. Comparisons of sequence data showed similarity with sequences of South Pacific group of BBTV. One sequence showed 98% homology to DNA-N of South pacific group. These results suggested that there was very little sequence heterogeneity among Pakistani isolates of BBTV. Phylogenetic analysis showed that these sequences belonged to the South Pacific group of BBTV. They showed the closest relationship with isolates from India and Pakistan (Fig. 2). South pacific group and Asian group showed greatest similarity, they showed only 5% sequence diversity at nucleotide level between groups and 3% sequence diversity within groups.

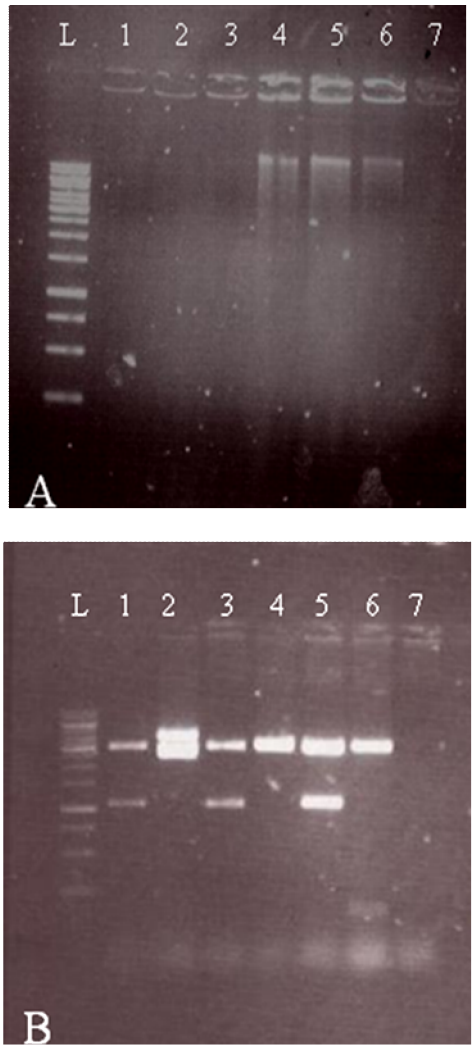

Fig. 1: (A) Rolling Circle Amplification of BBTV samples. Lane 4, 5, 6 represents amplification by RCA and in lane 1, 2, 3 samples remain unrestricted (B) Cloning of RCA product into pTZ57R. Lane 1, 3, 5 represents fragment of 1.1 kb released after restriction with enzyme.

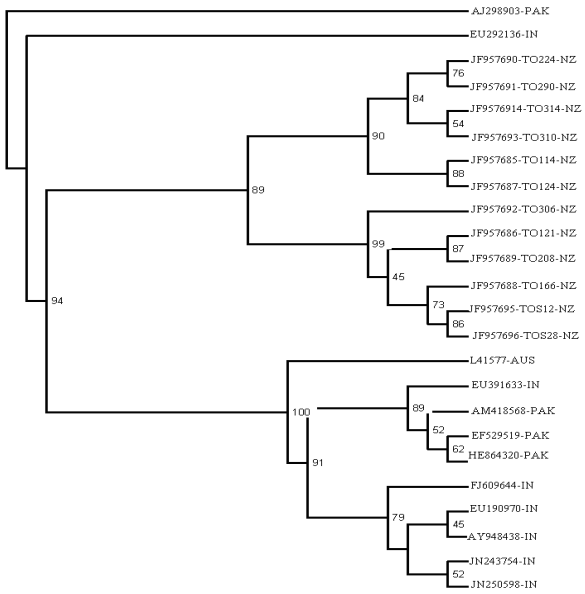

Fig. 2: Phylogenetic dendrogram based upon a nucleotide sequence alignment of reported BBTV DNA-N showing their relationship to the newly characterized component of BBTV (DNA-N) from Pakistan. Geographic origin is indicated by the country name.

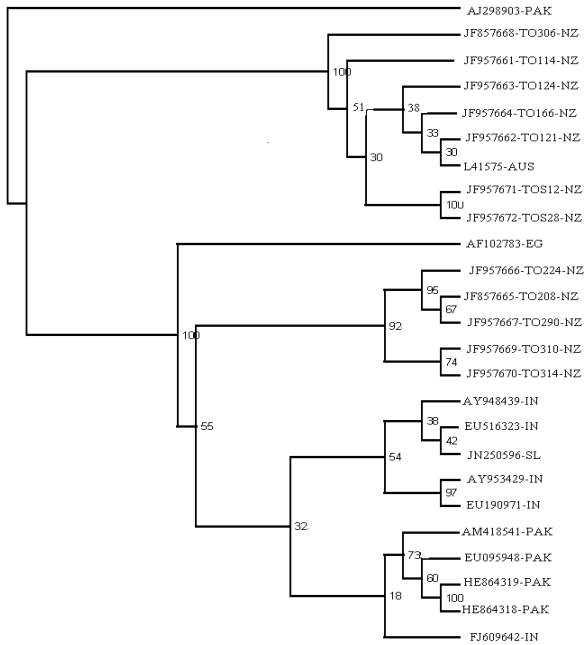

Fig. 3: Phylogenetic dendrogram based upon a nucleotide sequence alignment of reported DNA-M of BBTV, showing their relationship to the newly characterized component of BBTV (DNA-M) from Pakistan. Geographic origin is indicated by country name.

Table 1: Possible and actual sizes of bands with restriction enzymes in DNA-S (A), DNA-M (B) and DNA-N (C)

(A)

Component	Enzyme	Expected size (bp)	Size on gel (bp)	Southern hybridization (bp)
DNA-S	*EcoR*V	1075	1075	1075
	*Hpa*II	167,908	167,908	167,908
	*Mse*I	60,371,644	60,371,644	60,371,644
	*Mbo*I	520,555	520,555	520,555
	*Hae*III	176,801	167,801	167,801

(B)

Component	Enzyme	Expected size (bp)	Size on gel (bp)	Southern hybridization (bp)
DNA-M	*Kpn*I	1042	1042	1042
	*Hpa*II	349,693	349,693	349,693
	*Mse*I	64,118,162,239,459	64,118,162,239	64,118,162,239
	*Mbo*I	294,748	294,748	294,748
	*Hae*III	460,582	460,582	460,582

(C)

Component	Enzyme	Expected size (bp)	Size on gel (bp)	Southern hybridization (bp)
DNA-N	*Cla*I	1096	1096	1096
	*Hpa*II	25,1071	1071	1071
	*Mse*I	111,114,226,317,328	111,114,226,317,328	111,114,226,317,328
	*Mbo*I	79,202,245,570	202,245,570	202,245,570
	*Hae*III	137,164,795	795	795

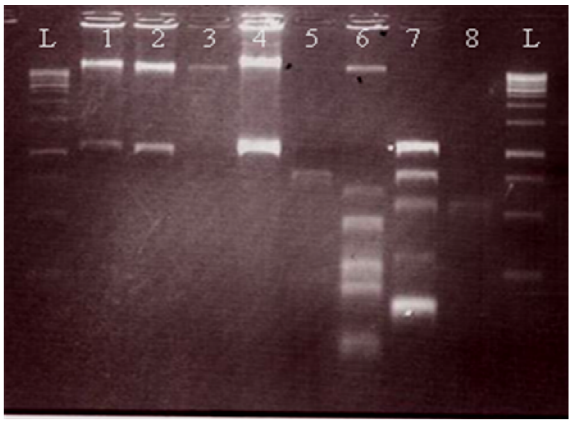

Fig. 4: RFLP analyzed restriction fragments of Phi product of BBTV, Phi product restricted with *Cla*I (1), *Kpn*I (2s), *EcoR*V (3), *Bgl*II (4), *Hpa*II (5), *Mse*I (6), *Mbo*I (7) and *Hae*III (8)

In the present study, two clones of DNA-M were sequenced. These clones also showed 99% homology to South Pacific group of BBTV. They showed 91% nucleotide sequence identity of already cloned component of DNA-M. Phylogenetic analysis also showed that DNA-M of Pakistani isolate was closely related to South Pacific group. Similarly, DNA-N clone also showed 98% homology to South pacific group of BBTV.

RFLP analysis of banana infected samples

Five to six components of BBTV have been characterized from both Asian group and South pacific group. South pacific group includes isolates of Egypt, India, Fiji, Australia, Tonga and Pakistan, while Asian group includes isolates from Taiwan, Vietnam, Philippines. DNA-R has also been investigated from both groups. While, additional Rep encoding components have been isolated and characterized only in Vietnam isolates, belongs to Asian group of BBTV isolates. These components were designated as DNA Y, W1 and W2 of approximately 1kb in size. So, in order to find out these additional Rep encoding components from South pacific group, we performed RFLP analysis of infected banana samples. For this purpose, the infected samples were amplified by RCA method. This RCA product was then restricted with tetracutters like *Hpa*II, *Mse*I, *Mbo*I, *Hae*III and their restriction pattern was checked. They gave the bands of different sizes (Fig. 4). We further checked restriction pattern with Southern hybridization with probes of already cloned BBTV components of DNA-S, DNA-N, DNA-M present in our lab. For this purpose, phi product was digested with hexacutters like *Kpn*I, *Cla*I, *EcoR*V, *Bgl*II, they gave the bands of 1.1kb. The restricted phi product was run on the gel and transferred to nylon membrane and these membranes were hybridized with probes of DNA-S, DsNA-N and DNA-M. All bands were hybridized with probe (Fig. 5 A, B, C). It means

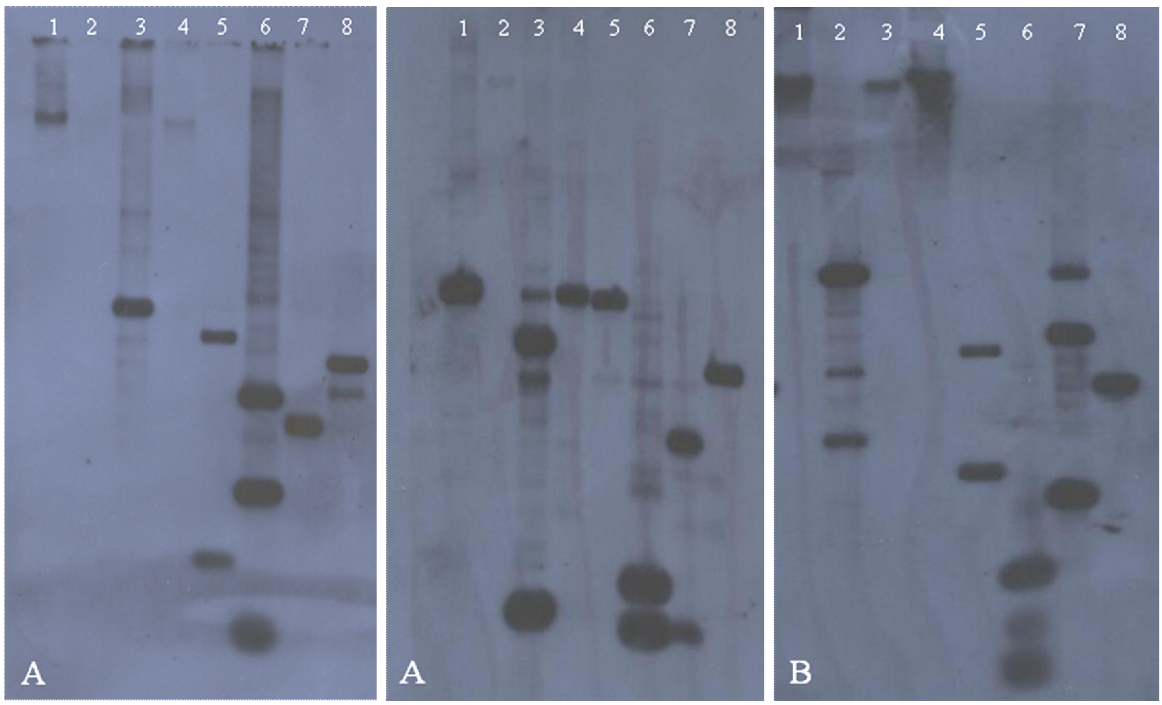

Fig. 5: (A) Southern hybridization of RCA products. RCA product was digested with enzymes *Cla*I (1), *Kpn*I (2), *EcoR*V (3), *Bgl*II (4), *Hpa*II (5), *Mse*I (6), *Mbo*I (7) and *Hae*III (8) and probed with DNA-S (A), DNA-N (B) and DNA-M (C)

that there is not new component in this sample. Rep-encoding satellite DNAs have been isolated from Asian isolate. On the basis of sequence analysis the present BBTV isolate in Pakistan belong to South pacific group. The restriction patterns and Southern hybridization reflected that a single homogenous population of virus is prevalent in Pakistan and no satellite molecules are associated with this isolate. So, there is very low level of genetic diversity in South pacific group than Asian group.

DISCUSSION

Banana bunchy top disease continues to cause heavy yield losses to banana production in Pakistan. Nanoviruses are responsible for many diseases in economically important crops. As FBNYV causes diseases in faba beans and BBTV infect banana plants. In Pakistan banana production is severely affected by BBTV since 1989. Banana is a monocot plant and is cultivated in southern coastal areas of Pakistan. However, due to increasing pressure of BBTV, the banana cultivation has moved upward in Sindh and adjoining areas of Balochistan to avoid BBTV. Previous molecular analysis of BBTV from Pakistan has shown that it is composed of five components (Amin et al., 2008) but another component (DNA U3) has also been cloned from Pakistan and this is the most diverse component of BBTV genome (Hyder et al., 2011). Six components of BBTV have also been characterized from geographically different isolates of banana (Karan et al., 1994). These components have been cloned by PCR method. In this study, we used a new technique, RCA for the amplification of unknown components of BBTV. Since RCA is a very useful technique for the molecular characterization of unknown components with circular genome. It can amplify the viral circular DNA of unknown identity. In BBTV, there is a need to find out whether any additional component is associated with the infectious unit or not. As BBTV is divided into two groups South Pacific group and Asian group. These additional components have been characterized from Asian group of BBTV. These were referred as DNA Y, W1, W2, S1, S2 (Horser et al., 2001). The isolate prevalent in Pakistan belongs to South Pacific group, so there was a need to identify these additional components from this group.

RFLP technique was used to find out these additional components. The results showed that there is no additional component associated with South Pacific group of BBTV. It is possible that these components are more widespread in Asian group as compared to South Pacific group or it is not detected by probes used in this study. Similarly another family of ssDNA *Geminiviridae* encodes only one Rep. In addition to

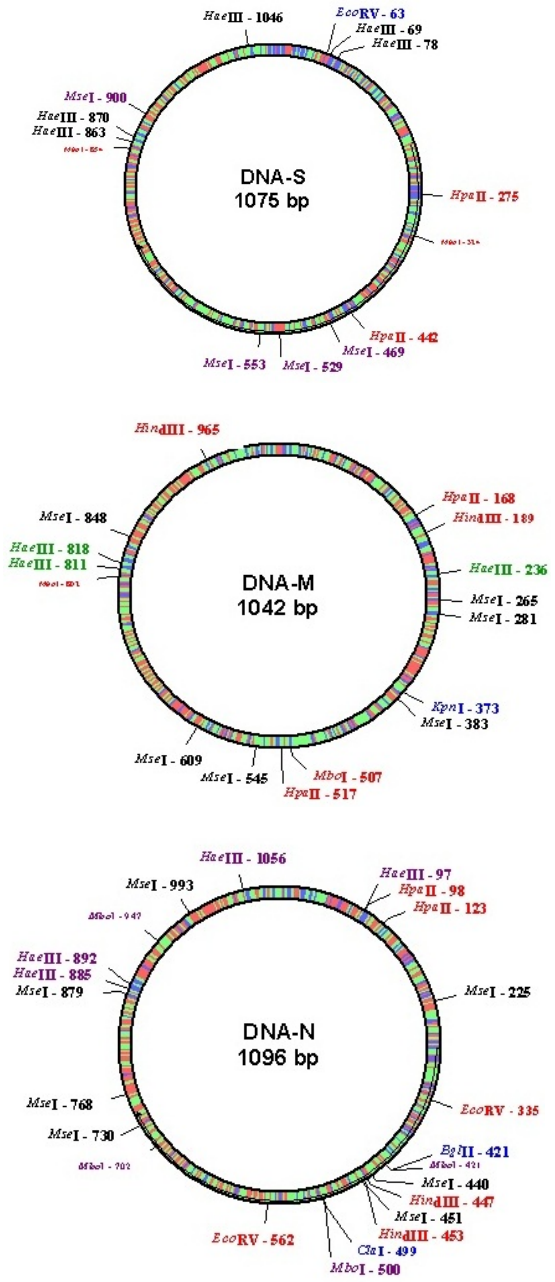

Fig 6: Schematic presentation of genome organization of BBTV DNA-S, DNA-M and DNA-N showing the position of tetracutters in the genome.

this; additional Rep encoding components have been identified for other members of family *Nanoviridae.* In FBNYV four additional Rep encoding components have been identified (Katul et al., 1998). In SCSV and MDV two and three additional Rep encoding components have been identified (Boevinck et al., 1995; Sano et al., 1998). Coconut foliar decay (CFDV) which is a possible member of *Nanoviridae* also showed one additional Rep encoding components (Rohde et al., 1990). These additional Rep encoding components are associated with nanoviruses and play an important role in infectivity of nanoviruses.

Phylogenetic analysis of BBTV also showed that there was very less diversity in BBTV components. Previous studies have also shown that genetic variability in South Pacific group of BBTV is less. These components showed 98% homology to South Pacific group. These isolates were closely related to Australian and Indian isolates of BBTV, which further confirmed the results that this disease was introduced in Pakistan from Australia or India to Pakistan. So there is single homogenous population of BBTV prevalent in Pakistan. This low level of diversity in Pakistan can be used for obtaining resistance to the virus by RNA silencing based technologies.

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