ANALYSIS OF BANANA BUNCHY TOP VIRUS GENE EXPRESSION RATIO IN BANANA CROP GROWN IN SINDH PROVINCE OF PAKISTAN

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ABSTRACT

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Banana bunchy top virus (BBTV) is a lethal virus, causal agent of banana bunchy top disease in banana plants. Present study was conducted to analyze gene expression ratio of BBTV in infected banana plants grown in Sindh Province of Pakistan. It involves establishment of the sequence of BBTV genomic components, genetic variability and expression of viral determined proteins in banana with reporter protein to know their intracellular localization. It also involves determination of the biochemical functions of BBTV M-Rep protein. The relative quantification of all the BBTV components in reference to the concentration of DNA-R in triplicates, suggested that DNA-U3 and DNA-N have highest (100 folds higher) concentration as compared to all other components in infected banana plants, while DNA-M is the least concentration(about 100 folds less) followed by DNA-S. The DNA-C has similar concentration to DNA-R. Similarly DNA-S and DNA-M have about 10 to 100 folds less concentration respectively. This is the first time, this kind of analysis of BBTV gene expression ratio was done in Sindh province of Pakistan. Result the present study showed that BBTV components were present in different concentrations in infected banana plants grown in Sindh province of Pakistan. The finding of the research may helpful for future researchers to establish inhibition based strategies against each BBTV component according to their ratio.

Key Words: BBTV, banana,gene expression, bunchy top disease.

INTRODUCTION

Banana is an important food crop having high nutritional values. It contains 35% carbohydrates, 6-7% fibers, fats and proteins (Marriot and Lancaster 1987). On Trial basis, farming of banana started inSindh province of Pakistan from 1913. Large numbers of banana varieties under different species were introduced in the Province from different countries like Bangladesh, Sri Lanka and India.Among all varieties, only *Musa actuminatacolla* also called "DwarfCavendish" (Basrai) i.e. Banana variety of Cavendish group was foundto be best adopted in the environment of Pakistan and was used for farming in 1950 (Bhatti 1991). In Sindh "Dwarf Cavendish" variety is mostly used for agriculture. William hybrid new banana specie was imported from Australia, during 1970s. It occupies simply 3-5% of the whole cultured area of Sindh (Khalid and Soomro 1993).Pseudo stem of bananas serve as host for variety of pathogens. These pathogens include viruses,bacteria, fungi and nematode(Jones2000). Among viruses, *Banana bunchy top viruse* (BBTV) shows severe pathogenicity on plants, and considered as the most dangerous virus (Jones 2000).Plant viruses belong to two families *Geminiviridae*and *Nanoviridae* containing circular single stranded DNA(cssDNA) as a genome. Family *Nanoviridae* is divided in to two genera,*Nanoviruses* and *Babuvirus* (Vettene*t al*. 2005*). Banana bunchy top virus* belongs to*Babuvirus.*In Pakistan bunchy top disease was first reported from district Thatta of Sindh in 1988. In 1991 banana bunchy top virus was detected from infected banana plant showing clear symptoms of bunchy top disease (Soomro*et al*.1992). Its sole vector for transmission is "*Pentalonianigronervosa*". Symptoms of Bunchy top disease include appearance of dark green streaks on leaves. These streaks are in the form of dashes and dots so called as "Morse code "or also termed as "ditz and dashes". Then subsequent leaves become narrow and develop marginalchlorosis or yellowing. Leaves become more upright and make bunch on the apex of stem so disease is termed as "bunchy top" or "banana bunchy top" disease. Bunchy top disease (BBTD) first reported in Fiji. It is also reported from India and Africa and various other

countries of the world including Pakistan (Khalid et al., 1993). BBTD is wide spread in Southeast Asia (Kagy *et al*. 2001).Bunchy top virus is 18–20 nm isometricvirion with circular single stranded DNA (ssDNA.). Genome of bunchy top virus contained six essential circular single stranded DNA components named as DNA–N, DNA -U3, DNA-S, DNA-R, DNA-C, and DNA-M, each one has a size of 1 kb.These viral genomic components determined number of proteins except DNA-U3 (Harding *et al*. 1993, Katul *et al*. 1995). Circular single stranded (ssDNA) DNA components possess two conserved regions which referred to as major common region and hair pin loop region (Burns *et al*.1995).Hair pin loop region contained total 69-70 and show 62% similarity with other integral components. Majorcommon region consist of 20-225 nucleotides showing 75-76% similaritieswith other BBTV genomic components. Genomic components of BBTV also contains reading frame (ORF) with poly adenylation signal and TATA boxes Fig. 2).DNA-R is of BBTV genome that involves in the replication of all other integral component of genome. Replication encoded by DNA-R of BBTV is termed as "master replication".During qPCR amplified product is accumulated unless it is not detected and product also measured when reaction is in progress. In qPCR reaction is added through a fluorescent molecule or real time dye that give information when amount of DNA increase which in turn detects the proportional increase in fluorescence. Sequence specific fluorescently labeled primers and SYBER green dyes used in qPCR. Specified thermo cyclers prepared with fluorescence and a variety of identification elements are utilized to supervise the fluorescence as strengthening occur. Detected fluorescence act as a sign of the total amplified product within each cycle.

The purpose of present work was to conduct real-time PCR assays to find out gene transcript analysis of BBTV, M-Rep, CP, MP, Clink and NSP genes.

MATERIAL AND METHODS

Collection of plant material used

Banana plants with banana bunchy top disease (BBTD) symptoms and apparently healthy plants used in the present piece of research were collected from banana fields located in different areas of Sindh province, Pakistan. Infected banana plants were pulled out; aphids are killed by spraying with kerosene oil on themand monitored carefully. Both types of plants were placed in a plant growth chamber for two weeks before sampling mid-rib tissues.

DNA Extraction and Polymerase chain reaction

DNAwas extracted from lamina of infected and normal leaves of banana plants by CTAB method. Normal banana plants were used as negative control. Exactly 50 ng of DNA was used as template with Taq polymerase pre mix (Fermentas -USA) and PCR amplification was performed. Primer Pairs of BBTV were used for the confirmation of presence and absence of BBTV infection in infected and normal plants (Table 1). PCR cycling conditions were 96 C for 20 sec, 52C for 20 sec and 72C for 40 sec with 35 cycles.

RNA extraction andreverse transcription-polymerase chain reaction

Total RNA was extracted from tissues of approximately 0.2 g mid-rib sample using SV Total RNA Isolation System (Promega USA). Purified RNA from each sample was transcribed to cDNA using Super Script III One Step Quantitative Reverse transcription PCR System (Invitrogen, USA). The cDNA was used as template with Taq mixand PCR amplification was performed. The pairs of primers were used in this reaction (Table 1). PCR cycling conditionswere 50C for 15 min for cDNA synthesis, 2 min for 23 hold at 95C subjected by 35 cycles of denaturing, 95C for 20 sec, 52C for 24 sec, 72C for 40 sec and more 20 min for final extension.

Real time PCR for BBTV genomic components

To quantitatively analyze the expression of BBTV genomic components, real-time PCR was performed using Step One Plus™ System (The Applied Biosystems® Life Technologies, USA with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, USA). PCR cycling conditions were 95C for 10 min, 96C for 20 sec, 52C for 20 sec, 60C for 1 min with 35 cycles and finally 60C for 20 min.

Purification of DNA and sequencing

PCR Clean-Up System Kit (Promega) and Wizard® SV Gel were used for the purification of PCR product. DNA was kept at -20C used for sequencing. Sequencing of DNA was carried out by using Macrogen Standard Custorm DNA Sequencing Services. Sequenced data was collected with the help of Blast 2 sequences for both thestrand individually BBTV genomic components. Results of sequencing were evaluated using DNA Dragon - DNA Sequence Contig Assembler Software (SequentiX - Digital DNA Processing, Germany).

Table 1. Sequences and properties of primer sets used in the study

List of Primers	Sequence 5'- 3'direction	Length of primer (bp)	Annealing Temperature used (C)
DNA-R F	GATGGCGCGATATGTGGTATG	21	52
DNA-R R	GTCTGTCGTCGATGATGATCTTG	23	52
DNA-U3 F	CAGATTAATTCCTTAGCGAC	20	52
DNA-U3 R	GACCGTTCATTCAACTTGAC	20	52
DNA-S F	GGAAGAATGTAACGGAGGTCG	21	52
DNA-S R	GTCAACACGGTTGTCTTCCTCAG	23	52
DNA-M F	GATGGCATTAACAACAGAGCG	21	52
DNA-M R	GTTAGCAGGGTCCTATTTATAGG	23	52
DNA-C F	GAATCGTCTGCTATGCCTG	19	52
DNA-C R	CCAGAACTCCATTTCTCTTC	20	52
DNA-N F	GATGGATTGGGCGGAATCA	19	52
DNA-N R	GCTTCTGCTTTGCTTTCGC	19	52
DNA-R RtF	GCGATATGTGGTATGCTGGATG	22	52
DNA-R RtR	GAGCTTCGTCTCTTCATCTCG	21	52
DNA-U3 RtF	CCGAAGGTCAAGGTAACCG	19	52
DNA-U3 RtR	AGCACAACCACCTTCACAG	19	52
16S rRNA F	GACGGGTGAGTAACGCGTAAG	21	52
16S rRNA R	C TTCCAGTACGGC[Y]ACCTTG	20	52
Actin F	ACTGTTCCTATATACGAAGG	20	52
Actin R	GAAAAGTGCTGAGCGAAG	18	52

RESULTS AND DISCUSSION

Presence and absence of BBTV in banana plants was confirmed using specific primers mentioned in Table 1, which indicated specific PCR products only from infected plants while no BBTV Specific products were obtained from normal plants. Positive controls were included to avoid DNA extraction/PCR failures which amplified from both infected and normal plants a specific band of about 0.6 kb of beta actin gene from banana nuclear and a band of 1.2 kb from chloroplast genome were used as control (Fig. 1).

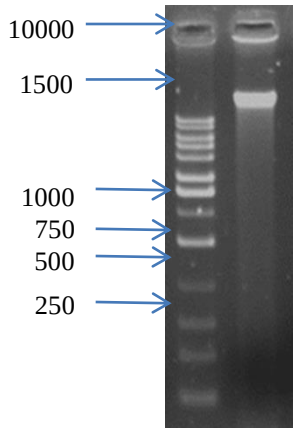


Figure 1. Agarose gel electrophoresis photograph of infected banana DNA

Total DNA was isolated from both healthy and infected banana plants, were amplified via primer sets capable to specifically amplify DNA-U3,-C, DNA-M and DNA-S. In addition β-actin genes from banana nuclear genome as well as 16s rRNA genes from chloroplastgenome were also amplified using specific primers as positive controls. Water was used as negative control instead of primers. BBTV specific bands of about 1.1 kb were amplified only in DNA from infected plants and there is no amplification in healthy banana for these primer sets. While bands of about 574nt with β-actin geneand band of about 1.2 kb with 16srRNA gene were produced from both infected and healthy plant DNA. For DNA extraction 1kb gene ruler is used and 0.5µl sample is loaded in 0.8% agarose gel is used (Fig. 2).

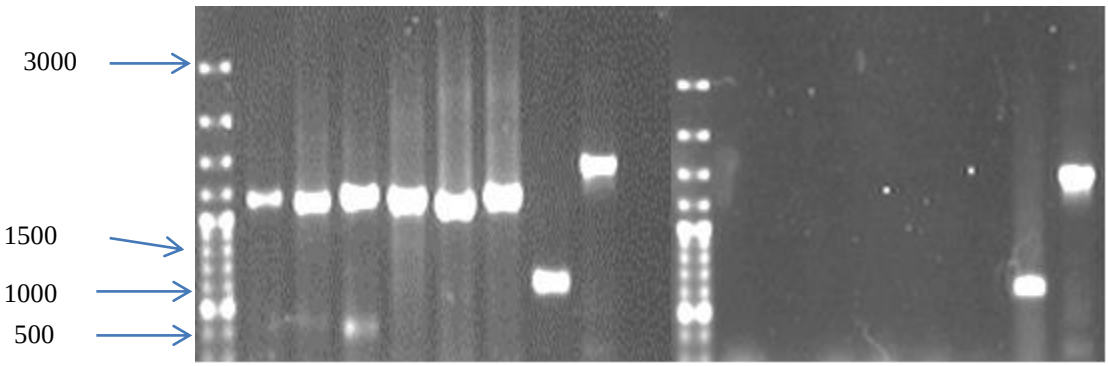


Figure 2. Agarose gel electrophoresis photograph of PCR amplified product of BBTV Infected and healthy banana plant [Lane 1, 100bp Plus Gene ruler (SM0323, Fermentas USA), Lane2, DNA-R; Lane3, DNA-U3, Lane4, DNA-S, Lane5, DNA-M, Lane6, DNA-C, Lane7, DNA-N, Lane8, β-actin, Lane9, 16sr RNA; Lane10, Negative control, Lane 11, 1 Kb gene ruler marker (Fermentas,USA), Lane12, DNA-R, Lane13, DNA-U3, Lane14, DNA-S, Lane15, DNA-M, Lane16,DNA-C, Lane17, DNA-N, Lane18, β-actin; Lane19, 16sr RNAs]

RNA isolation was optimized and extracted total RNA was subjected to cDNA synthesis and subsequently for amplification of BBTV Rep transcript and β-actin Gene transcripts The reverse transcribed PCR (RT-PCR) amplified BBTV Repencoding transcript along with banana nuclear transcript of β actin gene, indication of successful RNA extraction form infected banana, synthesis of cDNA and amplification of target transcripts. Total RNA were extracted from BBTV infected banana was converted into cDNA using DNA-R RtF and DNA-RtR primer set and banana actin gene using Actin F and Actin R primer set as positive control**(**Fig. 3**)**.. The RNA labeled lanes contains infected sample RNA first changed to cDNA and then treated with SuperScript III One Step Quantitative reverse transcribed PCR System (Invitrogen USA) kit for further amplification.A band of about 200 bp specific to BBTV Rep transcript was amplified from DNA-R while a band of about 575bp was amplified from banana actin gene transcript (Fig. 4). In lanes labeled DNA the total DNA extracted from the same plant was PCR amplified using the same primer sets which were used to amplify RNA. Same banding pattern was obtained by DNA amplification which was used as quality control Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas, USA) were used after reverse transcribed PCR for the quantification of BBTV genomic components. The relative quantification of all the BBTV components in reference to the concentration of DNA-R in triplicates, suggested that DNA-U3 and DNA-N have highest (100 folds higher) concentration of all the components in the infected banana while DNA-M is the lowest (about 100 folds less) followed by DNA-S. The DNA-C showed concentration comparable to DNA-R. Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, USA) were used for quantification of all BBTV genomic components in relation tothe concentration of DNA-R (reference control) in a given sample. The sample to sample variation was corrected using β-actin gene which was run for each DNA component separately as endogenous control having a coefficient of variation more than 99%. The amplification plots for BBTV genomic components and β-actin gene were shown separately for clarity(Fig. 5 and6). The relative concentration of BBTV DNA components indicates that DNA-U3 and DNA-N have about 100 folds high concentrations compared to DNA-R. Similarly, DNA-S and DNA-M have about ten to 100 folds less concentration, respectively while DNA-C has similar concentration as of DNA-R. The labels in amplification plots are A; DNA-R (as reference), B; β-Actin, C; DNA-R (as component), D; -U3 E; -S, F; -M, G; -C and H; DNA-N (Fig.7).

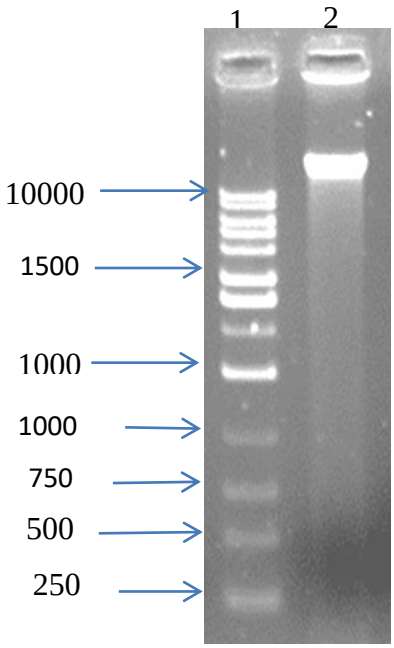


Figure 3.Agarose gel electrophoresis photograph of BBTV infected banana plant RNA [Lane1 1 Kb gene ruler (SM0313, Fermentas USA); Lane2, Infected plant RNA]

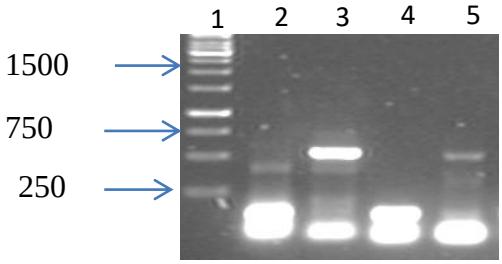
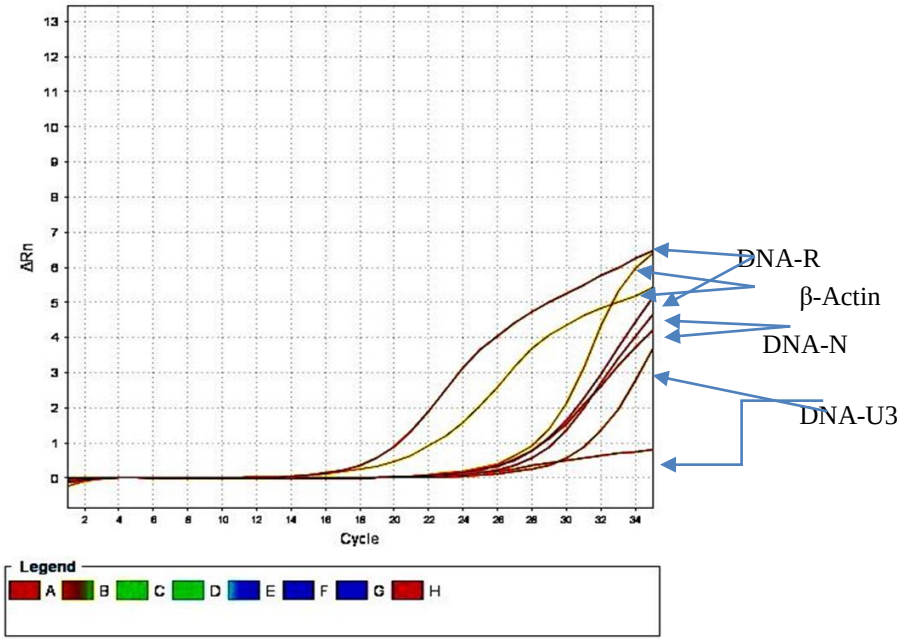


Figure 4.Agarose gel electrophoresis photograph of Amplification of cDNA of Rep gene along with banana β-actin gene[Lane 1, 1 Kb gene ruler marker (SM0313,Fermentas USA); Lane2, Rep gene; Lane3, β-actin gene; Lane4, Rep gene; Lane5, β-actin gene]

The current study provided evidence using Real-Time PCR that indeed all the components in BBTV genomes are not replicated in the same extent by BBTV Rep protein. All BBTV DNA componentshave a region of homology performing regulatory functions i.e. the stem-loop common region (CR-SL) (Burns *et al*. 1995). The CR-SL contains a stem-loop structure with an invariant loop sequence TA(G/T)TATTAC, found in all Nanoviruses, which functions as origin of replication(ori) for the proposed rolling-circle replication of these viruses (Hafner*et al*. 1997, Timchenko *et al*. 1999). Recently it has been shown that three iterons F1, F2 and R1of an iterated sequence GGGAC, are present in all the components of an Australian isolate of BBTV (Herra-Valencia *et al* 2006). By mutational studies these iterons have been shown to be the sites necessary for efficient replication of all the components by M-Rep, which binds with these iterons. Similar strategy is used by Geminiviruses where this process has been described in detail (ArguelloAstorga *et al*. 1994, Fontes*et al*.1994b).These evidences suggest that for replication of each DNA components, BBTV Rep has to interact through iterons with each component separately, and these interactions determine the extant with which an component is replicated in a cell. An analysis of CR-SL region (Hyder *et al*. 2011) suggested variations in CR-SL region which might explain the observed variations in BBTV component replication in the present study.



A; DNA-R, B; β-Actin, C; DNA-R (as component), D; -U3, E; -S, F; -M, G; -C and H;DNA-N

Figure 5. Amplification plot shows relative concentration of BBTV genomic components

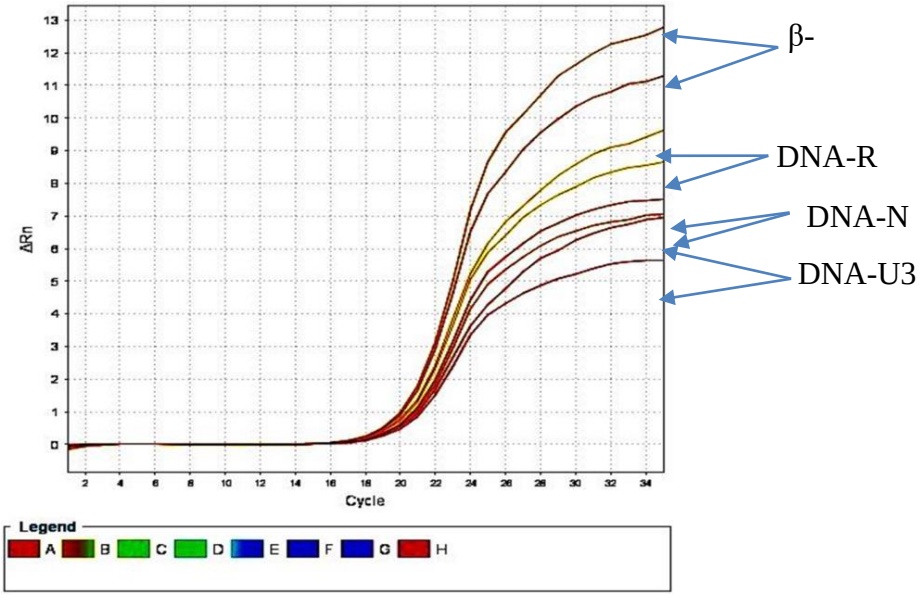


Figure 6. Amplification plot β-actin shows amplification plots for β-actin gene and BBTV genomic components

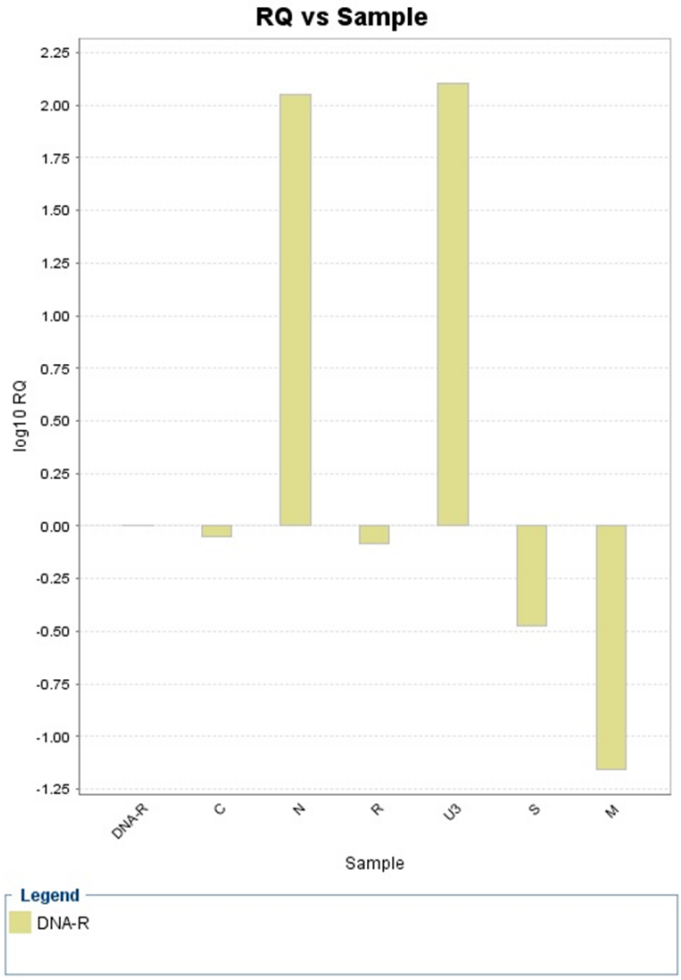


Figure 7. Relative Quantification of BBTV Genomic Components using Real-Time PCR.

Based on findings of the present study, it is obvious that BBTV rep protein is not only involved intranscription of the virus but also have important role in the tuning of other components of BBTV to make it a successful pathogen for disease. When viruspropagates to the other parts of the plant, BBTV rep protein helps nuclear shuttle protein to enhance its activity and also the movement protein as well.

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