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ORIGINAL ARTICLE

Molecular Characterization of Geographically Different *Banana bunchy top virus* Isolates in India

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Abstract Banana bunchy top disease (BBTD) caused by *Banana bunchy top virus* (BBTV) is one of the most devastating diseases of banana and poses a serious threat for cultivars like Hill Banana (Syn: Virupakshi) and Grand Naine in India. In this study, we have cloned and sequenced the complete genome comprised of six DNA components of BBTV infecting Hill Banana grown in lower Pulney hills, Tamil Nadu State, India. The complete genome sequence of this hill banana isolate showed high degree of similarity with the corresponding sequences of BBTV isolates originating from Lucknow, Uttar Pradesh State, India, and from Fiji, Egypt, Pakistan, and Australia. In addition, sixteen coat protein (CP) and thirteen replicase genes (Rep) sequences of BBTV isolates collected from different banana growing states of India were cloned and sequenced. The replicase sequences of 13 isolates showed high degree of similarity with that of South Pacific group of BBTV isolates. However, the CP gene of BBTV isolates from Shervroy and Kodaikanal hills of Tamil Nadu showed higher amino acid sequence variability compared to other isolates. Another hill banana isolate from Meghalaya state had 23 nucleotide substitutions in the CP gene but the amino acid sequence was conserved. This is the first report of the characterization of a complete genome of BBTV occurring in the high altitudes of India. Our study revealed that the Indian BBTV isolates with distinct geographical origins belongs to the South Pacific group, except Shervroy and Kodaikanal hill isolates which neither belong to the South Pacific nor the Asian group.

Keywords *Banana bunchy top virus* · *Babuvirus* · *Nanoviridae* · South Pacific · PCR · Hill Banana

Introduction

India is the largest producer of banana in the world, with a total production of 23.20 million tonnes from an area of 0.64 million hectares [13]. Banana is affected by many bacterial, viral, and fungal pathogens and they cause a significant yield loss [7]. Banana bunchy top disease (BBTD) caused by *Banana bunchy top virus* (BBTV) is a very serious disease in India. Since the 1970s, BBTD has devastated large plantations of cv. Virupakshi (AAB), an elite cultivar also known as "Hill Banana" in lower Pulney hills, Tamil Nadu State, India. The area under cultivation of this banana cultivar has been reduced from 18,000 to 2,000 ha (Kesavamoorthy [10] due to BBTD. A recent survey made during May 2009, in Pulney hills, Dindigul district of Tamil Nadu recorded 14–72% incidence of BBTD (Selvarajan unpublished). This increased incidence could be due to continuous propagation of bananas as intercrop in coffee plantations, planting new plantations with suckers from infected mother plants and the presence of banana black aphid vector (*Pentalonia nigronervosa*) round the year.

BBTV is a multi-component, circular single stranded DNA virus belonging to the genus *Babuvirus* and family *Nanoviridae*. The isometric virion measures 18–20 nm in diameter. It is transmitted by banana black aphid (*P. nigronervosa*) in a persistent manner. The genome of BBTV consists of at least six integral components each

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approximately 1 kb in size (BBTV DNA-R, -S, -M, -C, -N and -U) [3, 5, 17, 18, 21]. BBTV DNA-R encodes a master replication initiation protein (Rep) and DNA-S encodes a viral coat protein (CP) for encapsulation (Karan et al. [9]. BBTV isolates are categorized into two groups, namely the "South Pacific group" and the "Asian group" based on nucleotide sequence identity of DNA-R [8]. The mean sequence difference within each group was 1.9–3% and between isolates from the two groups was approximately 10% [8]. Wanitchakorn et al. [20] found significantly higher variability of 1.77% in the amino acid sequences of BBTV CP within the Asian group. They related this variability might be due to the presence of BBTV in Asian region for an extended period of time. In India, BBTV has been prevalent since 1943 and so it is possible that genetic variability such as that observed among isolates of the Asian group exists. Recently, the complete genomes of BBTV isolates occurring in Pakistan [1] and Lucknow, North India [19] were reported and found to belong to the South Pacific group.

In this context, we conducted molecular analysis of BBTV isolates infecting Hill Banana (HB-TN isolate) which is widely cultivated for more than four decades in the lower Pulney hills of Tamil Nadu. We have also cloned and sequenced the coat protein sequences from sixteen BBTV isolates and replicase sequences from thirteen BBTV isolates collected from the hill regions of Tamil Nadu, Arunachal Pradesh and Meghalaya and the plain regions of different states such as, Andhra Pradesh, Assam, Bihar, Delhi, Gujarat, Karnataka, Kerala, Maharashtra, Nagaland, West Bengal and Andaman to gain a comprehensive assessment of genetic variability of BBTV in India.

Materials and Methods

Leaf samples were collected from BBTV infected banana cv. Virupakshi (AAB) from lower Pulney hills, Tamil Nadu and from the hill and plain regions of different states of India during a survey conducted in 2005 (Table 2). Total DNA was extracted from the leaf samples by the method described by Gawel and Jarret [4].

Primers specific to different genomic segments of BBTV genome, CP and Rep gene were designed from published sequences available in GenBank. The sequences of these primers and the size of amplicon are furnished in (Table 1). PCR reaction mixture in a volume of 50 µl contained 5.0 µl of 10× PCR buffer containing 15 mM $MgCl_2$, 4.0 µl of 10 mM dNTPs, 1.0 µl (100 ng/µl) each of forward and reverse primer, 2.5 units of *Taq* polymerase (Genei, Bangalore) and 4.0 µl of total DNA. PCR was performed in a Master Cycler gradient PCR machine (Eppendorf, Germany) with initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 49–53°C for 1 min and extension at 72°C for 2 min with a final extension of 72°C for 10 min. The amplified products were resolved in a 1% agarose (1× TBE) gel and electrophoresed at 100 V for 1 h. The gels were visualized and documented using Alpha Imager (Alpha Innotech Corp., USA).

The PCR amplified products were purified using MinElute gel extraction kit (Qiagen, USA). The purified PCR product was ligated into pGEM-T Easy vector (Promega, Madison, USA) and transformed into a competent *Escherichia coli* (strain DH5α) using standard molecular biology methods [11]. The recombinant clones were confirmed with restriction digestion analysis. Complete nucleotide sequences of three full length clones for each component were determined by an ABI prism Big Dye Terminator Kit (Genei, Bangalore) and were aligned using CLUSTAL W [15]. The complete sequences of six components of HB-TN isolate, CP and Rep gene sequences of other BBTV isolates obtained in this study were deposited in NCBI GenBank and accession numbers are listed in (Table 2). Sequence identities were calculated from the "Sequence Identity Matrix" using BioEdit program version 7.05 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Phylogenetic analysis was done for aligned nucleotide sequences of all six genome components of Hill banana with available complete genome

Table 1 List of primers used in this study

Primers used in this study	Sequences for forward primer (5′–3′)	Sequences for reverse primer (5′–3′)	Size (bp)
DNA-R	CGAAGGCGATGAATAGCTG	GTCCATGCACTGAAGGATGTCTTC	1111
DNA-U	TCTAGAGAGGCGGCGAGGAAAC	TGCAGGTCGTTCCGACGATC	1060
DNA-S	AAGGTGAAGCCCGGAAGAAT	GCACACAAGAATCATAAACA	1075
DNA-M	AGACGAGATCAAGAACCGGCTG	GTCTTCCACAATACCTCTGCCG	1043
DNA-C	TATATTAACCCCTTAAGGGCCG	GCCCAATCAAATACCCGTACAT	1018
DNA-N	CTGCAGATGGATTGGGCGGAAT	GGATCCGCTTCTGCCTTC CGC T	1089
CP gene	ATGGCTAGGTATCCGAAGAAATCC	TCAAACATGATATGTAATTCTGTTC	513
Rep gene	TTGGATCCATGGCGCGATATGTGGTATGC	TCAGCAAGCAACCAACTTTATTCGA	861

Table 2 List of BBTV isolates used in this study

BBTV components	Accession no[a]	Place of origin	Reference or source[b]
DNA-R	IndEU140342	Tamil Nadu, India	This study
	AusNC_003479	Australia	Harding et al. [5]
	PakAM418536	Pakistan	Amin et al. [1]
	IndDQ285570	Lucknow, India	Vishoni et al. [19]
	ChiAY450396	Hainan, China	Tien et al. [16]
	VieAF416473	Dien, Bien, Phu: Vietnam	Bell et al. [2]
DNA-S	IndEU589459	Tamil Nadu, India	This study
	AusL41574	Australia	Burns et al. [3]
	PakAM418566	Pakistan	Amin et al. [1]
	IndEF687856	Lucknow, India	Vishoni et al. [19]
	ChiAF238877	Guangzhou, China	He et al. [6]
	VieAF148945	Vietnam	Wanitchakorn et al. [20]
DNA-M	IndEU190971	Tamil Nadu, India	This study
	AusNC_003474	Australia	Burns et al. [3]
	PakAM418541	Pakistan	Amin et al. [1]
	IndEU516323	Lucknow, India	Vishoni et al. [19]
	ChiAF349568	Zhangzhou, China	J. D. Sun et al.*
DNA-C	IndEU190969	Tamil Nadu, India	This study
	AusL41578	Australia	Burns et al. [3]
	PakAM418569	Pakistan	Amin et al. [1]
	IndEU051379	Lucknow, India	Vishoni et al. [19]
	ChiAY266417	China: Gaozhu	Y. Zheng et al.*
DNA-N	IndEU190970	Tamil Nadu, India	This study
	AusNC_003476	Australia	Burns et al. [3]
	PakAM418568	Pakistan	Amin et al. [1]
	IndEU391633	Lucknow, India	Vishoni et al. [19]
	ChiAY494787	China: Hainan	Tien et al. [16]
DNA-U	IndEU140341	Tamil Nadu, India	This study
	AusNC_003475	Australia	Burns et al. [3]
	IndEU402601	Lucknow, India	Vishoni et al.[19]
	ChiAY606084	Hainan, China	Tien et al. [16]
	IndGU085260	Kolli hills, Tamil Nadu	This study
	IndGU085261	Kodaikanal hills, Tamil Nadu	This study
	IndGU085262	Shervoy hills, Tamil Nadu	This study
BBTV CP	IndGU125406	West Bengal, India	This study
	IndGU125407	Delhi, India	This study
	IndGU125408	Gujarat, India	This study
	IndGU125409	Maharashtra, India	This study
	IndGU125410	Andaman, India	This study
	IndGU125411	Karnataka, India	This study
	IndGU125412	Andhra Pradesh, India	This study
	IndGU125413	Kerala, India	This study
	IndGU125414	Bihar, India	This study
	IndEU190965	Assam, India	This study
	IndEU190966	Nagaland, India	This study
	IndEU190967	Meghalaya, India	This study
	IndEU190968	Arunachal Pradesh	This study

Table 2 continued

BBTV components	Accession no[a]	Place of origin	Reference or source[b]
	IndEF584544	Maharashtra, India	M. N. Islam et al.*
	Ind FJ168538	Bihar, India	M. N. Islam et al.*
	Ind DQ996466	Bihar, India	M. N. Islam et al.*
	IndDQ515970	Lucknow, India	S. K. Raj and Vishoni*
	IndAY534140	Tamil Nadu, India	R. Selvarajan et al.*
	Ind FJ664270	Tamil Nadu, India	A. Chandrasekar et al.*
	Ind FJ664271	Tamil Nadu, India	A. Chandrasekar et al.*
	AusEF546810[c]	Australia	Sharman et al. [12]
BBTV replicase	IndGU125415	West Bengal, India	This study
	IndGU125416	Gujarat, India	This study
	IndGU125417	Andhra Pradesh, India	This study
	IndGU125418	Karnataka, India	This study
	IndGU085263	Kolli hills, Tamil Nadu	This study
	IndGU085264	Kodaikanal hills, Tamil Nadu	This study
	IndGU085265	Shervoy hills, Tamil Nadu	This study
	IndEU531473	Andaman, India	This study
	IndEU531472	Maharashtra, India	This study
	IndEU531471	Tamil Nadu, India	This study
	IndGU130586	Bihar, India	This study
	IndGU130587	Delhi, India	This study
	IndGU130585	Maharashtra, India	This study
	IndAY845437	Tamil Nadu	Harish et al.*
	IndEF584545	Bihar, India	M. N. Islam et al.*
	Ind DQ640742	Maharashtra, India	M. N. Islam et al.*
	IndDQ640741	Bihar, India	M. N. Islam et al.*
	IndDQ285571	Uttar Pradesh, India	S. K. Raj and Vishoni*
	IndDQ285570	Uttar Pradesh, India	S. K. Raj and Vishoni*
	IndAM055641	Karnataka, India	S. Rahman et al.*
	IndAY222303	India	S. B. Ghosh et al.*

[a] Accession numbers from NCBI GenBank are given after the three letter abbreviation of country or region names. *Ind* India, *Aus* Australia, *Pak* Pakistan, *Chi* China, *Vie* Vietnam

[b] *Asterisks* mark sequences found in GenBank database without reference

[c] *Abaca bunchy top virus* (ABTV)

sequences of BBTV isolates and also for aligned amino acid sequences of sixteen CP genes with available BBTV isolates using MEGA4, for constructing phylogenetic tree [14].

Results and Discussion

In this study, the complete genome of six components of BBTV isolate HB-TN was cloned and sequenced. The size of the DNA fragment of components DNA-R, DNA-U, DNA-S, DNA-M, DNA-C and DNA-N components of BBTV isolate HB-TN were 1111, 1060, 1075, 1043, 1018 and 1089 bp respectively. Sequence comparisons showed that the DNA-R component had 93–99% nucleotide (nt)/ 95–98% amino acid (aa) and 84–89% nt/92–94.4% aa sequence identity with of the corresponding gene of South Pacific group and Asian group isolates, respectively. The full-length DNA-S of the HB-TN isolate had 95–100 and 94% nt identity with isolates of the South Pacific group and Asian group, respectively. As functional protein is not encoded by DNA-U, the aa sequence identity was not analyzed. The DNA components M, C and N of the HB-TN isolate showed 94–99% nt and 95–100% aa sequence identity with the corresponding sequences of BBTV isolates of the South Pacific group. But the same isolate had 79–86% nt and 80–85.7% aa identity with the isolates of the Asian group. A Neighbor-Joining phylogenetic dendrogram for all six components of the HB-TN isolate based on full length nucleotide sequences is shown in Fig. 1. All the six components of HB-TN isolate clustered with

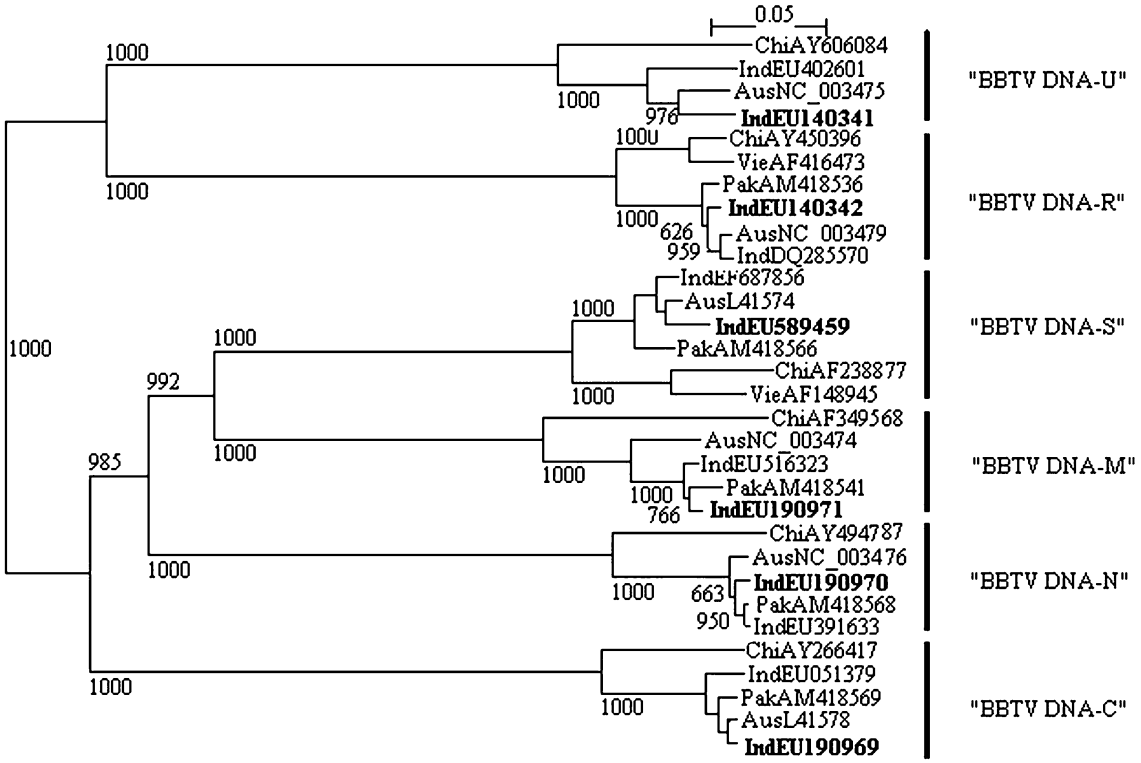

Fig. 1 Phylogenetic dendrogram based on nucleotide sequence alignment for all six components of BBTV HB-TN isolate (highlighted with *bold letters*). Bootstrap values for internal support of the branches are given along the branches. Accession numbers are preceded by abbreviations for the places of origin in Table 2

Table 3 Percent nucleotide and amino acid sequence identity of coat protein (CP) and replicase (Rep) gene of BBTV isolates with South Pacific and Asian group of isolates

BBTV isolates	Percent nucleotide/amino acid homology					
	Rep gene			CP gene		
	Hill isolates	Asian group	South Pacific group	Hill isolates	Asian group	South Pacific group
Rep hill isolates	–	93–95/91–93	97–100/98–99	–	–	–
Rep plain isolates	97–100/98–100	91–95/90–93	95–100/97–100	–	–	–
CP hill isolates	–	–	–	–	89–93/90–97	95–99/95–98
CP plain isolates	–	–	–	95–99/94–98	90–94/94–99	96–100/99–100

isolates of the South Pacific group and the isolates of Asian group clustered as separate clade. Our results corroborated with the results of Vishoni et al. [19] who have reported that the BBTV isolate occurring in Lucknow, North India was belong to the South Pacific group.

The species demarcation criteria for nanoviruses was based on amino acid identities of the CP gene and the members of distinct species should be <85% similarity (Vetten et al. 18). Karan et al. [8] characterized BBTV isolates into two distinct groups viz., Asian and South Pacific groups based on the sequences of DNA-R. We have cloned and sequenced both the CP (513 bp) and Rep (861 bp) genes from thirteen isolates and CP alone from three isolates collected from different geographic areas to assess genetic variability among the Indian isolates. When the CP gene sequences of 16 isolates were compared with isolates of South Pacific group, 97–100 nt/98–100% aa similarity was observed for 14 isolates. Interestingly, CP gene sequences of two isolates namely, Shervroy hill and Kodaikanal (Upper Pulney hill) of Tamil Nadu had high divergence when compared with either South Pacific group isolates (94–95 and 96–97% aa identity, respectively) or Asian group isolates (89–92 and 89–93% aa identity, respectively). This variability was observed only with the

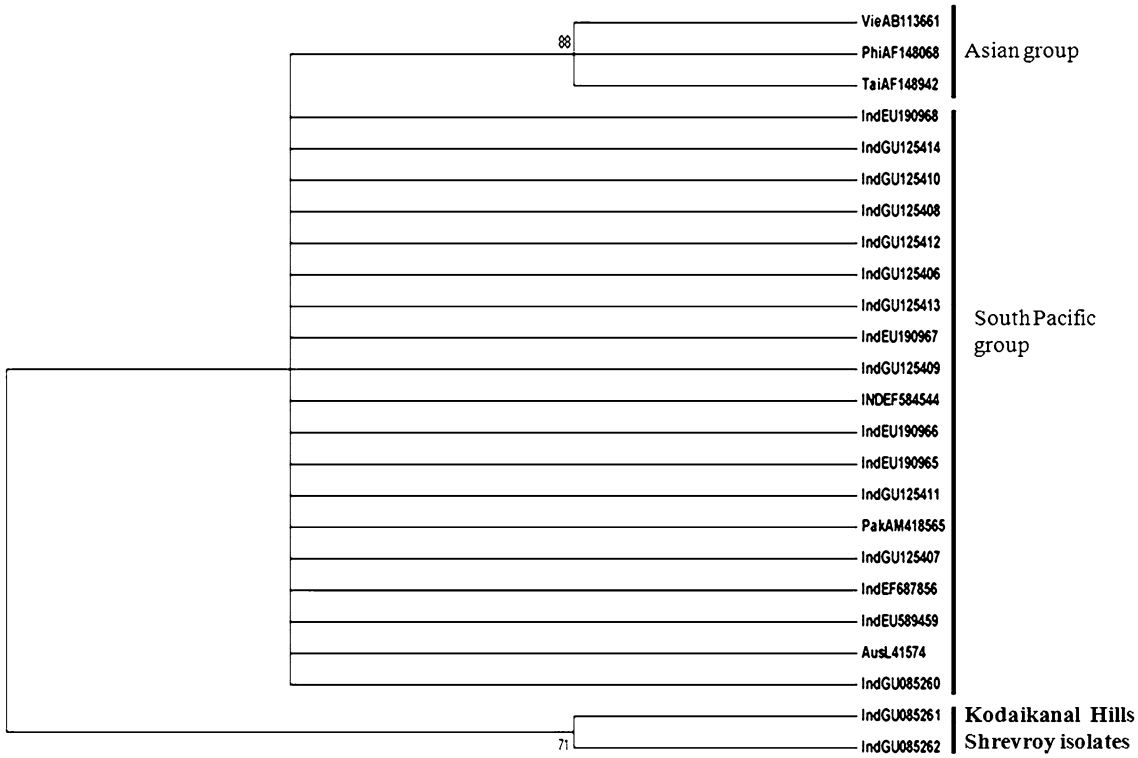

Fig. 2 Neighbour-joining dendrograms illustrating aa sequence of the CP gene of BBTV isolates. Bootstrap values for internal support of the branches are given along the branches. Accession numbers are preceded by abbreviations for the places of origin in Table 2

CP gene sequences but not with replicase sequences of the Shervroy and Kodaikanal hill isolates and this might be due to persistence of the virus in the cultivar Virupakshi (Syn: Hill banana) in the hill regions for long period. The replicase nt/aa sequences of the thirteen isolates had higher homology (97–100/98–100%) to the sequences of the corresponding gene of South Pacific group isolates (Table 3). In the case of BBTV isolate from Meghalaya, 23 nt substitutions were observed mostly in the third codon of the triplet leading to no change in aa composition.

A phylogenetic tree was constructed only using the aa sequence of the coat protein gene of BBTV isolates since variability was observed only with CP sequences of two BBTV isolates. The phylogenetic analysis indicated that the isolates of Shervroy hill, Kodaikanal grouped together and formed into a separate cluster (Fig. 2) whereas remaining Indian isolates formed the core of South Pacific group. Wanitchakorn et al. [20] reported that high degree of divergence in the sequence of CP gene of Asian group. Our study revealed that the Indian BBTV isolates from relatively distinct geographical origins belongs to South Pacific group except Shervroy and Kodaikanal hill isolates which neither belongs to the South Pacific nor the Asian group based on aa sequences of CP gene. Significance of this variability with respect to the CP gene of these two isolates is not known. This is the first report of molecular characterization of a complete genome of BBTV occurring in lower Pulney hills, Tamil Nadu, India. Based on our study with Indian isolates, we conclude that the amino acid sequences of BBTV Rep remain highly conserved, with a maximum of 2% sequence variation between all isolates in this study. However, there was a significantly higher degree of divergence (up to 4%) between amino acid sequences of BBTV CP among Indian isolates.

In India, annually more than 50 million tissue culture banana plants are produced. Virus indexing and certification for tissue culture plants is becoming mandatory in India. In this context, PCR-based diagnosis of BBTV targeting Rep gene would be helpful in certification of tissue culture plants. Further, we suggest that characterization of complete genome segments of more number of BBTV isolates originating from different agro-climatic zones of the country may be necessary to assess the extent of conservation of sequences for developing robust diagnostic assays for the detection of molecularly distinct isolates of BBTV.

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