Molecular Analysis of *Banana bunchy top virus* First Isolated in Bali, Indonesia

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Abstract: Banana (*Musa* spp.), locally known as 'Pisang' in Indonesia, is considered one of the principal crops consumed as food, either fresh, cooked or processed, or for religious purposes. This study was conducted mainly to determine the occurrence and molecular characterization of *Banana bunchy top virus* (BBTV) as well as possible detection of *Banana bract mosaic virus* (BBrMV) in Bali, Indonesia using both serological and molecular assays. Among the 8 banana isolates representing 2 banana cultivars, Pisang Sari (AAB) and Pisang Susu (AAA), we detected BBTV using specific antibody and primer pairs on 8 samples, whereas no samples were found positive to BBrMV. The full-length nucleotide DNA-R region and putative amino acid of the replication initiation protein gene were sequenced and showed 92.2 - 99.7% and 95.1 - 99.6% identities, respectively, with those previously isolated BBTVs from Indonesia and other isolates from the Asian group. The newly characterized BBTV isolates from Bali were grouped with Asian isolates based upon the constructed phylogenetic tree. Moreover, this study showed the first molecular diagnosis and characterization of BBTV in Bali and its rapid spread in the area was considered to be partly discussed due to importation of planting materials and presence of insect-vector aphids, *Pentalonia nigronervosa.*

Key words: banana, *Banana bunchy top virus*, *Banana bract mosaic virus*, Bali, Indonesia

Introduction

Banana bunchy top disease (BBTD) is the most destructive viral disease of *Musa* spp. including banana and abaca, caused by *Banana bunchy top virus* (BBTV). The disease in banana in particular was first reported from Fiji in 1879 (Magee 1927). Since then, it has spread to other countries worldwide (Dietzgen and Thomas 1991) and even to Hawaii (Hu *et al*., 1993). BBTV is considered the ultimate biological threat in banana production areas in many countries (Raymundo and Bajet 2000) because it can be easily transmitted persistently by aphids, *Pentalonia nigronervosa* Coq. in the field and also via vegetative propagation. BBTV can be retained in the aphids system during their life cycle of 15-20 days (Hu *et al*., 1996). BBTV is a phloem-limited virus which causes economic damage in both yield and quality. The disease can spread rapidly and can wipe out the whole plantation resulting in 100% yield loss (Dale 1987). BBTV is a member species of genus Babuvirus with at least 6 cssDNA components (Burns *et al*., 1995) in individual spherical virus particles, about 18-20 nm in diameter.

Isolates among BBTV occurring in different countries are known to be serologically indistinguishable. However, on the basis of DNA-R nucleotide sequence, two virus groups have been identified as South Pacific (Australia, Hawaii, Fiji, Egypt, Tonga, Pakistan, Burundi, and India) and Asian (China, Taiwan, Philippines, Vietnam and Japan) (Tahira Yasmin *et al*., 2005, Furuya *et al*., 2004).

Among various fruits, banana and plantain are one of the most important in Indonesia. They are grown on about 74, 751 ha with a total production of 4.5 million tons (Anonymous 2003).

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Their major diseases in Indonesia are Fusarium wilt (Panama disease), bacterial blood disease (BBD), Moko disease by *Ralstonia solanacearum*, *Banana streak virus* (BSV) (Furuya *et al*., 2005b), and BBTV. BBTV is the most devastating virus pathogen of banana which was first reported in Java Island, Indonesia in 1978 (Sulyo *et al*., 1978). BBTV is observed widely in Sumatra and Java Islands, and molecular characteristics were studied for BBTV in Java Island (Furuya *et al*., 2005a). In Bali Island, however, the official record of this virus was not made until now. In our survey, we first observed BBTV symptoms on some banana plants in Denpasar, Bali, and confirmed BBTV infection by ELISA in 2006 (data not shown). The banana plants with BBTV symptoms in Bali are commonly observed, recently.

Banana bract mosaic virus (BBrMV) on the other hand, is widespread in other Asian countries but not yet observed in Indonesia. BBrMV from the genus Potyvirus is ssRNA with an approximate virion size of 725 nm with 38 kDa coat protein (Thomas *et al.,* 1997). The virus was first reported in bananas in the Philippines (Magnaye and Espino 1990) and later on identified in India (Kokkan disease), Sri Lanka, Vietnam and West Samoa (Rodoni *et al.,* 1997; Lockhart 2002) which recorded 40% yield reduction (Magnaye and Espino 1990). In this study, BBTV Bali isolates were molecularly analyzed for the first time to compare other Indonesian and Asian isolates. Protection of BBTV in Bali was also discussed. Possible occurrence of BBrMV was also considered for quarantine purposes.

Materials and Methods

Plant material, virus isolates and serological assay

Two naturally infected banana cultivars, Pisang (P.) Susu (AAA non-Cavendish type) and Pisang (P.) Sari (AAB dessert type) were collected in Bali, Indonesia (**Fig 1**). All samples were tested for BBTV and BBrMV using enzyme-linked immunosorbent assay (ELISA). Commercially available antibodies and enzyme-conjugated antibodies (Agdia, Elkhart, USA) were used. ELISA was performed according to the manufacturer's protocol. 0.1 g leaf sample were homogenized in 1ml of the extraction buffer (Agdia, Elkhart, USA). 100µl of the crude extract were added into ELISA plate and allowed to react separately on BBTV- and BBrMV- specific antibody. 40 min after adding substrate solution, absorbance value at 405nm was measured using a BIO-RAD Model 680 (Bio-Rad Laboratories, Inc., Chantilly, USA). Samples were considered as positive when absorbance value was at least three times greater than the absorbance value of the healthy control.

Total nucleic acid extraction, primers and Polymerase chain reaction (PCR)

Total nucleic acid was extracted from 0.1 g of leaf samples using PhytoPure Plant DNA Extraction kit (GE®Amersham Biosciences, Little Chalfant, UK). To amplify the complete DNA-R component, D11 forward (5' - GGAAGAAGCCTCTCATCTGCTTCAGACARC – 3') and D12 reverse (5'- TTCCCAGGCGCACACCTTGAGAAACGAAAG – 3') set designed by Karan *et al.* (1994) was used. Total nucleic acid (2.5 µl) was mixed with 22.5 µl PCR cocktail consisting of 2.5 µl of 10x *Ex Taq* buffer, 2 µl dNTP mixture, 0.25µl of each primer (25pmol), 0.1 µl Takara Ex Taq™ (TaKaRa, Shiga, Japan) as the DNA polymerase, and 17.4 µl of double distilled water (q.s) containing 0.2% skimmed milk (De Boer *et al.,* 1995) to avoid possible latex interference during PCR detection. PCR program consisted of one cycle of 94°C for 4 min; 29 cycles of 94°C for 1 min, 61°C for 1 min, and 72°C 2 min; and final extension at 72°C for 10 min. PCRs were performed in a Gene Amp PCR system 9600 (Perkin Elmer, Norwalk, USA).

For BBrMV detection, immunocapture reverse transcriptase PCR (IC-RT-PCR) by Dassanayake (2001) was employed with the following modifications. Commercially available monoclonal antibody specific for BBrMV coupled with primer pairs Bract1 (5' GACATCACCAAATTTGAATGGCACATGG 3') and Bract 2 (5'CCATTATCACTCGATCAATACCTCACAG 3') by Rodoni *et al.* (1997) were used for more sensitive detection. Tubes were coated with anti-BBrMV capture antibody (Agdia) at 1:200 with coating buffer (Agdia) and incubated at room temperature for 3 h. Tubes were then washed with PBS-T three times at 3 min interval. Samples (0.1g) were homogenized in 0.5ml general extract buffer (Agdia) (pH 7.4) and centrifuged for 5 min at 14000 rpm. Fifty µl of plant extract was added in pre-washed tubes and incubated overnight at 4°C. After incubation, tubes were washed again 3 times with PBS-T and air-dried at room temperature for 5 min, centrifuged at 3000 rpm for 3 min. One µl of Oligo dT primer (0.5g/µl) (TOYOBO, Osaka, Japan) and 23µl of RNAse free water were added. Tubes were heated at 80°C for 10 min, chilled on ice for 5 min and spun down briefly. The following mixture was added to the tubes: 8µl 5x 1st strand buffer (TOYOBO), 4µl of 0.1M DTT, 1µl of ReverTra Ace™ reverse transcriptase (TOYOBO), 2µl of 10mM dNTPs, and 1µl RNAse inhibitor (TOYOBO). For reverse transcription, tubes were incubated at 42°C for 1 h, 75°C for 15 min, and chilled on ice for 10 min. For PCR, 3 µl of first-strand cDNA was mixed with 2.5µl 10xPCR buffer, 2µl of 25mM $MgCl_2$, 1µl of 10mM dNTPs (Invitrogen, Carlsbad, USA), 1µl each of 25pmol forward and reverse primers, 0.25µl of TaKaRa Ex Taq™ (TaKaRa), and 14.25µl and double distilled water (q.s). The PCR program consisted of one cycle of 94°C for 1 min; 32 cycles of 94°C for 30 sec; 56°C for 1 min and 72°C for 1 min; one

cycle of 72°C for 3 min and finally soaking at 4°C for 2 h prior to electrophoresis.

Analysis of PCR product

PCR products were separated by electrophoresis using 1.5% agarose gel (Roche, Mannheim, Germany) in 1xTAE (40 mM Tris-acetate, pH 8.0 plus 1 mM EDTA). The 100-bp DNA Ladder set (Promega, Madison, WI, USA) was included as size marker. After electrophoresis, the gel was stained with ethidium bromide (0.5 µg/µl), visualized and analysed by the AlphaImager 2000 Documentation & Analysis System (Alpha Innotech Co., San Leandro, USA) and DAS290 (KODAK,Tokyo, Japan).

Cloning, sequencing and analysis

Three samples; Bal-2 isolate from P. Susu in Tukad Badung II, Bal-4 from P. Susu in Tukad Petanu I, and Bal-6 from P. Sari in Sempidi I (**Table 1**) were used for cloning and sequencing. Approximately 1.1 kbp fragments by agarose gel electrophoresis were purified and eluted from the gel using a Wizard® SV Gel and PCR Clean-Up system (Promega) and ligated into pGEM® T-vector system (Promega) according to the manufacturer's instruction. The recombinant plasmids were transformed into cells of *Escherichia coli* strain JM109 (TaKaRa) and the potential recombinant clones were identified by screening on 2% 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) substrate. Purified plasmids were sequenced directly using LaboPass Plasmid Mini – Plasmid DNA Purification kit (Hokkaido System Science Co., Ltd., Sapporo, Japan), an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, USA or Tokyo, Japan), and a 3130/3130*xl* Genetic Analyzer System StdSeq50_POP7 v3 (Applied Biosystems). At least three clones of each PCR fragment were sequenced in both directions using Sp6 and T7 primers. Sequence data was analyzed using Blastn (http://www.ncbi.nlm.nih.gov) for homology search, and BioEdit (http://www.mbio.ncsu.edu/BioEdit) for consensus sequence determination. Multiple alignments for sequence comparisons were conducted with BBTV isolates from 16 other countries including isolates from Indonesia using ClustalW (http://clustalw.ddbj.nig.ac.jp). Phylogenetic tree was constructed using neighbor-joining (NJ) methods (Saitou and Nie 1987) with MEGA version 4 software (Tamura *et al.,* 2007). Genetic distance was estimated using Kimura's two-parameter distance method (Kimura 1980), with 1000 replications bootstrap analysis for the statistical validity.

Results and Discussion

Samples and serological assay

We detected BBTV in all banana samples except for Pisang Sari (Sempidi II) by ELISA (**Table 1**). All samples except Bal-7 (from P. Sari in Sempidi II) showed strong positive reaction against anti-BBTV antibody by ELISA. However, no samples reacted to anti-BBrMV antibody although positive control did. These results revealed that the banana samples collected from Bali, Indonesia were positive to BBTV and the occurrence of BBrMV has not yet been detected using serological assay.

Molecular detection and characterization of BBTV and BBrMV

A total of 8 banana samples were applied for molecular detection of BBTV and BBrMV (**Table 1**) by PCR and IC-RT-PCR, respectively. Specific primers D11/D12 by Karan *et al*., (1997) for major component DNA-R could amplify the target DNA from the samples with an approximate size of 1.1kbp. However, no band and weak band were observed from isolate Bal-7 (from P. Sari in Sempidi II) and Bal-8 (from P. Susu in Pegok I) (**Fig. 2**). No DNA amplification was observed from any samples using Bract1 and Bract2 primers which are known to amplify the coat protein (CP) region and the 3' untranslated region (UTR) of BBrMV. Sequences of BBTV DNA-R of 3 banana samples, Bal-2, Bal-4, and Bal-6 compared with two sub-groups (Asian and South Pacific) of BBTV isolated from banana. The DNA-R component of the 3 isolates comprised 1,104 nucleotides, and had similar genome composition with BBTV banana isolates belonging to Asian group. Low percent identities (88.7 – 89.5%) were obtained from the South Pacific group of BBTV isolates. The DNA-R of 3 isolates had fully conserved major common region (CR-M, from 693 to 774 nt) having 15 nucleotide GC-rich component (761-774 nt), and stem-loop common region (SL-R, 797-852 nt) with conserved loop sequence TATTATTAC (833-842 nt), polyadenylation signal (CATAA, 164-168 nt) and a potential TATA box (CTATAAATA, 872-880 nt) within Indonesian and Asian group BBTV isolates. The nt sequences of 3 isolates showed high percentage identities between Indonesian isolates (98.4 – 99.7%) and among Asian group including Philippines, Japan and Taiwan (98.2– 98.7%), and China (95.9 – 96.1%) and Vietnam (92.2%) isolates (**Table 2**).

Major open reading frame (ORF) was found on the DNA-R, ORF 1 of 861 nts (103 to 963 from the SL-R). The putative aa sequences of the ORF1 which codes the replication initiation protein (Rep) gene were also compared. High degree of identities between Indonesian isolates and among Asian groups (95.1 – 99.6%) was noted. In the deduced ORF 1 of DNA-R, a dNTP-binding motif, GGEGKT was also confirmed in all isolates. However, comparing the obtained 286 putative aa sequences of the replication initiation protein gene between the 3 BBTV isolates, leucine to arginine replacement was observed at aa

position 54 and lycine to threonine change in the next aa position of Bal-6 isolate (**Fig 3**). However, among BBTVs from Indonesia, these 3 BBTV isolates corresponded with the 2 previously characterized Indonesian BBTVs (IJs11 and IG64) at aa position 170 compared to IG33.

Phylogenetic analysis

To further support the close relationship of BBTV Balinese isolates with other Indonesian and Asian isolates, a phylogenetic tree on the basis of BBTV DNA-R nt was constructed. In the phylogenetic tree the full-length nt of DNA-R was obtained using the neighbor-joining (NJ) method and bootstrap with 1000 replicates for statistical validity. The Asian and South Pacific groups separated into two major clusters. Moreover, in the Asian group, isolates were divided into four clusters (**Fig 4**). Cluster A included the isolates from Bali along with two BBTV isolates previously identified in Indonesia (IJs11 and IG64), while one isolate from Indonesia (IG33), Philippines (Ph), Japan (JN4) and Taiwan (Tw) belong to cluster B. Clusters C and D corresponded to Guangdong, China (C-NSP) and Ho Chi Min, Vietnam (HCM-Vt), respectively. The 3 Balinese isolates analyzed in this study were therefore clustered with the 2 previously isolated BBTV from Indonesia and closely grouped along with IG33, Ph, JN4 and Tw.

In Bali, both table banana (banana) and cooking banana (plantain) are planted by small holders with minimum management. The varieties grown in Bali are Pisang Ambon (AAA), Pisang Hijau (AAA), Pisang Mas (AA), Pisang Kepok (ABB), Pisang Klutuk (BB), Pisang Raja (AAB), Pisang Sereh (AAB) as well as in other islands. Other banana varieties such as Pisang Andong (unidentified), Pisang Gancan (unidentified), Pisang Gedang (unidentified), Pisang Kayu (AAA), Pisang Ketip (unidentified), Pisang Sari (AAB) and Pisang Temaga (unidentified) are cultivated specifically in Bali mostly for religion purposes. As Bali is a tourist attraction area, Cavendish banana and Pisang Raja are grown and also imported from Lampung (Sumatra) and Java. In Bali, there is no commercial banana farm. Most of bananas are produced in the back yard gardens of small scale farmers and consumed domestically while some are sent to the local markets. The farmers input least fertilizer and chemicals to have considerable harvest by minimum pest and disease control. Currently, banana Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (Foc) is recognized as a serious problem in Bali (Suprapta *et al*., 2005). To protect bananas and to substitute for dead plants, disease-free banana seedlings were produced in Bali. The projects named PERSADA were conducted by the Bali government in 2002. The purpose of the project was to grow banana protected from wilt disease by application of a mixture of *Gleocladium* spp., an avirulent strain of *Fusarium oxysporium*, *Streptomyces* spp. and *Bacillus* spp.

In contrast, for BBTV, the symptoms were observed in Denpasar, Bali, in 2006 (data not shown). Now, only about four years after the first observation, the disease can be found anywhere around Bali. The fast spread of the disease in Bali may not be surprising because *Pentalonia nigronervosa*, an effective insect vector of this virus, can be found colonized on banana plants year-around. However, at present, no BBTV protection measure has been taken officially. The BBTV in Bali is likely brought from Java Island via BBTV infected banana seedlings. This suggestion is based on the fact that the production level of banana in Bali is much lower than the demand of about 150,000 tons a year. This high demand for banana, especially for religious activities in Bali, is estimated to be more than 70% of the total demand. And such banana fruits are mostly supplied from Java (Suprapta, 2005). This observation was also confirmed by the results of this study showing the close phylogenetic relatedness between Java and Bali isolates.

In this study we confirmed the presence of BBTV among banana cultivars Pisang Sari (AAB) and Pisang Susu (AAA) in addition to other banana genomes susceptible to BBTV in Bali using both serological and PCR assays. Based upon our molecular comparison, these isolates belong to Asian group along with Indonesia, Philippines, Japan and Taiwan rather than with China and Vietnam. High identity both in the full-length DNA-R nt and deduced aa sequence of the Rep gene between Balinese isolates and isolates of Asian group were obtained. Despite the minor change in the 286 aa sequence at positions 54, 55 and 170 observed between and among Indonesian isolates, the newly characterized isolates showed high percentage identity with the Asian group (IJs11, IG64, Ph, JN4 and Tw). The motifs including the TATA box (CTATAAATA), polyadenylation signal (CATAAA) and GC-rich box are believed to be important in the viral replication and promoter elements were highly conserved between all isolates (Wanitchakorn *et al.,* 2000). Moreover, variation in the sequences of BBTV genome has been reported among isolates from the same country of origin due to different sizes of PCR products or the occurrence of additional amplicons (Su *et al*., 2003). These results were further supported by the phylogenetic analysis based on the nt sequence of the full-length DNA-R component. The analysis revealed that Balinese isolates formed clusters with isolates from Indonesia, Philippines, Japan and Taiwan. Hence, BBTV isolates from these countries have a single origin and evolved independently through time. The distant relationship of Balinese isolates along with other Asian isolates to China and Vietnam was rather due to high mutation and divergence of the later two isolates. The NSP isolate from China in particular, is known to be more prone to high mutation than any other isolate (He *et al*., 2000), whereas in Vietnam, BBTV isolates consist of 8% divergence (Bell *et al*., 2002).

On the other hand, BBrMV was not detected in any of the banana samples from Indonesia using serological and IC-RT-PCR. However, the disease poses quarantine risk like BBTV since it is quite difficult to identify because of confusing symptomatology and infectivity to other banana.

Considering the significance of banana production in Bali, both as a staple and in the local economy, occurrence of BBTV in Bali is very serious. In Bali, banana plants infected by BBTV can be the source of the virus transmission by aphids or by use of suckers from infected mother plants. To protect further spread of BBTV, early diagnosis of the disease and eradication of the infected plants are requested. In commercial banana production in some countries, the replacement of virus free seedlings is conducted. Recently, BBTV resistant banana line is produced by mutation breeding (Damasco *et al*., 2005). Until such a resistant variety becomes available, the use of local resistant variety is recommended. For example, one banana variety present in Bali and also in other area of Indonesia, Pisang Klutuk, is not affected by the bunchy top disease symptom. This banana variety may have some degree of resistance to BBTV.

For the breeding of resistant varieties and other research activities, development and use of molecular diagnosis is requested. Although serological assay in the form of ELISA can be used in a large number of samples, its reliability can be compromised by low virus titer. BBTV used to occur in low concentration in banana-infected plant or plant parts (Harding *et al*., 1991). Thus, the use of PCR is necessary for early and accurate diagnosis of BBTV even in the symptomless young plants. Molecular analysis also gives the understanding from where BBTV was brought to Bali. At present, based upon the phylogenetic analysis in this study, BBTV Bali was shown to share very high homology with other Indonesian BBTV isolates. This technology will be also useful to find BBTV imported newly from foreign countries at the time of importation of new planting materials such as tissue cultured bananas.

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インドネシアバリ島で初めて分離したバナナバンチートップウイルスの分子生物学的解析

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要約；バナナ（*Musa* spp.） はインドネシアのバリ島において果物としてだけでなく調理用としても重要であり、また、宗教儀式でも多用される。4 年ほど前までバリ島においてバナナバンチートップウイルス（*Banana bunchy top virus*; BBTV）による被害はほとんど認められなかったが、近年、急速に被害が広がったことを観察した。そこで、バリ島のバナナにおいて、BBTV と、同じくバナナの重要ウイルスである *Banana bract mosaic virus* (BBtMV)の検出を血清学および分子生物学的技術で実施した。その結果、2 種のバナナ品種 Pisang Sari (AAB)と Pisang Susu (AAA)から BBTV が 検出され、その DNA-R の全塩基配列を解析したところ、アジアグループに属し、また、ジャワ株、フィリピン株などとの類縁度が高いことが明らかになった。しかし、BBrMV は検出されなかった。多くのバナナがジャワ島からバリ島に移入されており、また、病害に対する特段の防除が行われておらず、さらに BBTV の媒介虫（*Pentalonia nigronervosa*）の存在が、BBTV の侵入と拡大に寄与したと考えられた。

キーワード：バナナ, *Banana bunchy top virus, Banana bract mosaic virus,* バリ島, インドネシア

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