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Virus Detection from Local Banana Cultivars and the First Molecular Characterization of *Banana bunchy top virus* in Indonesia

By

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Summary : For banana plants (*Musa* spp.), occurrence of viruses is serious threat not only for production but also in conservation of banana species/cultivars as genetic resources. In this study, we collected 68 samples of 38 banana cultivars during 3 years of survey and carried out the detection of viruses by serological and/or molecular methods in a banana germplasm garden of central Java, Indonesia. *Banana bunchy top virus* (BBTV) and *Cucumber mosaic virus* (CMV) were confirmed from 21 samples and 3 samples, respectively, though *Banana bract mosaic virus* (BBrMV) was not detected in this survey. Thus it was made clear that the major constraint virus is BBTV in this germplasm garden where it should be free from any virus infection. The occurrences of BBTV were confirmed in 12 cultivars and of CMV were confirmed in 3 cultivars among 38 cultivars along with viral symptoms though all of tested cultivars were grown under the same condition for long time. Based upon this results, it was supposed that these 12 and 3 cultivars are more sensitive to BBTV and CMV infection, respectively. Two BBTV isolates (BBTV-IG33 and IG64) in this germplasm garden and one isolate of BBTV (BBTV-IJs11) from a village nearby the germplasm garden were sequenced for their DNA-1 and DNA-3 and shown to have high homologies among them in full-length of each component (98 to 99% and 99 to 100%, respectively). In major common region (CR-M) of DNA-1, the Indonesian isolates showed higher homology obviously with isolates from Japan, Taiwan, the Philippines, China and Vietnam than with isolates from Australia and Fiji (93 to 95% and 63 to 65%, respectively). Therefore, molecular characters of these Indonesian BBTV isolates, which were classified into the Asian group (KARAN *et al.*, 1994), were firstly reported and analyzed in this study.

Key words : virus detection, Indonesia, banana cultivar, *Banana bunchy top virus*, nucleotide sequence

Introduction

Banana (*Musa* spp.) is one of the most important tropical and subtropical crops and its origin is known as Malay Peninsula and the neighborhood. In Indonesia, since geographic location is close to the origin of banana, the diversity of banana plants is observed in local regions. Consequently, various cultivars of both dessert- and cooking-type bananas were produced total 3.7 million tons, approximately 10% share of it in Asia, in a year 2003 mainly for domestic market (FAO). In

Indonesia, under the supervision of Gadjah Mada University, more than 300 cultivars of banana have been collected from all over the country and grown for conservation of biological resources. However several virus-like symptoms have been observed in the germplasm collections.

Two banana viruses have been previously reported prevalent in Indonesia¹⁰. Among them, *Banana bunchy top virus* (BBTV), genus *Babuvirus*, is the most destructive banana virus in the world except American region and causes banana bunchy top disease. The isometric

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virions of 18–20 nm in diameter contain at least six circular ss-DNA components of approximately 1 kb each and are transmitted by banana aphid, *Pentalonia nigronervosa* Coq. Another virus, *Cucumber mosaic virus* (CMV), belongs to *Cucumovirus* and has spherical particles 28–30 nm in diameter including tripartite genome with ss-RNA. CMV has worldwide distribution and is a causative virus of banana mosaic disease. Host range of CMV is wide and is transmitted by more than 60 species of aphids. Moreover, in Indonesia, it has been known one more disease, banana streak disease, only by symptoms¹⁰. However, in this study, we did not work on *Banana streak virus* (BSV), genus *Badnavirus*, because the characterization of BSV is under development due to integration to host genome and high serological and genomic variability^{6,4,7}. Additionally two potyviruses, *Banana bract mosaic virus* (BBrMV) and *Abaca mosaic virus* (AbaMV), are known to occur on banana in some parts of the Asia¹⁰, though the occurrence of them has not been reported in Indonesia.

This work reports the occurrence of viruses in local banana cultivars detected by serological and/or molecular methods to show the viral epidemiology in Indonesia and find sensitive cultivars against viruses. The sequences of DNA-1 and DNA-3 of isolates of BBTV are also studied as the first report of molecular analysis of Indonesian BBTV.

Materials and methods

Collection of banana cultivars

We collected banana leaves showing symptoms of viral diseases like bunchy top, mosaic, and mottle or no symptoms in the banana germplasm garden at Yogyakarta District, Yogyakarta Special Territory, Indonesia once a year from 1998 to 2000. Along with sixty-eight samples of 38 cultivars, 1 sample, observed typical bunchy top symptoms in a village nearby the banana germplasm garden was stored in -30°C until use. Among them, BBTVs which were detected from three samples of banana (IG33, IG64 and IJs11) were used for molecular analysis. Virus isolates IG33 and IG64 were obtained from banana cultivars of Mas and Ambon Warangan, respectively, and IJs11 was from a banana plant of unidentified cultivar. These banana samples from Indonesia and one abaca plant from the Philippines were imported under the permission of the Ministry of Agriculture, Forestry and Fisheries, Japan.

Detection of BBTV, CMV and BBrMV by ELISA

Three ELISA kits (Agdia, USA) for three banana viruses (*Banana bunchy top virus*; BBTV, *Cucumber*

mosaic virus; CMV and *Banana bract mosaic virus*; BBrMV) were used according to the manufacturer's instructions. The sample which showed 3 times higher absorbance value at 440 nm than healthy banana sample was evaluated as positive to each of the viruses.

Detection of BBTV by PCR

Total DNA was isolated from small pieces of banana midribs (0.1 g) using a PhytoPure DNA extraction kit (Nucleon, UK). Primers FPCR4 (5' TTC CCA GGC GCA CAC CTT GAG AAA CGA AAG 3', nts 284–313) and F3 (5' GGA AGA AGC CTC TCA TCT GCT TCA GAG ARC 3', nts 287–258) designed by KARAN *et al.*¹¹ and TaKaRa Ex Taq™ (TaKaRa, Japan) were used in PCR to amplify complete BBTV DNA-1. PCR conditions were 4 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C and finally an extension time of 10 min at 72°C in a thermal cycler (Gene Amp PCR System 9600, Perkin Elmer). To avoid possible interference by latex or other banana leaf components during PCR reaction, 0.1% skimmed milk was added to the PCR cocktail³.

Detection of potyviruses by RT-PCR

RT-PCR detection by potyviruses universal primer sets was conducted on banana samples which showed positive reaction to BBrMV ELISA kit (Agdia, USA). After small pieces of banana leaves (0.1 g) were ground with sterilized PBS, the crude sap including putative virus particles depend on sample was incubated in PCR tube to be trapped to the wall. Total nucleic acids were derived by washing with sterilized PBST and heat shock. First-strand cDNA was synthesized using ReverTra Ace® (TOYOBO, Japan) according to the manufacturer's instructions with Oligo d(T) primer, as the initial primer. PCR was carried out using TaKaRa Ex Taq™ (TaKaRa, Japan). Primers Poty 5' (5' GGA TCC GGB AAY AAY AGY GGD CAR CC 3') and Poty d (T) 3' (5' CAC GGA TCC CTT TTT TTT TTT TTT TTT V 3'), from Gibbs and Mackenzie⁵ with minor modification, were used to amplify approximately 1.7 kbp nucleotides including coat protein gene of *Potyviridae*. One abaca (*Musa textilis*), infecting BBrMV-aP87 from the Philippines, was used as a positive control.

Cloning, sequencing and homology analysis of BBTV DNA-1 and DNA-3

Complete DNA-1 and DNA-3 of three BBTV isolates (IG33, IG64 and IJs11) were cloned and sequenced. Amplification of full-length DNA-3 was carried out under the same PCR condition for DNA-1 except for an annealing temperature of 49°C and the specific primers¹⁶:

CBT3F.PRI (5' GGT ATT TCG GAT TGA GCC TAC 3', nts 356–376) and CBT3R.PRI (5' TTG ACG GTG TTT TCA GGA ACC 3', nts 355–344). PCR reactions were subjected to electrophoresis in agarose gel and stained with ethidium bromide. Each approximately 1.1 kbp fragment was recovered using a QIAquick™ Gel Extraction kit (QIAGEN, Germany). Three isolates (IG33, IG64, IJs11) of BBTV DNA-1 and DNA-3 were ligated into the pGEM-T Vector (Promega, USA) and transformed into *E. coli* DH5 α (Life Technologies Inc., USA). The positive clones were selected by X-gal and IPTG screening. At least three clones of each PCR fragment were sequenced in both directions using an automated ABI PRISM 377 DNA Sequencer with an ABI Prism BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Sequence alignment and homology analysis were carried out using AssemblyLIGN™ 1.0.9 c (Accelrys, USA) and CLUSTAL W package¹³⁾ with Mac Vector 6.5.3 (Accelrys, USA).

Sequences obtained in this study were submitted to DDBJ/EMBL/GenBank database with accession numbers from AB186924 to AB186929. The reported sequences of BBTV DNA-1 and DNA-3 in various parts of the world were obtained from DDBJ/EMBL/GenBank database and used for comparisons: Japan (JN4: AB108452/AB108449, JK3: AB108453/AB108450, JY1: AB108456/AB108451), Taiwan (Tw: AF416468/AF148942), Philippines (Ph: AF416469/AF148068), China (C-NS: AF238874/AF238876, C-NSP: AF238875/AF238877), Vietnam (V6: AF113659/AF113661, V14: AF113660/AF113662), Australia (Au: S56276/L41574) and Fiji (Fj: AF416466/AF148944).

Results

Detection of viruses by ELISA and PCR and/or RT-PCR

BBTV was detected from 21 samples of 17 cultivars, by ELISA and PCR, and CMV was detected from 3 samples of 3 cultivars, by ELISA (Table 1). Mix infection of BBTV and CMV was not confirmed in these samples. Eight samples which were slightly reacted to BBrMV in ELISA were not amplified by RT-PCR for Potyvirus universal primers even though an approximately 1.7 kbp band of BBrMV-aP87 was shown in BBrMV positive sample.

Among 17 cultivars from which BBTV were detected, there were 5 cultivars (Kepok Gabu, Raja Entos, Raja Trunpong, Rejang and Tanduk Hijau) without viral symptoms such as bunchy top observed in other 12 cultivars. As for CMV, mosaic symptom, but not with necrosis, was observed in all 3 cultivars (Klutuk Susu, Raja Bandung, Turi). While both BBTV and CMV were not detected, rosette and chlorosis symptoms which had probability of virus infection were observed on 'Gading' and 'Jiwe!', respectively. Among total 38 cultivars used in this study, BBTV and CMV were not detected from 16 cultivars. Especially from 5 cultivars (Barly, Klutuk Warangan, Koja Srimentak, Pinang and Raja Polo), the viruses were not detected during consecutive survey for 3 years.

Sequence analysis of BBTV

BBTV DNA-1 and DNA-3 of two isolates (IG33 and IG64) from the germplasm garden and one isolates (IJs11) from neighbor village were amplified by PCR and

Table 1 BBTV and CMV detection in the germplasm garden in Indonesia

Virus	Cultivars	
	without any viral symptom	with viral symptoms
BBTV	Kepok Gabu, Raja Entos, Raja Trunpong, Rejang, Tanduk Hijau	Ambon Kuning, Ambon Warangan, Emas Besar, Giwangan Rejan, Jambe Saat, Klutuk, Kongo, Lilin Biotik, Mas, Sri Nyona, Palembang, Ujung Jangkung
CMV		Klutuk Susu, Raja Bandung, Turi
Not detected	Barly, Cavendish 60, Empat Puluh Hari, Kelpak, Kepok Kuning, Klutuk Warangan, Koja Srimentak, Mas Bunga, Mas Raja, Mas Troping, Pinang, Raja Polo, Raja Talun, Sobo Kerik, Sobo Londo, Turi Oring	Gading*, Jiwe! **

* The cultivar with rosette symptom

** The cultivar with chlorosis symptom on the leaf

sequenced after cloning. All DNA-1 and DNA-3 fragments were consisted of 1103 to 1104 nts and 1058 nts, respectively. In both components, the stem-loop sequences were observed in all isolates. Two open reading frames (ORFs) were found on the DNA-1, ORF 1 of 858 nts and ORF 2 of 126 nts, and one ORF of 510 nts was found on the DNA-3 of the Indonesian isolates as previously reported for other BBTVs^{1,15}.

Sequences of all isolates were compared with previously reported sequences. Among Indonesian isolates, though 4 nucleotides of IG33 in DNA-1 were differ from IG64 and IJs11 in approximately 90 nucleotides in major common region (CR-M) of DNA-1, nucleotide sequence in CR-M of DNA-3 were same completely, and they were apparently close to the Asian group than the South Pacific group (Fig. 1).

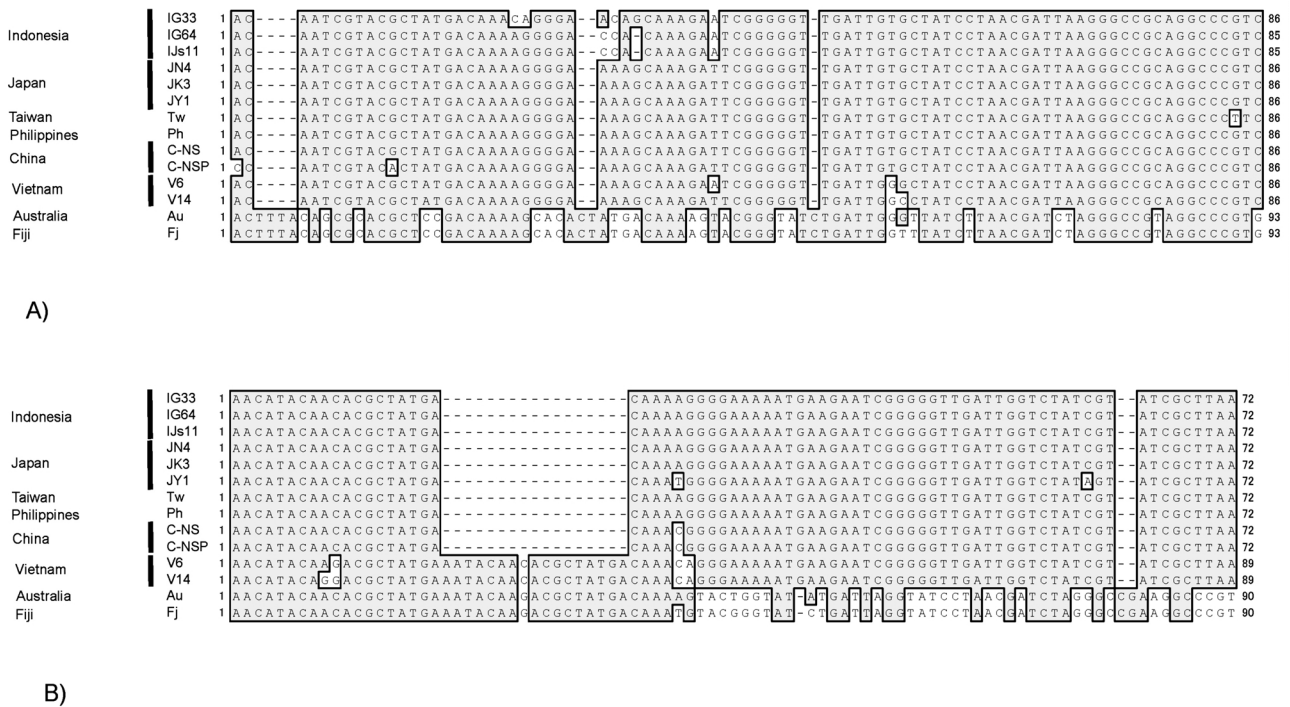


Fig. 1 Comparison of nucleotide sequences in CR-Ms of BBTV : A) DNA-1, B) DNA-3. Dashes mean gaps introduced to maximize alignment

Table 2 Sequence homologies (%) of Indonesian BBTV isolates and their homologies with the other geographical isolates in DNA-1 and DNA-3

		among	between	between
		the Indonesian isolates	the Indonesian isolates and the Asian group	the Indonesian isolates and the South Pacific group
DNA-1	full-length (nt)	98-99	95-99	89
	CR-M(nt)	95-100	93-95	63-65
	master-Rep(nt)	98-99	95-99	91-92
	master-Rep(aa)	99-100	95-99	94
DNA-3	full-length (nt)	99-100	92-99	85-86
	CR-M(nt)	100	76-100	45-47
	CP(nt)	100	95-99	93-94
	CP(aa)	100	94-100	98

nt : nucleotide, aa:amino acid, CR-M: Common Region -Major, CP: coat protein

The three Indonesian isolates were shown to have high homologies within them in full-lengths of DNA-1 and DNA-3 (98 to 99% and 99 to 100%, respectively, Table 2). In CR-M of DNA-1, the nucleotide sequence of Indonesian isolates had a high homology with isolates of the Asian group (93 to 95%) but a low homology with the South Pacific group (63 to 65%, Table 2). Amino acid sequence in CP gene of three Indonesian isolates agreed completely in each other and it was also same even though in a nucleotide level (Table 2). When compared with foreign BBTV in amino acid sequences, Indonesian isolates showed a high degree of homology with foreign isolates except C-NSP from China (98 to 100% and 94%, respectively).

Discussion

In Indonesia, banana bunchy top disease has occurred since 1978 and been considered to reach epidemic level in most banana growing provinces^{12,14}. On CMV, though the occurrence on tobacco was known since 1972, it is said that infection in banana plantings is localized and seldom causes serious outbreaks^{12,14}. However the epidemiology of the causal viruses in local banana cultivars based on serological or molecular measures and the molecular characterization of them have not been surveyed. This study revealed that viruses from bananas with or without viral symptoms were identified from 38 local cultivars in Indonesia, and the partial genetic status of BBTV was determined and evaluated.

In the germplasm garden surveyed, viruses detected were only two species, BBTV and CMV, and the population of BBTV was 7 times higher than that of CMV in this garden. Therefore it was shown that the major constraint virus is BBTV in this germplasm garden. Though BBrMV was not detected in this study, it is necessary to continue taking precautions against it because BBrMV is reported to occur on banana and abaca in Southeast Asia¹⁰.

On BBTV, Damarjati² has cited that Purnomo (1996) confirmed the disease incidence on 3 cultivars (Mas, Ambon Kuning and Ambon Hijau). And Nurhadi and Setyobudi¹² have referred that Muharam (1984) showed 'Ambon Jepang' and 'Ambon Putih' are very susceptible to the virus, meanwhile SULOY *et al.* (1992) reported 4 banana cultivars (Klutuk, Jimbluk, Kapas and Seribu) showed resistance to BBTV out of 30 cultivars tested. However, the methods of virus detection in these studies were not precisely reported. Our study using ELISA and PCR showed that the occurrences of BBTVs in 17 cultivars and CMVs in 3 cultivars among 38 cultivars and this is particular interesting

because they were grown under the same condition for long time and occurrence might reflect the resistance of cultivars against virus infection. Among the cultivars shown to be infected of BBTV or CMV by serological and/or molecular detection, viral symptoms were observed on 12 cultivars out of 17 cultivars for BBTV and 3 cultivars out of 3 cultivars for CMV. Thus it was supposed that these 12 and 3 cultivars are more sensitive to BBTV and CMV infection, respectively, than other latently infected cultivars. Further study by artificial inoculation test is essential for accurate screening of virus resistant cultivars.

We selected DNA-1, which encodes the master-Rep protein gene⁹, and DNA-3, which encodes the coat protein gene¹⁵, and determined and analyzed their sequences of Indonesian BBTV isolates. In our study, the Indonesian BBTVs had a common structure with previously reported BBTVs on their nucleotides in DNA-1 and DNA-3. And the translated CP genes, which were coded in DNA-3, had a high homology (over 98%) with the all isolates from Japan, Taiwan, the Philippines, Vietnam, Australia and Fiji, and the NS isolate from China, but not with NSP isolate from China which was reported to have a unique biological character⁸. Karan *et al.*¹¹ identified that BBTV forms two major groups genetically. They are the Asian and South Pacific groups by comparisons of nucleotide sequences and the Asian group included isolates from three countries (the Philippines, Taiwan and Vietnam) as opposed to the South Pacific group included isolates from seven countries (Australia, Burundi, Egypt, Fiji, India, Tonga and Western Samoa). Because of geographical and historical complex backgrounds in Indonesia, it was expected to detect BBTV isolates with molecular diversity including both the Asian and South Pacific groups. Additionally, the banana plants in the germplasm garden have been collected from many different regions in Indonesia, the detection of different virus strains might be possible. In this study, however, all three Indonesian BBTVs tested belonged to the Asian group with high homology and the genetic difference among isolates from the germplasm garden and a village nearby the garden were not detected. Therefore, it was suggested that a single strain of BBTV, classified to the Asian group, has been introduced and spread in this germplasm garden and the neighborhood.

In this study, as we firstly analyzed Indonesian BBTV isolates for their molecular characterization, further study became possible based upon these data including development of specific primers for detection of BBTV in Indonesia.

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インドネシアにおけるバナナ在来種からの ウイルス検出およびバナナバンチートップウイルスの 分子生物学的初解析

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要約：バナナ (*Musa* spp.) にとって、ウイルスの存在は、生産の場だけではなく、遺伝資源としての種や品種の保存においても重大な脅威である。本研究では、インドネシアのジョグジャカルタ特別州に位置するバナナ品種保存園において、38 品種 68 株のバナナを 3 年間に渡って採集し、血清学的・分子生物学的手法の両方または一方を用いて、ウイルスの検出を行った。その結果、キュウリモザイクウイルス (CMV) は 3 株のみで感染が確認されたのに対し、バナナバンチートップウイルス (BBTV) は 21 株で認められたことから、本来はウイルスフリーであるべき本品種保存園においても BBTV は蔓延していることが示された。また、調査を行った 38 品種は長期間同じ環境条件下で栽培されていたが、BBTV は 12 品種で、CMV は 3 品種でそれぞれ病徴を伴って感染が確認された。このことから、これらの 12 品種と 3 品種はそれぞれ BBTV と CMV に対して、より感受性であると思われる。バナナ品種保存園に発生した BBTV2 分離株 (BBTV-IG33, -IG64) とその近郊の農村に発生した BBTV1 分離株 (BBTV-IJs11) について DNA-1 と DNA-3 の塩基配列を決定した結果、それぞれのコンポーネントの全長で 3 分離株は高い相同性を有していた (98~99%, 99~100%)。また、DNA-1 の CR-M 領域 (major common region) では、既報のオーストラリアおよびフィジーの分離株よりも、日本・台湾・フィリピン・中国・ベトナムの分離株と、より高い値を示した (63~65%, 93~95%)。したがって、インドネシアにおける BBTV アジアグループ (KARAN ら, 1994) の発生とそれらの分子生物学的性状が本研究によって初めて明確に示された。

キーワード：ウイルス検出・インドネシア・バナナ品種・バナナバンチートップウイルス・塩基配列

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