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First Report and Molecular Characterization of Exogenous Banana Steak Mysore Virus from Banana in Indonesia

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ABSTRACT

In Indonesia, yellow streak and necrosis symptoms were observed on banana plants. By species-specific PCR, infection with both Banana streak Mysore virus (BSMyV) and/or Banana streak OL virus (BSOLV) was suspected. To confirm whether the DNAs were derived from viral particles or banana endogenous viruses (BEVs), we used combination of two methods: PCR-based assay using long and semi-nested PCR and southern hybridization assay using total DNA. Both of those detection methods revealed that these two samples were infected only by exogenous BSMyV but not BSOLV. This study is the first report of the identification of BSMyV in Indonesia.

Key words: *Badnavirus*, banana streak virus, endogenous virus, exogenous virus, Indonesia

INTRODUCTION

Banana (*Musa* spp.) is one of the important crops in many tropical countries. Banana streak disease (BSD), an emerged-viral disease, is considered to be a significant constraints to banana production worldwide. This disease was first found in the Ivory Coast in 1958 and is known to have spread to more than 43 countries in various region of the world until 2000 (Jones, 2000). The causal viruses of BSD are considered to be members of the genus *Badnavirus*, the family Caulimoviridae. These include Banana streak GF virus (BSGFV), Banana streak Mysore virus (BSMyV) and Banana streak OL virus (BSOLV) and many tentative species such as Banana streak IM virus (BSIMV), Banana streak RD virus (BSRDV) and Banana streak Cav virus (BSCavV). The genus *Badnavirus* is characterized by viruses with a single circular and double-strand DNA genome of 7.4-8 kbp which is encapsidated in bacilliform particles of 130-150×30 nm in size. These badnaviruses cause various symptoms from inconspicuous chlorotic fleck to lethal necrosis of the infected banana leaves and are transmitted by mealybugs (Jones, 2000).

The badnaviruses occurring in banana (hereafter, banana badnaviruses) are serologically and genetically very diverse (Lockhart and Olszewski, 1993). Moreover, as the genus *Badnavirus* is a pararetrovirus, the nucleotide sequences of BSOLV and BSMyV are partially and discontinuously integrated into the genome of banana (Harper *et al*., 1999b; Geering *et al*., 2001; Geering *et al*., 2005b). Such integrated viruses are known as Banana Endogenous Viruses (BEVs) and the nucleotide sequences of total 36 distinct BEVs were found in banana genomes (Geering *et al*., 2005a).

Although the occurrence of BSD in Asia has been reported from 9 countries (Jones, 2000), there have been no studies on characterization of BSD pathogens in Indonesia. Recently, we found BSD in Java Island, Indonesia. This study reports the first detection by combined assay and molecular characterization of the causal viruses obtained from bananas showing BSD in Indonesia.

MATERIALS AND METHODS

Plant materials: Two banana plants, IBoGB2 and IBoGB4 (cv. unknown), with yellow streak and necrosis were collected at the same farm in Bogor, Indonesia (Fig. 1). For further comparison, two Indonesian banana cultivars, cv. Barangan Kuning (AA) described as BK and cv. Ambon Kuning (AAA) described as AK were used as healthy control. Banana samples from Indonesia were imported under the permission of the Ministry of Agriculture, Forestry and Fisheries, Japan (15Y69415).

ELISA assay: Double-antibody sandwich (DAS)-ELISA test systems (Agdia, USA) for Banana streak virus and for Sugarcane bacilliform virus, which were known the limitation of the detection for some banana badnaviruses, were used following the manufacturer's instructions. PCR assay.

All DNA samples were isolated using a PhytoPure DNA extraction kit (Nucleon, UK) and PCR was conducted with *TaKaRa Ex Taq*™(TaKaRa) and *TaKaRa LA Taq* (TaKaRa) for long PCR with an initial denaturation at 94°C for 4 min, 30 cycles (94°C for 1 min, 55° to 57°C for 1 min, 72°C for 1 to 5 min) and a final extension at 72°C for 10 min in a thermal cycler (Gene Amp PCR System 9600, Perkin Elmer). Two sets of long PCR with specific primers for each of virus species (BSMyV: Mys/GB1F and Mys/GB1R, Mys/GB2F and Mys/GB2R, BSOLV: OE/B2F and OE/B2R, OE/B4F and OE/B4R) were followed by semi-nested PCR using primers (BSMyV: Mys/GB2F and BADNA T, BSOLV: OE/B2F and BADNA T) (Table 1).

Sequencing analysis: DNA fragments amplified were purified using a QIAquick™ Gel Extraction kit (QIAGEN, Germany). Sequences were determined using ABI PRISM377 DNA Sequencer and analyzed with AssemblyLIGN™ 1.0.9c (accelrys, USA) and Mac Vector 7.2 (accelrys, USA). Phylogenic trees were constructed using neighbor-joining methods with PAUP* 4.0 beta version.

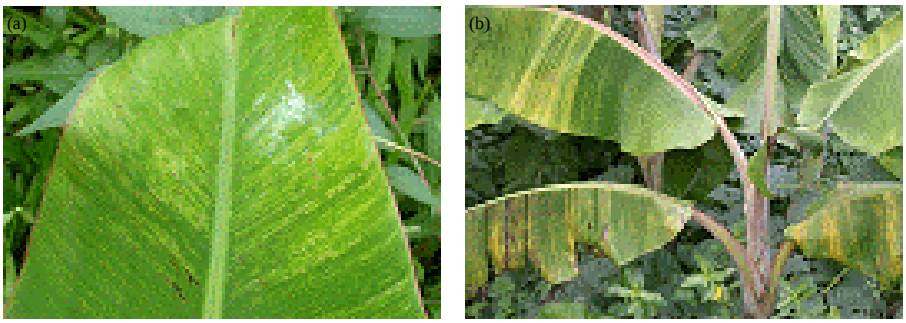

Fig. 1(a-b): Symptoms of naturally banana badnaviruses (a) IBoGB2 and (b) IBoGB4 infected bananas collected in the same field in Bogor, Indonesia, Yellow streak and necrosis were observed

Table 1: Sequences of primers used in this study

Target virus	Primer	Primer sequence	Size of product	Reference
BSMyV	L8238-F	5' CCC AGG AAT AAA CAC GAT TAT CAG TC 3'	2.1 kbp	Geering *et al*. (2000)
	BADNA T	5' CAC CCC CGG GMY MWN GCT CTG ATA CCA 3'		
BSOLV	BSV4673	5' GGA ATG AAA GAG CAG GCC 3'	0.6 kbp	Harper *et al*. (1999a, b)
	BSV5317	5' AGT CAT TGG GTC AAC CTC TGT CCC 3'		
BSOLV, BSRDV,	BADNA 3	5' ATH ATH ATH GAR ACY GAY 3'	1.3 kbp	Lockhart and Olszewski (1993)
BSCavV and BSGFV	BADNA T	5' CAC CCC CGG GMY MWN GCT CTG ATA CCA 3'		
BSMyV	Mys/GB1F	5' TTC CTG GCT AAC AAT GAG CTC TAT G 3'	Full-length	This study
	Mys/GB1R	5' AAG CCG TCC ATG GGA TGG ATT CA 3'		
	Mys/GB2F	5' CTA CTG TCG GCA AGG CAC AAG AA 3'	Full-length	This study
	Mys/GB2R	5' GTC CAC TTA CAG ACG CCG C 3'		
BSOLV	OE/B2F	5' GGA GAA AAA TCA GGT GAA ATG TAG 3'	Full-length	This study
	OE/B2R	5' CCA ATG TTT GAG AGG TTC TTC TC 3'		
	OE/B4F	5' GTT CTA GAG GGG AAA TTT CTG AA 3'	Full-length	This study
	OE/B4R	5' CTC TCA GCT GCA CAT ATG TTA TAG T 3'		

Bootstrap consensus tree was generated from 1000 replications. The nucleotide sequence data reported in this paper were deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers AB252636 and AB252637.

Southern hybridization: Individual total DNA was directly separated on 1% agarose gel in TAE buffer. Each of PCR products was labeled and detected using ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare Biosciences) according to the manufacturer's instructions. The DNA fragment of 1.3 kbp obtained by the semi-nested PCR of IBoGB2 were used as a probe for BSMyV and the DNA fragment of 0.6 kbp obtained by the species-specific PCR of IBoGB2 were used as a probe for BSOLV.

RESULTS AND DISCUSSION

In ELISA test, while a positive control BSIMV, strongly reacted with these antibodies, two Indonesian banana samples IBoGB2 and IBoGB4 did not show positive reaction in any of the tests serologically.

Using two sets of species-specific primers to BSMyV and BSOLV and one set of common primers to detect 4 banana badnaviruses, BSOLV/BSGFV/BSRDV/BSCavV, the samples were checked by conventional PCR (Table 1). Two bands of target sizes (2.1 and 0.6 kbp) were observed in IBoGB2 and IBoGB4 with BSMyV and BSOLV species-specific primers. Viruses of the family Caulimoviridae have two or three gaps or discontinuities at specific sites on the genomic DNA. These gaps or discontinuities, however, are sealed at the replication of the virus, then, the DNA forms covalently closed dsDNA. To confirm whether the banana *Badnavirus* DNAs detected from IBoGB2 and IBoGB4 were episomal viral DNA or integrated DNA, we carried out two sets of long PCR with specific primers for each of virus species (BSMyV: Mys/GB1F and Mys/GB1R, Mys/GB2F and Mys/GB2R, BSOLV: OE/B2F and OE/B2R, OE/B4F and OE/B4R) followed by semi-nested PCR. By these primers, approximately 7-7.5 kbp of DNA fragments were amplified as a possible full-length *Badnavirus* DNA (Fig. 2a, b). A clearly recognizable fragment (300 bp) smaller than the fragment with the expected size (2.1 kbp) was detected by semi-nested PCR using primers of BSOLV (OE/B2F and BADNA T; Fig. 2d). On the

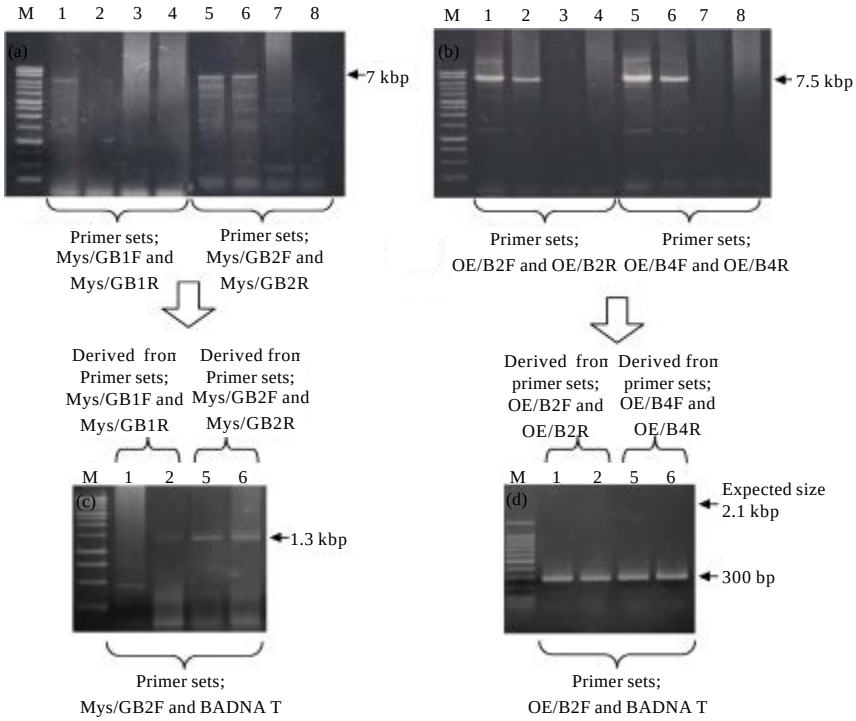

Fig. 2(a-d): Semi-nested-PCR following long-PCR with two sets of primers: long-PCR for the detection of (a) BSMyV and (b) BSOLV and semi-nested-PCR for the detection of (c) BSMyV and (d) BSOLV, Lane M: DNA ladder (Promega), Lane 1 and 5: IBoGB2, Lane 2 and 6: IBoGB4, Lane 3, 4, 7 and 8: Healthy banana plants

other hand, the expected fragment (1.3 kbp) were amplified from the 7 kbp fragment using primers of BSMyV (Mys/GB2F and BADNA T; Fig. 2c).

To confirm whether the BSMyV DNA was derived from episomal DNA or not, the total DNAs extracted from IBoGB2 and IBoGB4 were directly applied to electrophoresis and subjected to the southern hybridization assay. Bound to the banana DNAs, the probe for BSMyV exuded characteristic signal at the smaller position than that of the plant DNA (Fig. 3). As a result of sequencing and the analysis of 2.1 kbp DNA fragment amplified from IBoGB2 by PCR using BSMyV specific primers, it showed the highest homology (99%) with BSMyV (AF214005; Geering *et al*., 2000) isolated from Australia.

For further characterization, we sequenced RNaseH genes, the suitable gene for molecular phylogenetic analysis among Badnaviruses, of the Indonesian BSMyV-IBoGB2 and BSMyV-IBoGB4 using primers 1F (5' CTT GAA ACA GAC GGT TGC ATG GAA 3') and BADNA T (Table 1) (Geering *et al*., 2000) and estimated the putative amino acid sequences. RNaseH genes of these Indonesian isolates were 375 nt and their amino acid residues were 125 aa. A phylogenetic tree based on the putative amino acid sequences of RNaseH genes of Indonesian isolates and 11 isolates of the related species is presented in Fig. 4. Viruses are split into 6 groups in accordance with their original host plants. BSMyV-IBoGB2 and BSMyV-IBoGB4 were made dense sub-clusters with BSMyV isolated in Australia.

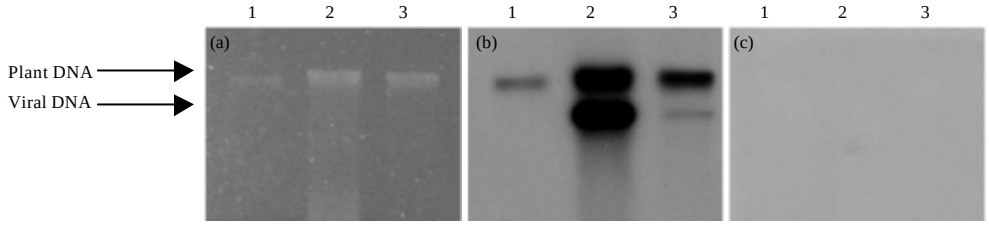

Fig. 3(a-c): Southern hybridization of total DNAs (a) Agarose gel, (b) Probe for BSMyV and (c) Probe for BSOLV, Lane 1: Symptomless banana plant, Lane 2: IBoGB2, Lane 3: IBoGB4

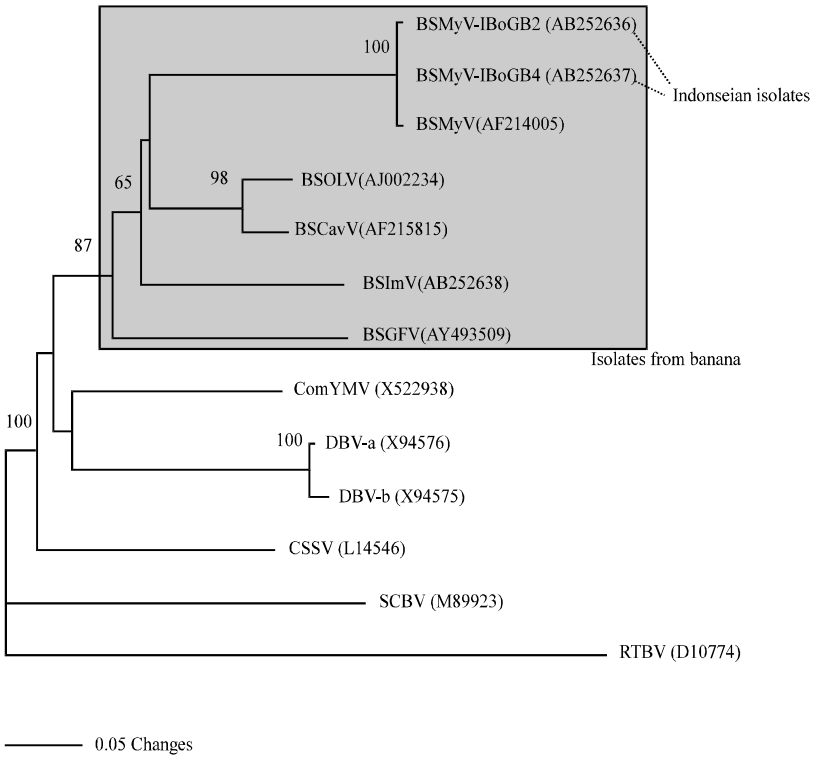

Fig. 4: Phylogenetic tree depicting the relationships of BSMyV-IBoGB2, BSMyV-IBoGB4, 5 BSV isolates and the related species, based on the putative amino acid sequences of RNaseH using neighbor joining method, The dendrogram was bootstrapped 1,000 times (scores are shown at nodes), The shaded box indicates badnaviruses isolated from banana. ComYMV: *Commelina yellow mottle virus*, DBV: *Dioscorea bacilliform virus*, CSSV: *Cacao swollen shoot virus*, SCBV: *Sugarcane bacilliform virus*, RTBV: *Rice tungro bacilliform virus*

For the reasons that the genome of banana badnaviruses has been integrated into the banana genome and their serotypes are very diverse, their identification and classification are not simple. In this study, we attempted the identification of the causal viruses of two banana plants showing BSD like symptoms in Indonesia using a combined detection assay; PCR-based and southern

hybridization. Both PCR-based assay and southern hybridization assay showed the same result: these two banana samples IBoGB2 and IBoGB4 were thought to be infected with exogenous BSMyV. To date, Immuno-Capture Polymerase Chain Reaction (IC-PCR) and Rolling-Circle Amplification (RCA) have been basically used for the detection of episomal banana badnaviruses (Harper *et al*., 1999a; James *et al*., 2011). By IC-PCR, antibodies were, however, not able to detect all kinds of banana badnaviruses, although a peptide that interacts with BSV using a phage display system has been developed (Heng *et al*., 2007) and RCA method is not supposed to have enough credibility in case of using alone. This combined detection assay of PCR-based and southern hybridization can be precisely available, even though when the virus detection is required from serologically unique and minor strain. For their further identification, this combined assay introduced in this study would be useful to detect and identify the wide spectrum of banana badnaviruses.

In Indonesia, occurrence of Banana Streak Disease (BSD) was already reported based on symptom observation (Diekmann and Putter, 1996) and the mealybug transmission was demonstrated in our preliminary research (Suastika *et al*., 2004). This study utilized the molecular characters to describe the causal pathogens of BSD in Indonesia for the first time.

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