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Begomovirus Associated with Pepper Yellow Leaf Curl Disease in West Java, Indonesia

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Chili pepper (*Capsicum frutescens*) showing yellow leaf curl from West Java were analysed for the presence of a geminivirus. Detection using specific primers for geminivirus was conducted following whitefly (*Bemisia tabaci* Genn.) transmission. A DNA fragment of 1.6 kb was amplified in the polymerase chain reaction using primers which anneal within the replication initiator protein gene and coat protein gene. DNA clone of the polymerase chain reaction product was then sequenced. The stem loop region was found, included the conserved nonanucleotide sequence TAATATTAC present in all geminivirus. The geminivirus associated with yellow leaf curl disease in pepper showed the highest sequence identity 93 and 98% with *pepper yellow leaf curl Indonesia virus* from *C. annuum* and *pepper yellow leaf curl Indonesia virus* from *Lycopersicon esculentum*, respectively.

Key words: Begomovirus, common region, leaf curl disease

Geminivirus is a family of plant viruses that contains a circular single-stranded DNA (ssDNA) genome encapsidated in geminate particles. They are classified into four genera, i.e. Mastrevirus, Curtovirus, Begomovirus, and Topocuvirus, based on their vector relationship, host range and genome organization (van Regenmortel 2000). Members of the genus Begomovirus are transmitted by the whitefly *Bemisia tabaci* Genn.(Hemiptera : Aleyrodidae) and infect dicotyledonous plants. Diseases caused by whitefly-transmitted geminiviruses (WTGs) have become a serious constraints to crops in tropical and subtropical areas throughout the world (Idris & Brown 1998; Samretwanich *et al.* 2000). Polston and Anderson (1997) reported that geminivirus infection had caused significant damage to tomato production in Mexico, Venezuela, Brazil, Florida, Central America, and Caribia.

Geminiviral diseases in chilli pepper is caused by several distinct species of begomoviruses, such as *Serrano golden mosaic virus* (Brown & Poulos 1990), and *Texas pepper virus* (Lotrakul *et al.* 2000). In the pepper growing area at Bogor, West Java yellow leaf curl symptoms have been observed in the last five years. It becomes an important disease following a long dry season in 2002 throughout Java and Sumatra. The causal agent of the disease could be transmitted by grafting and whitefly, but it was not mechanically transmitted. Based on the type of symptoms and its transmission by whitefly, geminivirus infection was suspected. Polymerase chain reaction (PCR) using specific degenerate primers, PALIv1978 and PARIc715 was successfully amplified a 1.6 kb DNA fragment from infected plants (Rusli *et al.* 1999).

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This research was initiated to clone the geminivirus causing pepper yellow leaf curl in West Java and to study its relationship with other characterized geminiviruses. As the complete nucleotide sequences of many geminiviruses have been determined, virus identification based on DNA sequencing of viral genomes appears to be the most useful and reliable.

MATERIALS AND METHODS

Geminivirus Isolate. Virus-infected chillipepper (*C. frutescens*) with characteristic symptoms were collected from field at Segunung, Bogor, West Java. The virus was transmitted using whitefly (*Bemisia tabaci*) to *Nicotiana benthamiana* plants. After geminivirus detection using PCR method, virus cultures was maintained in tomato plants by whitefly transmission (Aidawati *et al.* 2002) for further studies.

PCR-based Detection. Total DNA was extracted from fresh young leaf tissue according to the method developed by Dellaporta *et al.* (1983). The DNA pellet was resuspended in 50 ul of sterile distilled water. A pair of degenerate primer designed for the amplification of the DNA-A genomic component, pALIv 1978 (5'GCATCTGCAGGCCCACAT YGTCTTYCCNGT 3') and pARIc 715 (5' GATTTCTGCA GTTDATRTTYTCRTCCATCCA 3') (Rojas *et al.* 1993), was used. PCR was carried out in a 25 ul reaction mixture containing 1 ul of sample DNA solution and 0.2 uM of each primer using Ready To Go PCR kit (Amersham Life Science). PCR was performed in thermalcycler Gen Amp PCR System9700 (Perkin Elmer) with 30 cycles of melting, annealing and DNA extension at 94C for 1 min, 55C for 2 min, and 72C for 2 min, respectively. Amplified DNA fragments were analysed by electrophoresis in 1% agarose gels in Tris-buffer EDTA.

Nucleotide Sequence Analysis. Amplified DNA fragments of approximately 1.6 kbp was digested with Pst I, then cloned into the same restriction sites of pGEM- T Easy vector (Biorad) and sequenced by the dideoxy nucleotide chain termination method. To obtain complete nucleotide sequences of 1.6 kbp viral DNA fragment, additional primers were designed based on the previous nucleotide sequences. A 1.6 kbp viral DNA fragment was completely sequenced on both strands.

Sequence data of the pepper yellow leaf geminivirus clone was compared with those of other whitefly-transmitted geminiviruses available in Genebank using Clustal W program version 1.82 European Bioinformatics Institute (EMBL-EBI : www.ebi.ac.uk/serve/clustalW). Phyllogenetic analysis was conducted with UPGMA approach using PAUP program version 4.0 b.

RESULTS

The virus that was collected from chillipepper at Segunung, West Java was successfully transmitted from infected chillipepper to healthy *N. benthamiana* plants by whitefly *B. tabaci* of the B biotype. All inoculated *N. benthamiana* plants began to develop symptoms 7-10 days after inoculation. The infected and healthy *N. benthamiana* plants were tested by PCR to determine the presence of geminivirus. The primer designed for DNA-A was successfully amplified geminivirus specific DNA fragments of 1600 bp from infected plants. The 1600 bp DNA product obtained from the PCR using pAL1v 1978 and pAR1c 715 sets of primer was cloned into the PGEM-T Easy vector. Following screening of recombinant DNA, a clone identifed as pPL#8 was selected for viral sequence analysis. Nucleotide sequences from base 1 to 1491 of pPL#8 was determined (Gene Bank Accession No. AB 246170). The nucleotide sequence of 31 base stem loop region was found in the sequence of pPL#8 (Figure 1). The conserved nonanucleotide sequence TAATATTAC, which has been found in all geminiviruses sequenced so far (Ikegami *et al.* 1988) was also evidenced in the sequence of pPL#8. The repetitive sequences (5' - GGAGACA- 3') known as an iteron was found in three nucleotide positions. The iteron is known located in intergenic region (IR) and is assumed as specific-binding site of the geminiviral replication-associated protein (Arguello-Astorga *et al.* 1994). The isolate of geminivirus associated with pepper yellow leaf curl disease in Segunung, West Java was then tentatively called *pepper yellow leaf curl Indonesia virus - Bogor* (PYLCIV-Bgr).

Relationship between PYLCIV-Bgr and other selected begomoviruses was evaluated by generating phylogenetic tree based on the common region of pPL#8. It was found that PYLCIV-Bgr was ciustered with PYLCIV-LBI and PYLCIV-LBI.I (Figure 2). Analysis of sequence identity using Clustal W program revealed that nucleotide sequence identity between PYLCIV-Bgr and selected begomoviruses ranges from 26 to 98% with the highest nucleotide sequence identity was found with PYLCIV-LBI and PYLCIV-LBI.I (Table 1). The two viruses were collected from the same geographic location, i.e. Lembang, Bandung, West Java but from different host plant, i.e. *L. esculentum* and *C. annuum*, respectively (Table 2).

5'- CATTTGGAGTGTCGTTTTGTATTGGAGACAATCACTTCTATCCC

TATGTATTGGAGACAGGAGACA TATATATAA GTCCTATAAT

GGCTTTTAAGTAATTTTGTACACCATTGAATGGTTAAAGCGGCA

CTCGTATAATATTACCGAGTGCCGCGAAAATATTTAAATGTGGT

CCCCCAAGCCAGCCTTTTGACTGACCA - 3'

Figure 1. Nucleotide sequences of the intergenic region of Pepper yellow leaf curl geminivirus from Segunung, West Java (PYLCIV-Bgr) derived from pPL#8 showing TATA sequences (boxed sequences), repeat sequences (bold letters), and the stem-loop region (underlined sequences).

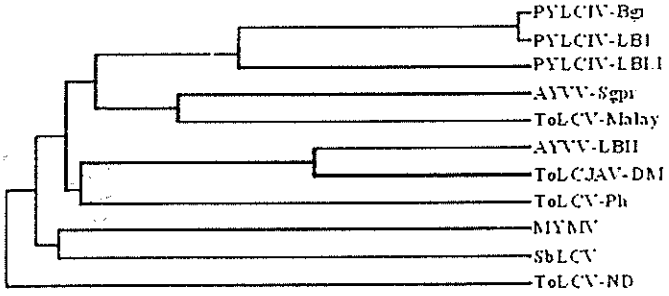

Figure 2. Phylogenetic tree based on the alignments of nucleotide sequences of common region of Pepper yellow leaf curl geminivirus from Segunung, West Java (PYLCIV-Bgr) with other selected begomoviruses as listed in Table 1. The tree was generated with UPGMA approach using PAUP program version 4.0 b

Table 1. Comparison of nucleotide sequence identities (%) between Pepper yellow leaf curl geminivirus from Segunung, West Java (PYLCIV-Bgr) and other geminiviruses based on common region sequence using ClustalW program (http://www.ebi.ac.uk/clustalw/index.html)

	SbCLV	PYLCIV Bgr	AYVV-LBII	AYVV-Sgpr	ToLCV-Ph	MYMV	PYLCIV-LBI.I	ToLCV-ND	ToLCJAV-DM	ToLCV-Malay
PYLCIV-Bgr	53									
AYVV-LBII	62	63								
AYVV-Sgpr	38	36	25							
ToLCV-Ph	41	41	35	66						
MYMV	22	26	30	6	9					
PYLCIV-LBI.I	53	93	63	34	15	31				
ToLCV-ND	37	55	60	23	6	9	54			
ToLCJAV-DM	62	62	91	22	33	30	63	58		
ToLCV-Malay	42	35	34	74	80	9	19	6	33	
PYLCIV-LBI	53	98	62	36	41	26	95	56	62	22

Table 2. List of geminiviruses (Begomovirus) used for relationship analysis

Gene Bank Accession number	Organism	Nucleotide's length (bp)	Geography origin	Host plant	Acronim
AB189845	*Pepper yellow leaf curl Indonesia virus*	1563	Indonesia: Bandung, Lembang	*Lycopersicon esculentum*	PYLCIV-LBI
AB189913	*Ageratum yellow vein virus*-[Indonesia]	1557	Indonesia: Bandung, Lembang	*Ageratum conyzoides*	AYVV-LBII
AB189848	*Tomato leaf curl Java virus*-[Magelang]	1562	Indonesia: Magelang, Dukun	*L. esculentum*	ToLCJAV-DM
AB246170	*Pepper yellow leaf curl Indonesia virus*	1491	Indonesia: Segunung,Bogor	*Capsicum frutescens*	PYLCIV-Bgr
AB189850	Pepper yellow leaf curl Indonesia virus	1555	Indonesia:Bandung, Lembang	*C. annuum*	PYLCIV-LBII
L11746	*Tomato leaf curl virus*	1518	India	*L. esculentum*	ToLCV-ND
X74516	*Ageratum yellow vein virus*	2741	Singapore	-	AYVV-Sgpr
AF327436	*Tomato leaf curl Malaysia virus*	2754	Malaysia: Klang	*L. esculentum*	ToLCV-Malay
AF136222	*Tomato leaf curl Philippines virus - [LB]*	2744	Philippines: Los Banos	*L. esculentum*	ToLCV-Ph
E00957	Mungbean yellow mosaic virus	2715	-	-	MYMV
AB020977	*Soybean crinkle leaf virus*	1242	-	-	SbCLV

DISCUSSION

Geminivirus associated with yellow leaf curl disease of chili pepper was detected using polymerase chain reaction with DNA-A geminivirus-specific degenerate primers. Genome type of the geminivirus causing yellow leaf curl disease in chilli pepper in Segunung, West Java remained to be identified wether belong to monopartite or bipartite group. Using specifically designed primers to amplifiy DNA-B of Asian tomato geminiviruses, DNABLC1/DNABLV2 and DNABLC2/DNABLV2, Tsai *et al.* (2006) was not able to detect the DNA-B from infected chillipepper collected from Bogor, West Java, Indonesia. On the other hand, Ikegami (unpublished data) was able to amplify DNA-B fragment from isolate of *pepper yellow leaf curl Indonesia virus* (PepYLCIDV) using similar primer pair (DNABLC1/DNABLV2) (Green *et al.* 2001). Based on this evidence we may expect that several distinct begomoviruses are present in chillipepper with yellow leaf curl symptoms in Indonesia, i.e those with monopartite and bipartite genomes.

A 31-base stem loop region was found in the nucleotide sequence of PYLCIV-Bgr in which eight GC pairs and two AT pairs formed the stem structure, while the loop consists of 11 nucleotides including the sequence TAATATTAC. This stem loop region was found in all geminiviruses sequenced so far (Kheyr-Pour *et al.* 1991) and known as part of common region (Harrison 1985; Lazarowitz 1987). In contrast to this sequence conservation, the common regions of different geminiviruses are not very similar. A 33-base stem loop region was found in ageratum – infecting begomoviruses and *tomato leaf curl Java virus* (ToLCJAV), whereas a 35-base stem loop region was found in *tomato leaf curl Indonesia virus* (ToLCIDV) (Sukamto *et al.* 2005). Furthermore, direct repeat sequences was varies among different geminiviruses. In *bean golden mosaic virus-Guatemala* (BGMV-GA, M91064) and BGMV-Puerto Rico (BGMV-PR, M10080, M10070, D00200, D00201) there are direct repeats of the sequences TGCGAGTGTCTCCAA whereas in BGMV-Dominican Republic (BGMB-DR, L01035, L01636) the repeat sequences are GTGTCTCCATT. Sukamto *et al.* (2005) reported that clones of tomato infecting begomovirus originated from Bandung has identical repeat sequences with PepYLCIDV. Interestingly this repeat sequence, GGAGACA, turned out identical with those found in PYLCIV-Bgr.

To investigate the relationship between PYLCIV-Bgr and other selected begomoviruses from Asia, phylogenetic tree was generated using nucleotide sequence of the common region. The sequence comparison showed that PYLCIV-Bgr has the highest similarity with PYLCIV-LBI (98%). Padidam *et al.* (1995) proposed that virus isolates displaying more than 90% sequence identity should be considered as isolates or strains rather than different viruses. On the other hand, Howarth and Vandemark (1989) showed two different phylogenetic trees when nucleotide sequence of two different specific region of geminivirus genome was used. They proved that a phylogenetic tree based on coat proteins was correlated with vector specificities of the virus, and a tree based on replication-associated proteins was correlated with viral host specificities. Sukamto *et al.* (2005) reported that when full nucleotide sequence was used for the analysis, the tomato-infecting begomoviruses from Java fell into three groups. Similar results were obtained when they used the amino acid sequence of the coat protein. Although our geminivirus isolate have high nucleotide sequences similarity with PYLCIV-LBII, and PYLCIV-LBI, it could not be considered as the same virus yet. Availability of full genome sequence will provide comprehensive and complete analysis to come up with such conclusion.

The seriousness of geminiviral diseases in Indonesia, especially in those tomato and chilli pepper, requires collaboration to exploit all possible management measures. Genetic diversity of geminiviruses that infect tomato and chilli pepper in Indonesia should be considered in developing disease control strategies.

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