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PCR BasedCloning of Pre Coat Protein(V2) Gene Pepper Yellow Leaf Curl Virus From Chili Pepper(*Capsicum annuum* L.).

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ABSTRACT

Movement process, cell to cell transportation, and development of systemic symptom in Pepper Yellow Leaf Curl Virus (PepYLCV) was affected by the activity of V2 gene, known as pre coat protein. The development of broad spectrum virus resistance based on the concept of pathogen-derived resistance (PDR) could be achieved by utilizing this gene. The objective of this study was to isolate the V2 gene from a PepYLCV population collected from West Sumatera. Cloning was performed by PCR-based cloning, started with primer combination specifically designed for West Sumatera isolate WS-PYLCV-387 FR and continued with two others primer combinations in order to cover the complete sequence of V2 gene region. Sequence analysis based on BLAST tool verified that the cloned gene is a V2 gene encoding pre coat protein, composed of 354 bp in length. The gene showed high similarity with other PepYLCV V2 genes from several areas in Indonesia. Predicting of its amino acids exhibited that the V2 gene has isoelectric point at 6.93 and molecular weight of 13,404.36 Dalton.
Keywords: *Capsicum annuum*, geminivirus, V2 gene, West Sumatera, Pathogen derived resistance

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INTRODUCTION

Pepper yellow leaf curl virus (PepYLCV) is one of devastating pathogen that most commonly found in chili pepper and several horticulture crops such as tomato [1-2], tobacco [3] and eggplant [4-5]. The virus belongs to the family *Geminiviridae* and genera of *Begomovirus* [6-7]. Infection of the disease in chili pepper can cause high losses by decreasing yields and damaging the plant itself. Many controlling techniques against PepYLCV have been developed, but those have not given satisfactory result yet.

In order to improve genetic resistance against some viruses, a pathogen-derived resistance (PDR) approach is suggested. The PDR is a concept of resistance engineering using pathogen's genetic material as source of resistant gene[8]. In this case, V2 gene encoding pre coat protein of PepYLCV which has main role in virus particle movement isrationally tought to be used to improve the crop resistance against PepYLCV in chili pepper.

V2 gene, referred as pre coat protein is one of important genes that affect the virulence and infection successfullness of many Begomoviruses [9]. Furthermore, this gene influences viral accumulation and symptoms development that will affect the severity level of infected plants [10]. Availability of V2 gene as a resistance source is prerequisite in order to develop resistant plant against PepYLCV by inhibiting cell to cell viral movement that finally block the development of systemic symptom. It also may lead to broad spectrum resistance and seemed to be effective against distantly related viruses as reported by [11]. Based on that reason we cloned the V2 gene by means of PCR based cloning approach. Furthermore we analyzed its structure characteristic and compared with the existing V2-gene sequence in NCBI database.

MATERIALS AND METHODS

Plant Material

PepYLCV infected chili pepper leaves were collected from Tanah Datar Regency, one of the chilli pepper cultivation center available in West Sumatera in 2012. Sample material was labelled as TD-24 and kept in -80^{o} C prior to be used for DNA preparation.

DNA Isolation and Molecular Assay

DNA virus was isolated together with genomic DNA of chili pepper leaf. Extraction of genomic DNA was performed as described previously by [6]. A number of amplification reactions were done by applying primer combinations listed in table 1.

Table 1. Primer combinations used in the study. All primers were specifi-cally designed for this study.

Primer ID	Sequence 5' → 3'	Note
WS-PYLCV-387 F	GCTCCCTGRAWGTTHGGATGGAA	West Sumatera universal primer
WS-PYLCV-387 R	GAGCTAARTCMAGYTCCGAYGTCA	West Sumatera universal primer
TD21-PYLCV-455 F	CGTGCAGACGTATTTCCCTTCGAAT	Tanah Datar specific primer
TD21-PYLCV-455 R	CAACAGATTCTTCGACCTGGTAT	Tanah Datar specific primer
V2-TD21-*BamHI* NT	CATGGGGATCCATGTGGGATCCGCTT	V2-gene specific primer
V2-TD21-*SmaI* NT	CTAATCCCGGGCTGCTCCTTCGACCC	V2-gene specific primer

Cloning of V2 Fragment into*Escherichia coli*

The PCR product generated by primer combination of V2-TD21-*Bam*HI NT and V2-TD21-*Sma*I NT was cloned into pGEM-T® Easy Vector (Promega-USA). The recombinant plasmid was transformed into competent *Escherichia coli* strain BL21 by using heat shock method [12]. Transformation cocktail was composed of 5 µL recombinant plasmid and 50 µL competence cells and then incubated on ice for 2 minutes. Heat shock treatment was done by subsequent incubation in a waterbath at 42^{o}C for 45 seconds followed by incubation

on ice for 2 minutes. Liquid LB medium containing 34 µgmL^{-1} Chlorampenicol was added and then incubated in 37^{o}C agitated with 150 rpm for an hour. Afterward, pellet was harvested by centrifugation at 14.000 rpm for 6 minutes. Fivehundred microliter of LB medium was used to dissolve the pellet. The bacterial suspension was then plated on solid LB medium supplemented with 34 µg mL^{-1}Chlorampenicol and 50 µg mL^{-1} Ampicilin and finally incubated at 37^{o}C for 18 hours.

Selection of Putative *E. coli* Recombinant

The recombinant *E. coli* containing V2 gene was analyzed by several ways, for instance direct colony PCR[13], DNA digestion, DNA plasmid amplification and sequence verification using sequencing technique. The colony PCR analysis was applied by using primer T7 (5' TAATACGACTCACTATAGGGCGA 3') and SP6 (5'ATTTAGGTGACACTATAGAATAC 3') available in plasmid sequence. PCR condition was run in 25.0 µL reaction containing 5.0 pmolµL^{-1} of forward and reverse primer, 1 µL DNA Polymerase, 2.5 µL 10x PCR Buffer, 2.5 µL dNTPs and filled with 13 µL of PCR grade H_2O. PCR condition was run in two loops and initiated at 95^{o}C for 3 minutes. The first loop was run at 94^{o}C for 1 minute as initial denaturation, followed with primer annealing at 55^{o}C for 1 minute, and primer extension at 72^{o}C for 1 minute 30 seconds. The second loop containing 26 cycles started with denaturation at 94^{o}C for 1 minute, annealing at 57^{o}C for 1 minute, and extension at 72^{o}C for 1 minute 30 seconds. Additional extension was applied at 72^{o}C for 5 minutes.The PCR product was separated in 1% agarose gel, compared with 1 kb DNA ladder as size marker and lambda DNA 50 ngµL^{-1}.

Plasmid DNA principally was extracted byusing Quick and Dirty method described by [14]. The extracted plasmid DNA was treated similarly as described above by using T7 – SP6 primers. Verification of inserted V2 gene in the plasmid was done with V2-TD21-*Bam*HI NT and V2-TD21-*Sma*I NT primer combination. PCR products were then analyzed by using 1% agarose gel electrophoresis accompanied with 1 kb DNA ladder as size marker and lambda DNA 50 ng µL^{-1} as concentration reference.

Insert Verification and Sequence Analysis

One of positive PCR products from previous selected transformants was double digested with *Bam*HI and *Sma*I to verify insert integrity. Final confirmation was done by sequencing of positive PCR product by applying bidirectional sequencing using both T7 and SP6 primers. Sequence data obtained was analyzed and edited manually to verify the sequence data validity. Homology analysis was performed by running BLAST tool provided by NCBI(National Centre of Biotechnology Information) at: https://blast.ncbi.nlm.nih.gov/Blast.cgi [15]. Sequence analysis was also further continued with sequence alignment using CLUSTALtool, available at: http://www.ebi.ac.uk/Tools/msa/clustalo/ [16]. Additional analysis was also performed by translating the V2 gene nucleotide sequence into amino acids and predicting its pI (isoelectric point) and Mw (molecular weight) value at http://web.expasy.org/compute_pi/ [17-19].

RESULTS AND DISCUSSION

Symptome Variation

A B

Figure 1. Morphology variation of PepYLCV infected chili leaves collected from Tanah Datar Regency. A, yellow leaf symptoms; B, curly leaf symptoms

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In total, eight chili pepper leaves showing PepYLCV infection symptom samples were used in this study. All sampleswere collected from Tanah Datar – West Sumatera. The collected samples in fact showed symptom variation although grown in the same area (Fig. 1). Symptom variation can occur because of different habit condition and the presence of interaction between virus and other factors, such as growing stage of plant, cultivar, genotype, and nutrient status of plant. Other environmental factors, such as level of soil fertility and agroclimate also can cause different symptom of virus infection [20].

Identification of PepYLCV Strain TD-24

Genomic DNA of chilli pepper containing PepYLCV nucleic acid was successfully extracted from all 8 samples (Fig 2A). DNA concentration extracted from this step ranged between 25 ng/µl-250 ng/µl (data not shown), meaning that analysis could be proceed to the next step.

The presence of PepYLCV DNA was succesfully identified by the presence of about 2.700 bp fragments after PCR assay by meant West Sumatera universal primer combination (WS-PYLCV-387 FR) (Fig 2B). However, only 6 out of 8 samples successfully produced about 2.700 bp fragment.Two samples (2-3) were failed to produce expected fragment (Fig 2C).

Eventhough, analysis was proceed to identify V2 gene from the all 8 samples of extracted DNA. All 8 samples was positively proven containing V2 gene specific from Tanah Datar (Fig. 3). This was confirmed by a single fragment of about 370 bp in size.

Primer used to identify this Tanah Datar strain 24 specific V2 gene is non-terminator extension primer. This primer has been modified from primer used in the previous studies by adding the recognition sequence of *Bam*HI and *Sma*I in its sequences (Table 1.) and replacing stop codon (TGA) encoded sequences found in the previous primer. The addition of recognition sites of *Bam*HI and *Sma*I was adjusted with the cloning sites area in pBI121 binary vector and purposed to simplify the releasing of target gene after being transformed into *E. coli*. In addition, the replacement of stop codon encoded sequence is purposed to avoid the inhibition of biomarker gene expression located successive in the downstream of multi cloning site (MCS) of the cloned gene.

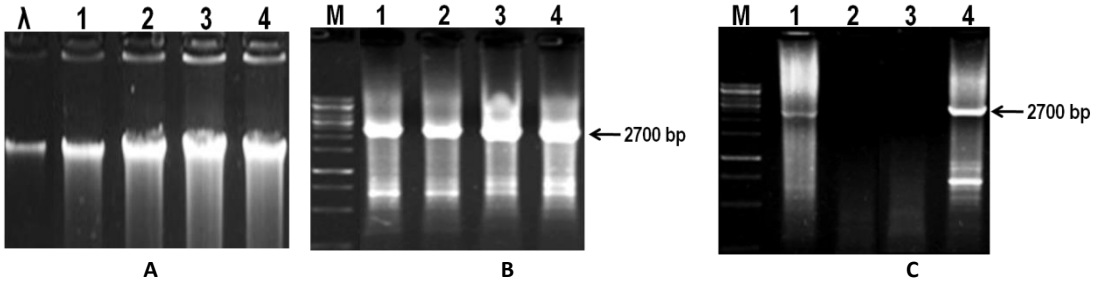

Figure 2. Steps of PepYLCV strain Tanah Datar identification. A. Representative of DNA isolation of four leaf samples, B. PCR product generated with West Sumatera universal primer combination (WS-PYLCV-387 FR), C. PCR product generated with Tanah Datar specific primer combination TD21-PYLCV-455 FR. λ . Lambda DNA 50 ng/µl, M. 1 kb size marker.

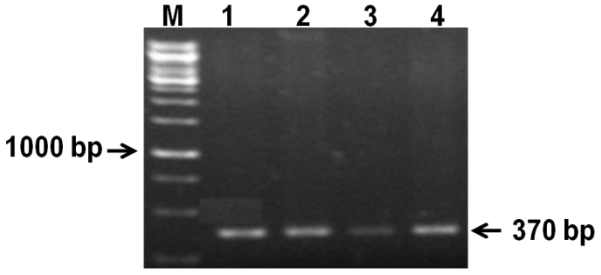

Figure 3.Representative PCR analysis of TD isolates with Tanah Datar specific V2 gene primers V2-TD21-*Bam*HI NT- V2-TD21-*Sma*INT.

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Cloning of Putative Pre Coat Protein(V2) Gene into*E. Coli*

Cloning of V2 gene from sample TD-24 into *E. coli*strain BL21 was done by heat shock method [12]. Transformants selection successfully produced 22 white colonies regarded as *E. coli* transformant containing inserted plasmid. All putative transformant colonies were tested by colony PCR using T7-SP6 primers. Twelve of 22 colonies could produce PCR product with expected size (about 520 bp) indicating the presence of plasmid containing V2 gene sequence. The other 10 colonies generated PCR product less than 250 bp and therefore regarded as unexpected transformants. In order to verify the 12 positive clones, PCR assay with V2-TD21-*Bam*HI-NT/V2-TD21-*Sma*I-NT primer combination was performed. All 12 putative transformants successfully generated single fragment of about 370 bp in size. This results confirmed that the 12 putative transformants contained V2-gene sequence.

Sequence Analysis of Putative V2-Gene

Sequencing result successfully recovered complete sequence of cloned V2 gene. The sequence is flanked by V2-TD21-*Bam*HI-NT/V2-TD21-*Sma*I-NT primer combination, composed of 373 nucleotides. After editing the adaptor sequence in both linker left and right side, the V2 gene sequence was fixed by 351 nucleotides, started with start codon ATG and ended with codon CAG. The codon terminator TGA was omitted, enabling fusion with variety of marker genes in future application. Translating nucleotide sequences into amino acid sequences produced 117 amino acid residues, started with methionin (M) amino acid residue and ended with glutamin (Q) residue (Figure 5B.). Further analysis of pI/Mw (isoelectric point and molecular weight) value at http://web.expasy.org/compute_pi/ exhibited 6.93/ 13,404.36 Dalton.

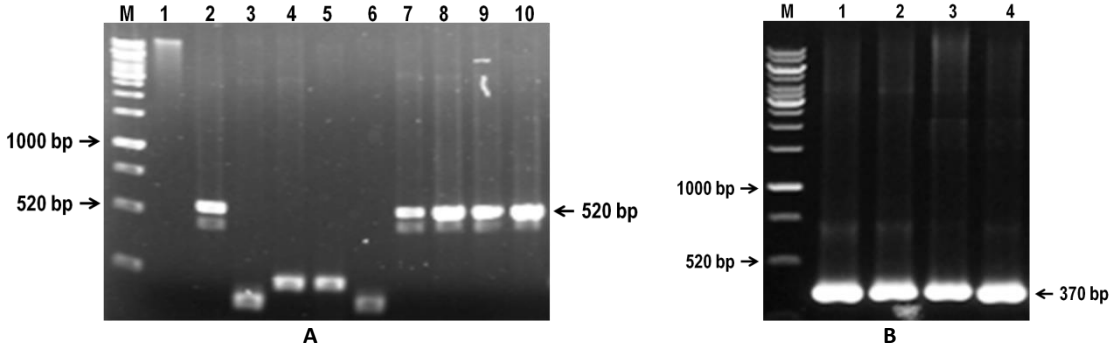

Fig. 4. Colony PCR of representative *E. coli*transformantsusing T7-SP6 primers (A) and PCR product generated from 4 representative DNA plasmid amplified with V2-TD21-*Bam*HI-NT/V2-TD21-*Sma*I-NT primer combination (B). M: 1 kb size marker

BLAST analysis in nucleotide level with 100% query coverage exhibited 94-99% homology. Homology of 94 % was achieved with V2 gene of Pepper yellow leaf curl Indonesia virus (AB189850.1), while 99% homology was achieved with V2 gene of Pepper yellow leaf curl virus TDWS-21 segment DNA-A (KT809346.1) published by [6]. Significant homology (99%) with V2 gene of Pepper yellow leaf curl virus TDWS-21 segment DNA-A is as expected, since both viruses originated from similar location. This result was also in accordance with previous published V2 gene of Pepper yellow leaf curl virus PSSWS-14 segment DNA-A originated from Pesisir Selatan (KT809345.1) published by [7], that in this analysis showing 95% in homology. Homology comparison with other V2 genes isolated from North Sumatera newly available at NCBI nucleotide database, for instance LC051114.1, LC051115.1, and LC051113.1 [21] showed highly significant homology up to 99%.

Meanwhile, BLAST analysis in amino acid level with 100% query coverage exhibited homology in range of 70 to 93%. The highest homology was detected with protein number BAU21325.1 (LC051113.1), BAU21331.1 (LC051114.1) and AMO26187.1(KT809346.1) The two first isolates were collected from Northern Sumatera [21], while the latter isolate was collected from Western Sumatera [6]. Another putative V2 protein deduced from PepYLCV isolate collected also from Western Sumatera AMO26186.1 (KT809345.1) [7] showed homology only 94% and was detected as unique protein compared to other putative V2 proteins. Recombination detection program (RDP) [22] also failed to identify any recombination event, indicating a

highly conserved motif among V2-gene of PepYLCV existing in West and Northern (Sumatera) as well as in Java island.

Further detailed domain analysis showed that our putative V2 protein has two conserved domain, i.e. gemini-V2-Superfamily motif and WCCH superfamily motif. The gemini-V2 motif was believed to be associated with movement and systemic transportation of virus particle in PepYLCV [10]. Mutation of the V2 gene in Tomato yellow leaf curl virus (TYLCV) inhibited its ability to accumulate and systemically infect tomato plants, suggesting that the V2 gene product is required for successful infection of the host [10]. The specific function of WCCH domain is not yet identified, and was identified to be present in retrotransposon and many geminiviruses [23].

CONCLUSION

Theputative V2 gene sequence was successfully cloned spanning 354 nucleotides or 117 amino acids. The gene encodes protein with molecular weight of about 13,404.36 Dalton and its pI value is predicted to be 6.93.

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