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Submission date: 15-Feb-2022 10:26AM (UTC+0700)
Submission ID: 1762654588
File name: 6_Jurnal_DNA_Mutation_in_CVPDr_DNA_Fragment_IGA_Diah_Yuniti.pdf (267.96K)
Word count: 3298
Character count: 17904

International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 08, 2020
ISSN: 1475-7192
DOI: 10.37200/IJPR/V24I8/PR281131

DNA Mutation in CVPD^r DNA Fragment of Bali Citrus

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***Abstract*---**The CVPD^r DNA fragment was identified previously and found in some citrus plants in Bali. This DNA fragment was said to be a resistant factor against citrus vein phloem degeneration (CVPD), or citrus greening, or also called huanglongbing disease. However, the CVPD^r DNA fragment also found in some other citrus plants which susceptible to CVPD disease. In this study we analyzed the DNA sequence of CVPD^r DNA fragments from 13 citrus plants. The results of this study found some mutations in some citrus plants comparing to the CVPD^r DNA fragments of *Triphacia trifolia* which considered to be resistant to the disease. The mutation included deletion, insertion, transvertion, and transition on the DNA sequences of each sample. These mutations may affect the character of CVPD^r DNA fragment and resulted in susceptible to the disease. The dendogram derived from this sequence analysis showed the genetic distance/similarity of each citrus plants to *T. trifolia*. The citrus plants which cluster near *T. trifolia* showed rather tolerance to CVPD disease.

***Keywords*---**Citrus plants, CVPD^r DNA fragment, DNA mutation

Introduction

Citrus spp. is one of the most important horticultural crops in Indonesia. Citrus production in this country was fluctuated during the period 1970-2012. During 2015 to 2016citrus fruit

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Manuscript submitted: 09 March 2020, Manuscript revised: 18 April 2020, Accepted for publication: 01 May 2020

production in Bali was decreased 35% compares to the previous period (Ministry of Agriculture, 2016). This reduction was mainly due to the attack of Citrus Vein Phloem Degeneration (CVPD) or - Citrus Greening disease. CVPD has became a serious threat to the sustainability of citrus production in many provinces (Tirtawijaya, 1981, Wirawan, et al, 2004, Wirawan, et al., 2015). It iscaused by *Candidatus liberibacter asiaticus*, abacterium which propagates through the bud asulusor transmitted by an insect vector, viz. *Diaphorina citri* (Tirtawijaya, 1981, Sandrine & Bove, 1996, Wirawan, et al., 2004, Secor, 2009).

The CVPD symptom is very distinct. The leaves of the infested plant are yellowing, with irregular green color, while the dark green veins are bulging. The leaves of the heavily infested plants are small and thick. The symptoms resemble that of particular nutrient deficiency (Mead, 1998; Knapp *et al.* 1999). The pathogen cannot be cultured *in vitro* but can be detected by PCR using 16S rDNA primer (Sandrine &Bove, 1996) and observed under electron microscopy (Hoy,1998).The whole genome of strain A4 of *L. asiaticus* was already sequenced by Zeng et al.(2014).

Almost all of the cultured citruses are susceptible to CVPD. Wirawan et al. (2004) found severalplants that show resistance to CVPD suh as *Citrus aurantifolia* and *Triphasia trifolia*. The considered resistant or tolerant plants are called $CVPD^r$ and harbor the $CVPD^r$ DNA fragment (Wirawan, 2016). This $CVPD^r$ DNA fragment has been cloned into the plasmid vector, pWR27, and patented with the ID number of P 0020148. In the study of the distribution of $CVPD^r$ DNA fragment among the citrus plants, we found that this fragment are also distributed in the susceptible plants (Wirawanet al.,2004, Wirawan and Juliasih,2015). This finding rise a question on the validity of the $CVPD^r$ DNA fragment itself, whether it can be regarded as a gene that bore the resistant factor to CPVD. Therefore, we conducted the study to analyse the polymorphism of $CVPD^r$ DNA fragment in citrus from Bali and discuss the differences of the resulted DNA sequence compare to $CVPD^r$ DNA fragment of *T. trifolia* which is considered to be resistant to CVPD.

Materials and Methods

Sampling sites and plant material

The sampling sites werethe regencies and city in Bali as shown in Fig. 1. Topographically, the sites were located at the altitude of 800 meters above sea level, with the tropical climate,the average annual rainfall between 2000 and 2500 mm, and the temperature of 24^{o}C to 32^{o}C. The samples were taken from citrus cultivated in Buleleng, Karangasem, Bangli, Badung, Tabanan,and Gianyar Regencies, as well as Denpasar City. Leaves were collected from CVPD resistant citrus (*T. trifolia* and *C. aurantifolia* seedless) and susceptible citrus viz. *Citrus nobilis*, *Citrus reticulate* slayer, *C.reticulate* keprok, and *Citrus grandis*. The leaves are selected from the second leaf below the growing point, wrapped in plastic and taken to the lab for further DNA isolation by using liquid nitrogen. Samples were taken from leaf bones using the NucleoSpint®kit.

Figure 1. The sampling sites of the citrus leaves used in the study: 1) Buleleng (Singaraja), 2) Bangli, 3) Karangasem, 4) Tabanan, 5) Denpasar, 6) Gianyar Regency, and 7) Badung.

DNA Isolation and PCR Condition

Isolation of total DNA was conducted using Mini Kit Plant from nucleoSpin® Plant II (Marchery-Nagel) following the company's instruction. The amplification of the $CVPD^r$ DNA fragment was done using GACTAGGTGGTAATAACTACTTTT and CCTTTTTGGTCTATCTTTACTTAG primer pairs (Wirawan et al., 2016) that targeted 841 bp DNA. PCR reaction contained 1 ng of DNA sample, 100 p mol of each primer of WR-F and WR-R, 2µl dNTP, 2µl PCR Buffer (10X), 0,2 µl Taq polymerase (5 U/µl) and H_2O to reach a total volume of 20 µl.The PCR products were then sequenced using the Ilumina Next Generation Sequencer. The PCR condition used by Wirawan et al., (2014),was employed: one cycle of pre-denaturation at 92ºC for 40s, 40 cycles of denaturation at92ºC for 60s, annealing at 60ºC for 40s, elongation at 72ºC for 90s. This was followed by one cycle of final extension at 72ºC for 90s. The PCR products were examined using1% agarose gel electrophoresis in TAEbuffer. The products were then sequenced in Genetika Science Indonesia (GSI) Jakarta.

Data Analysis

The resulted DNAsequences were edited using UGENE (Okonechnikov et al, 2012). The alignments were processed using MUSCLE algorithm as integrated in UGENE to find the differences in base pairs in each sample. Mutation calculation was carried out in a program (C++ Programming) that was run in C++ programming language. This was aimed to spot the differences in base pairs and specify the types of mutations (insertions, deletions, transitions, or transversion). A dendrogram was then derived from UGENE employing the Maximum Likelihood algorithmon both the DNA and the aminoacid translation of the sequences (Vinga & Almeida, 2003; Mulder & Apweriler, 2007).

Results and Discussion

Gel documentation system records a single DNA band of 841bp from almost all of the citrus samples processed with WR primer pairs (Fig. 1).Only one sample that did not produce a band, that was the *C. nobilis* Petang. Two citrus species used as controls were considered resistant or at least tolerant to the CVPD disease, they were *T. trifolia*, and the seedless *C. aurantifolia*. The rest of the samples, viz. 11 of them, were previously identified to be susceptible to CVPD (Yuniti, 2017).

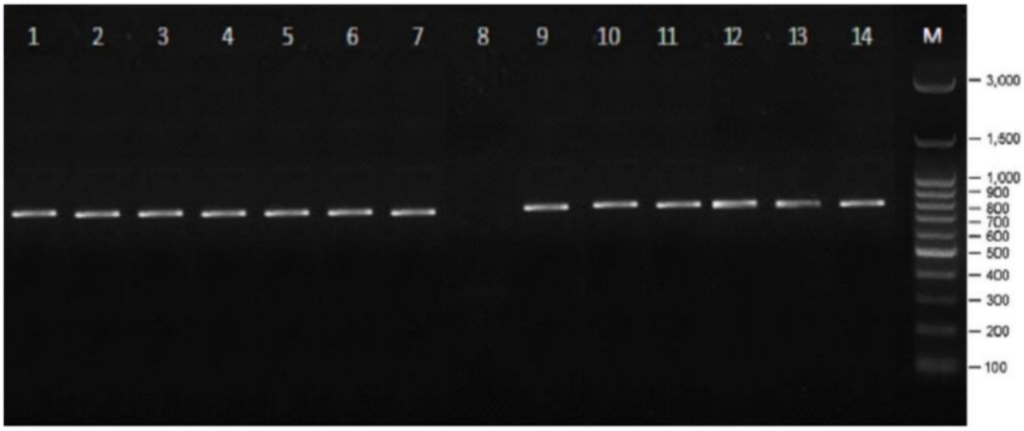


Figure 2. CVPD[r] DNA fragments (841 bp) of Bali citrus. Samples identity from 1-14 were: *C. nobilis* Denpasar, *C. reticulate* keprok Kintamani, *C. nobilis* Tabanan, *C. nobilis* Buleleng, *C. nobilis* Pecatu, *C. nobilis* Gianyar, *C. reticulate* selayer Kintamani, *C. nobilis* Petang, *C. aurantifolia* (control), *C. nobilis* Payangan, *C. nobilis* Karangasem, *C. reticulate* keprok Gianyar, *C. reticulate* selayer Buleleng, and *T. trifolia* (control).

Table 1. The mutations of CVPD[r] DNA fragments in Bali citrus. All newly generated sequences were compared to that of *T. trifolia*.

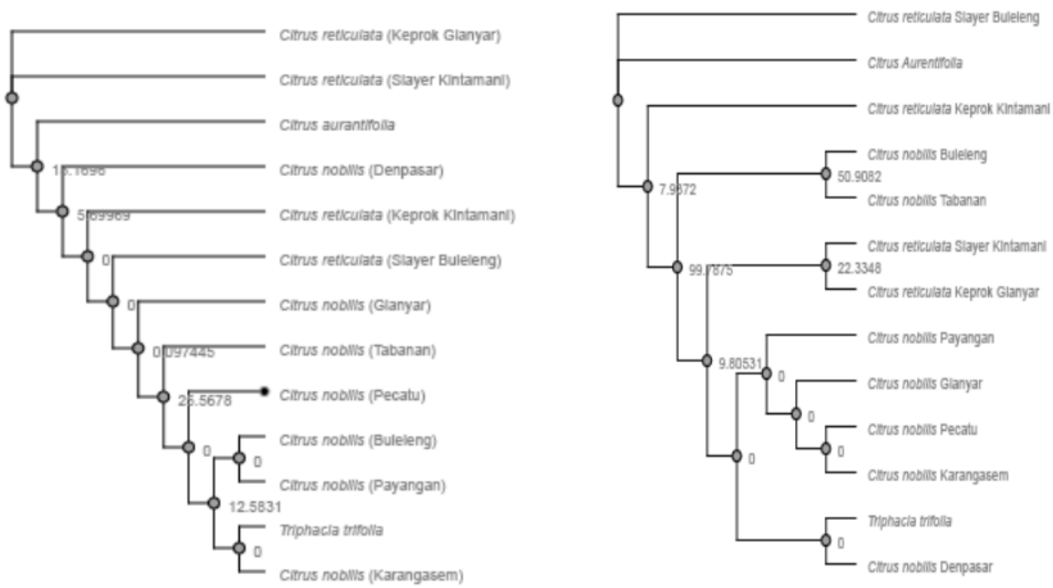
Species	Location	Mutation (Site)				Similarity (%)	Total Mutation
		Insertion	Deletion	Transversion	Transition		
C. nobilis	Denpasar	0	3	2	3	99	8
	Tabanan	2	1	0	2	95	5
	Buleleng	1	0	1	0	95	2
	Pecatu	0	0	1	1	94	2
	Payangan	0	0	0	0	91	0
	Karangasem	0	0	0	0	89	0
	Gianyar	2	0	1	3	93	6
C. reticulata	Kintamani (keprok)	0	4	2	3	99	9
	Kintamani (slayer)	0	0	1	4	93	5
	Buleleng	160	125	103	196	45	584
	Gianyar	0	0	0	3	61	3
C.	-	25	3	6	23	91	57

aurantifoli a

The 11 plants produced CVPD^r DNA fragments are susceptible to CVPD disease. This result indicated that CVPD^r DNA fragment in these citrus plant species or varieties did not work properly. Whether this is caused by this mutation or by other resistant genes is still needed to beconfirmedfurther in other study. The results are shown in Table 1.

There were nine sequences that showed the DNA mutations, while two sequences did not show any mutations (Table 1.).The two unchanged sequences were *C.nobilis* from Payangan and Karangasem. *C.nobilis* Denpasar and *C.reticulate* Kintamani, contained no insertion, but shown the deletion, transversion, and transition of DNA. Both sequences had eight and nine total mutations consecutivelyand shown 99% homology with *T. trifolia*. *C.nobilis* Tabanan however, underwent insertion, transition, and deletion with a total of 5 mutations. *C.nobilis* Buleleng was only detected to has two insertions and two transversions. *C. nobilis* of Tabanan and Buleleng shown 95% similarity to *T. trifolia*. On the other hand, *C.reticulate* Buleleng had a total of 584 mutations with only 45% similarity to *T. trifolia*. This result indicated that the CVPD^r DNA sequences were totally differed from the CVPD^r DNA sequence found in *T. trifolia*.

There were 124 DNA polymorphisms found in the CVPD^{r}DNA fragmentof the newly sequenced samples. The dendrogram derived from the sequence data showed that the *T. trifolia* located in the same cluster with *C. nobilis* Denpasar, while another sample located in different cluster (Figure 3). This result indicated that polymorphisms in the CVPD^r DNA fragment caused the different resistance of citrus plants to CVPD disease. In addition, based on our observation on the field those differences may lead to the resistant, rather tolerant, susceptible and most susceptible to CVPD disease. *C. nobilis* Petang which contain no CVPD^{r}DNA fragment become the most susceptible (Figure 3 and Table 2).



A **B**

Figure 3. Maximum likelihood dendrogram of the DNA (A) amino acid sequences (B)

Table 2. Tolerance response of Bali citrus to CPVD

	Tolerance		
Tolerant	Moderate tolerant	Sensitive	Susceptible
T. trifolia	*C. nobilis* Payangan	*C. reticulate* Buleleng	*C. nobilis* Petang
C. nobilis Denpasar	*C. nobilis* Gianyar	*C. aurantifolia*	
	C. nobilis Pecatu	*C. reticulate* keprok Kintamani	
	C. nobilis Karangasem	*C. nobilis* Buleleng	
		C. nobilis Tabanan	
		C. reticulate slayer Kintamani	
		C. reticulate Gianyar	

The study showed that there were many DNA polymorphisms detected among the samples in CVPD^r DNA fragment. Gene polymorphism occurs because of a change in the nucleotide arrangement of a gene. Changes in the composition of the gene are influenced by several factors such as natural or artificial selection, mating, and mutation. These changes can affect the phenotypic changes of an organism (Frankham, et al., 2002). Polymorphism is a variant in DNA sequences that can cause changes in protein function. Protein, when detected, can estimate the gene owned by the individual, because one type of protein is a representation of the locus owned by the individual concerned. (Ayala and Kiger,1980).

Theoccurrence of polymorphisms in a gene, indicating the presence of some differences in DNA sequences in citrus plants sampled with different species and the same. The difference in DNA sequences is due to the presence of deletion, insertion, recombination, low random mating and selection in the population (Schleif, 1993).

The CVPD^r DNA fragments, which were previously identified in *T. trifolia* and *C. aurantifolia* var. seedless lime, were also found to be distributed in other citrus plants. The most susceptible plants to CVPD disease, *Murraya paniculata* and *C. nobilis* Petang did not contained CVPD^r DNA fragment. This result indicated that the CVPD^r DNA fragment was a resistant or tolerant factor against CVPD disease.

Previous studies support this analysis. In animal studies, the Major Histo compatibility Complex (MHC) polymorphism was characterized by the presence of many alleles at each locus and the difference in the number of amino acids in each allele. This variability was correlated with the diversity of T cell receptors, which contributes to differences in immune responses and resistant to diseases in each individual (Sommer, 2005). Other study reported that twelve types of native Portuguese cattle were used to investigate polymorphisms of microsatellite loci of BoLA gene and non-BoLA microsatellite locus as additional data to compare the genetic / polymorphic variation found in both genes. Ammer et al. (1992) reported that polymorphisms were found in both exon and intron regions of the BoLA-DRB gene. Ellegren et al., (1993) also found a strong correlation between the sequence of ex-BoLA-DRB3.2 exon polymorphisms and simple repeat microsatellite polymorphisms in the intron 2 region of this gene. It has also been reported that polymorphic microsatellite in the intron 2 region of BoLA-DRB1 (DRBP1) pseudogene and this gene is not genetically related to the BoLA-DRB3 (DRB3) microsatellite (Gwakisa et al., 1994). Using two molecular markers of microsatellite BM1815 and RM185, it was reported that there was a correlation between microsatellite loci of BoLA class II gene with resistance or susceptibility to *Boophilus microplus* livestock infection in cattle (Acosta-Rodriguez et al., 2005). The nature of the BoLA gene polymorphism causes each individual's ability to react to different and very specific antigens.

Citrus that looks healthy has a possibility to contain CVPD pathogenic, because the period of CVPD pathogens incubation in plants host were ranges from three to five months (Tirtawidjaja and Suharsojo, 1990). So that we need a precise and fast way to detect the presence of CVPD pathogens in citrus seedlings. There are citrus plants types that are resistant to CVPD disease and here in after referred to as $CVPD^r$. The $CVPD^r$ gene was found in Siam Kintamani oranges, which were susceptible to CVPD. Polymorphism was carried out to confirm the the$CVPD^r$ gene presence in all citrus plants. The $CVPD^r$ DNA fragments Polymorphism in citrus plants. These results indicate that $CVPD^r$ DNA fragments are a factor that is resistant to CVPD disease. Although there has been genetic material change (DNA) mutations have not occurred yet. Further research needs to be done whether it is true that a mutation has occurred, even though the results of sequencing have found many nucleotide sequences that caused proteins dis-function. The cells Exists were un-able to tolerate the protein inactivity, it will cause death (lethal mutation).

DNA polymorphism in $CVPD^r$ DNA fragments caused citrus plants resistance differences to CVPD disease. Another factor is caused by the lack of nutrients, which is due to phloem tissue infection by *L. asiaticum* bacteria. As a result, it inhibits the phloem cells transport mechanism, so the mineral elements are not transported properly to plant cells that's has a function as photosynthesis. The low levels of Zn and Mg in the objects are also thought to be the result of harvesting crops and other agricultural crops, so that nutrients were reduced. The condition of water content, vitamin C levels and antioxidant levels due to the inhibition of nutrient condition absorption which needed by plants. Lack of root ability to absorb nutrients can result in deficiencies of Fe, Zn, and Mn, which makes a chlorosis symptom in plant leaves (Zekri M and Obreza, 2012).

Conclusion

The difference in polymorphism of $CVPD^r$ DNA fragment resulted in difference position in the dendogram. Citrus plants studied could be classified as citrus resistant among citrus plants. In the *T. trifoliate* dendrogram that were resistant to CVPD disease classified in the same group with *C. nobilis* Denpasar which consider being relatively tolerant to the CVPD disease. On the other hand, citrus plants which were sensitive to CVPD disease located in different group. This result indicated that the $CVPD^r$ DNA fragment is a resistant factor against CVPD disease and the resistant respond of citrus plants depended on their polymorphism in $CVPD^r$ DNA fragment.Thepolymorphism changes, adds, or reduces nucleotides than it will change the amino acid in the protein which it encodes. This amino acids changecertainly willchange the protein character or function, which in turn it willchanges the phenotype, especially the phenotype which differentiates resistance to CVPD disease.

Acknowledgements

We thank the Faculty of Agriculture and the Laboratory of Genetic Resources and Biomolecular, Udayana University, Denpasar, Bali for the support and facilities.This study was funded by Udayana University research grant, LoA no. 383-5/UN14.4.A/LT/2018.

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