INTERNATIONAL JOURNAL OF BIOSCIENCES AND BIOTECHNOLOGY • Vol. 7 No. 2 • April 2020
eISSN: 2655-9994
pISSN: 2303-3371
https://doi.org/10.24843/IJBB.2020.v07.i02.p05

PRIMER DESIGN OF CVPD^r DNA FRAGMENT SEQUENCES THAT AMPLIFY SPECIFIC FRAGMENTS TO DISTINCT THE RESISTANT FRAGMENT FROM *Triphasia trifolia* (Burm. F.) P. Wils. AND THE SUSCEPTIBLE FRAGMENT FROM *Citrus nobilis* Lour.

Ni Made Ayuratih Utami*, I Gede Putu Wirawan, and I Ketut Suada
Department of Agroecotechnology, Faculty of Agriculture, Udayana University
*Corresponding author: ayuratihutami@gmail.com

ABSTRACT

CVPD^r is a DNA fragment that indicates that plants are resistant to CVPD. Previous research using primers that amplified 841 bp CVPD^r fragment was able to amplify the fragment from *Triphasia trifolia* that considers being a resistant plant, *Citrus aurantifolia* var. seedless which considers being a tolerant plant, and some susceptible citrus plants to CVPD disease. In this study, we designed some primers that amplified only CVPD^r DNA fragment from *T. trifolia* which consider as the resistant plant and a primer that amplified only DNA fragmen from *Citrus nobilis* which consider as the susceptible citrus plants. The primers for CVPD^r on *T. trifolia* are TCATCTGCATGGGATACC for forward primer and GCCTTGAGCTTGTAAGTG for reverse primer which turned out to amplify the DNA of *T. trifolia* and also the *C. nobilis* cultivar Denpasar and only succeeded in not amplifying the *C. nobilis* cultivar Gianyar. The primers for CVPD^r on *C. nobilis* are GAATGGCTTAGCAGAAAGG for forward primer and GGTTGTAGATGGACATAGG for reverse primer turned out can not only amplify the DNA *C. nobilis* but also amplify *T. trifolia*.

Keywords: CVPD^r fragment, Primer Design, *Citrus nobilis, Triphasia trifolia*

INTRODUCTION

Citrus fruit production in Bali has fluctuated over the past five years. In 2016, citrus production in Bali from the previous year decreased by 45,133 tons based on various factors decreasing citrus production (Badan Pusat Staristik, 2018), one of which was an attack of Citrus Vein Phloem Degeneration (CVPD). Some popular plants, some non-commercialized citrus plants and some citrus relatives, are proven to be resistant to CVPD.

CVPD is caused by *Liberibacter asiaticus* which develops in shoots and is transmitted by vector insect *Diaphorina citri* (Tirtawijaya, 1983; Jaqoueix et al., 1994; 1996; Wirawan, et al., 2004). In addition to oranges, CVPD can be transmitted to several members of the oranges (*Rutaceae*) tribe such as *Poncirus trifoliata* (L) Raf, Kemuning / *Murraya paniculata* (L) Jack, *Swinglea glutinosa* and *Clausena indica*.

Martasari et al. (2004), stated that many citrus farmers complained about the condition of their oranges which were

attacked by various diseases both by bacteria and viruses. All citrus plants cultivated are susceptible to CVPD disease attacks (Mahayani, 2013). In several cases several types of citrus plants were reported, notably commercialized citrus plants and some of their relative plants, known to be resistant to CVPD. Among them are seedless lime, Tahiti lime, *Triphachia trifolia* (kinkit orange), and *Poncirus trifoliata* (karatachi). Citrus plants that are resistant to CVPD are thought to contain genes that produce a trait that is able to break the pathogenic CVPD infection or is able to resist transmission of pathogens carried by vector insects (Wirawan et al., 2004).

Previous studies was done using DNA fragments isolated using RAPD and mutations from *T. trifolia*, these fragments were indicated to be resistant factors to CVPD disease and then referred to as CVPD^r DNA fragments. Yuniti et al. (2018) found polymorphisms in CVPD^r DNA fragments in several citrus species (*Citrus*, spp) and 12 types of citrus plants that were sampled were plants that had CVPD^r DNA fragments. Mutations that occur in some CVPD^r DNA fragments cause changes in bases in DNA fragment sequences so that repairing sequences of some of these plants can be distinguished according to their level of sensitivity to CVPD disease.

The purpose of this study is to develop primers that only amplify DNA fragments from resistant plants (*T. trifolia*) and susceptible plants (*Citrus nobilis*). This primer is expected to be a specific primer that can be used to detect CVPD^r in resistant plants (*T. trifolia*) and susceptible plants (*Citrus nobilis*).

MATERIALS AND METHODS

The study was conducted in two stages, namely in *silico* and *in vitro*. Research conducted in silico or using a computer is a bionformatics research to design the desired primer.

The results of *C. nobilis* DNA sequencing from seven regions in Bali and *T. trifolia* DNA (Yuniti et al., 2018) were converted to FASTA format. The sequences in the FASTA format are then aligned using the Ugene (Okonechnikov, *et al.*, 2012) application with the MUSCLE algorithm. Sequences are sought for conservative regions which are then used as templates for primer designs.

The primer design is done with *in silico* method using the Clone Manager program through several stages. First install the Clone Manager Pro 9 Program. Prepare the sequential data in FASTA format. Open the Clone Manager Pro 9 program, select the primer menu on the menu bar, then choose design. In the dialog box, enter the desired

primer and reverse primer lengths (18-24 bases); Click the criteria menu then enter the desired primer parameters. Then select next. Enter the data sequence that has been previously saved and the target region is equipped with the desired base position as the start and end of the target region, select finish. In the dialog box primer results will appear based on the primer criteria and sorted by rank.

The Clone Manager program will display several primer candidates according to the required criteria. The best primers candidates then tested for their annealing place using the Ugene application.

In vitro research was carried out after the primer design was completed, which is the primer test using the PCR method in the laboratory.

RESULTS AND DISSCUSSION

Triphasia trifolia Primer

The CVPD^r fragment sequence from kinkit orange is input into the Clone Manager application along with the desired primer parameters. The Clone Manager application then display several suitable primer candidates and sorted by rank. There are five candidate pairs of *T. trifolia* primers who fit the proposed criteria. Information about the five primer pairs can be seen in table. 1.

Table. 1 *T. trifolia* primers candidate from the Clone Manager Program

Primer	Jenis Primer	Primer sequence	GC %	Tm (°C)	Length	3' End Dimer	Any Dimer	Product Length
Primer Kinkit 1	F	TCATCTGCAT-GGGATACC	50	57	18	2	4	302
	R	GCCTTGAGCT-TGTAAGTG	50	57	18	1	4	
Primer Kinkit 2	F	GTCATCTGCA-TGGGATACC	52	59	19	2	4	303
	R	GCCTTGAGCT-TGTAAGTG	50	57	18	1	4	
Primer Kinkit 3	F	GGATTGGTCA-GCCTACAAAC	50	60	20	2	3	401
	R	GCCTTGAGCT-TGTAAGTG	50	57	18	1	4	
Primer Kinkit 4	F	GGTCAGCCTA-CAAACTTTGG	50	60	20	2	4	396
	R	GCCTTGAGCTT-GTAAGTG	50	57	18	1	4	
Primer Kinkit 5	F	GGTCATCTGCA-TGGGATACC	55	62	20	2	4	304
	R	GCCTTGAGCTT-GTAAGTG	50	57	18	1	4	

From the five pairs of primer candidates produced by Clone manager suite 9, primers were selected that best suit their needs. The selected forward kinkit primer then written as F-Kinkit and the reverse primer is written as R-Kinkit.

F-Kinkit: TCATCTGCATGGGATACC

R-Kinkit: GCCTTGAGCTTGTAAGTG

The results of the analysis and primer information can be seen in Fig. 1 and Fig. 2. Based on these pictures it is known that the primer pair has fulfilled good primer criteria.

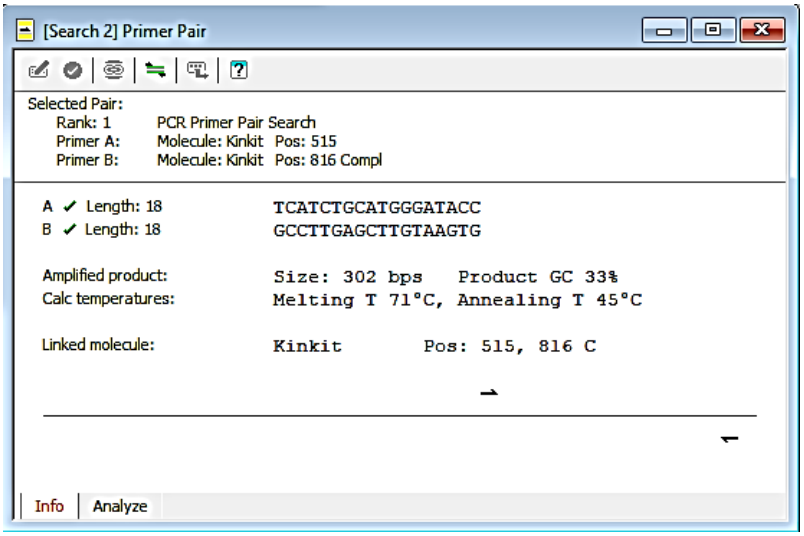

Fig. 1. F-Kinkit and R-Kinkit primer information

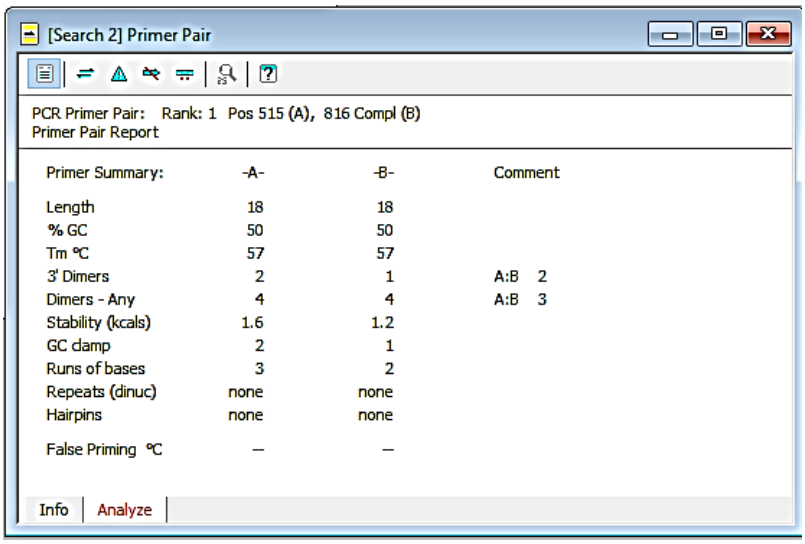

Fig. 2. The results of the analysis of the primer design of F-Kinkit and R-Kinkit

The length of the F-Kinkit and R-Kinkit is 18 bases. Each has a GC percentage of 50% and Tm 57℃. The general rule that is followed by most primer design programs is to use a base percentage of G and C between 40% to 60% (Dinda Eling, 2014).

Primers may not contain complementary base sequences (palindromes) on the same strand. This can result in the formation of hairpins. The primers will naturally fold and produce unproductive priming. The best primers chosen are primers that do not or have little hairpin structure, dimers, and cross dimers (Breslauer et al., 1986).

Based on the analysis results in the program (Figure 4.2), the two primers do not form a hairpins structure. The selected primer pair has a dimer at the end of 3 'ie two on the primer forward and one on the reverse primer. The other dimers formed each bind four base strands. Cross dimers are also formed between the two primers, which are at the end of 3 'by two bases and the other dimer by five bases.

Primer attachment position was again tested using the Eugene program. Primers that have been made stick to the sequences of Kinkit Oranges (*T. trifolia*) and Denpasar Siem orange (*C. nobilis*). The position of the primer attachment in each sequence is as follows:

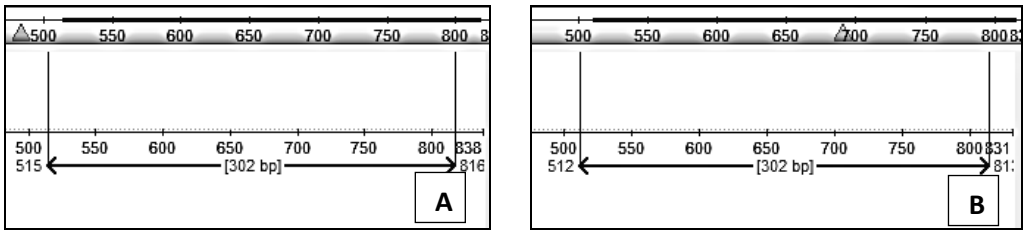

Fig. 3. Position of primer attachment to (A) Kinkit Oranges (*T. trifolia*) and (B) Denpasar Siem Oranges (*C. nobilis*)

The primers attach to the *Triphasia trifolia* sequences and *Citrus nobilis* cul. Denpasar because the two oranges have a similar sequence of bases.

***C. nobilis* Primer**

Primer for *C. nobilis* was obtained after aligning seven samples of *C. nobilis*

cultivar in several regions in Bali so that the same base sequence can be seen. The sequence is then used as a template for primer design. Data sequence of the seven samples of siem orange has different lengths, so the extra bases on the front and back of the sequence are removed so that the sequences of the seven samples are the same length. There were 733 bases after the sequence length of the seven sampled samples were then analyzed with CM suite 9 to obtain the desired primer pair.

There are 59 primer pairs that match the proposed criteria. Four of the 59 primer pairs were ranked first based on ratings in the Clone Manager application. Primer information with the first rank can be seen in table. 2.

Table. 2 *C. nobilis* primers candidate from the Clone Manager Program

Primer	Jenis Primer	Primer sequence	GC %	Tm (°C)	Length	3' End Dimer	Any Dimer	Product Length
Primer Siem 1	F	AGAATGGCTTAG-CAGAAAGG	45	59	20	1	3	144
	R	TGGTTGTAGATG-GACATAGG	45	58	20	1	3	
Primer Siem 2	F	GAATGGCTTAGC-AGAAAGG	47	57	19	1	3	142
	R	GGTTGTAGATGG-ACATAGG	47	56	19	1	3	
Primer Siem 3	F	GAATGGCTTAGC-AGAAAGG	47	57	19	1	3	189
	R	GTTTGTAGGCTG-ACCAATC	47	57	19	2	3	
Primer Siem 4	F	AGAATGGCTTAG-CAGAAAGG	45	59	20	1	3	191
	R	AGTTTGTAGGCT-GACCAATC	45	59	20	2	3	

F-Siem: GAATGGCTTAGCAGAAAGG
R-Siem: GGTTGTAGATGGACATAGG

Compared to the first and fourth primer pairs, the selected primer pairs have a higher GC percentage of 47%. Tm F-Siem is 57°C, while R-Siem is 56°C. The primer pair has a Tm difference of 1°C which is tolerable.

The primer pair does not form a hairpin structure. Each primer has a dimer at the end of 3 'of one base and the other dimer of three bases. Cross dimers are also expected to be formed, which is one end 3 'and three bases complementary to each other.

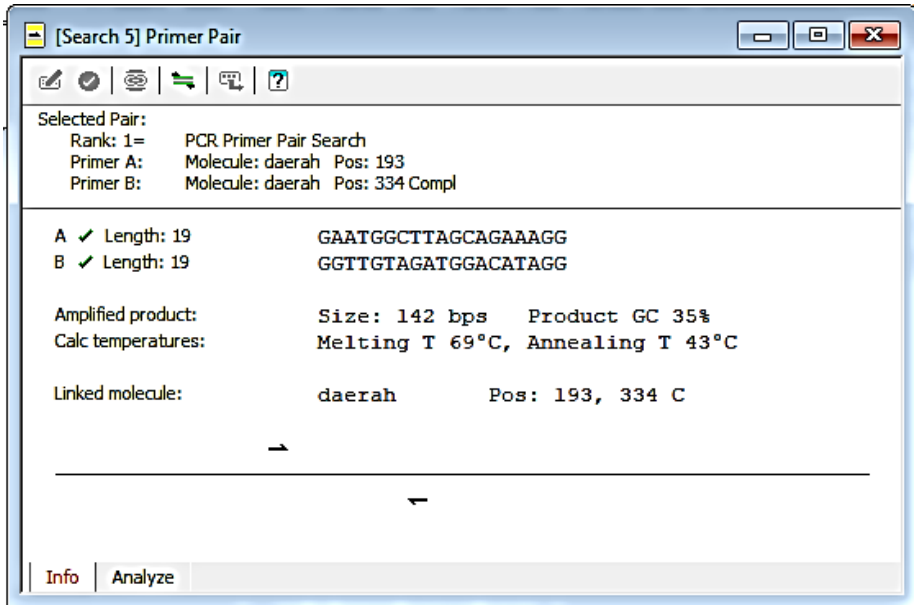

Fig. 4. Primer information of F-Siem dan R-Siem

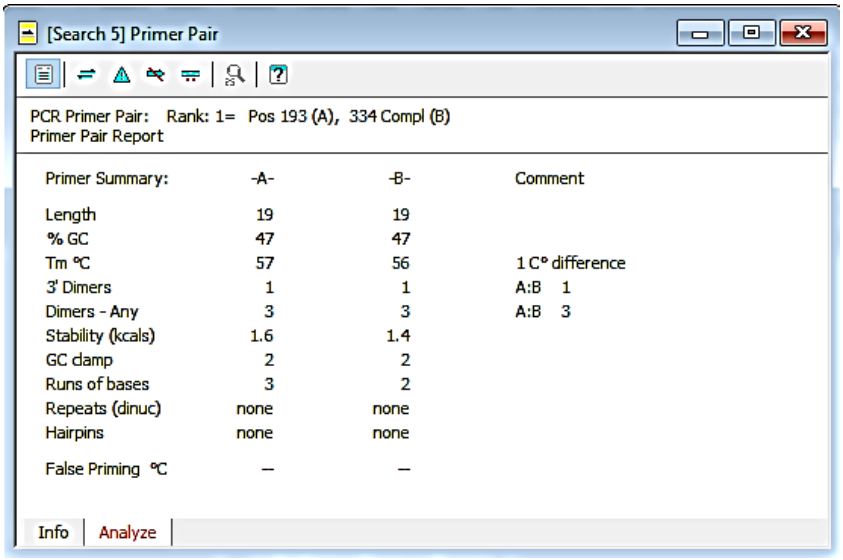

Fig. 5. The results of the analysis of the primer design of F-Siem and R-Siem

The primer attachment sites for each *C. nobilis* sample in the Ugene application are as follows:

Table. 3 Primer attachment sites F-Siem and R-Siem on each sample *C. nobilis*

Orange cultivar	Attachment sites	Product length
Siem	193-334	142
Siem Denpasar	244-385	142
Siem Buleleng	235-376	142
Siem Gianyar	240-381	142
Siem Payangan	202-343	142
Siem Pecatu	236-377	142
Siem Tabanan	249-390	142

Primer Design Test Results by PCR (Polymerase Chain Reaction) Method

Pair of kinkit primer and siem orange primer design results were tested to find out whether the primer succeeded in amplifying the DNA of oranges that had been isolated. Testing is done by PCR method. Each primer was tested on the three DNA samples namely DNA *Triphacia trifolia, Citrus nobilis* cul. Denpasar, and *Citrus nobilis* cul. Gianyar.

DNA isolation from the leaves of siem orange and kinkit orange was carried out to test the primer that had been made. Total DNA isolation was carried out using the Mini Genomic DNA Kit from Genaid. The success of the DNA isolation stage can be seen from the visualization of agarose gel electrophoresis on the UV transilluminator (Fig. 6). The success of the total DNA isolation process is evidenced by the presence or absence of DNA bands in agarose gels during electrophoresis.

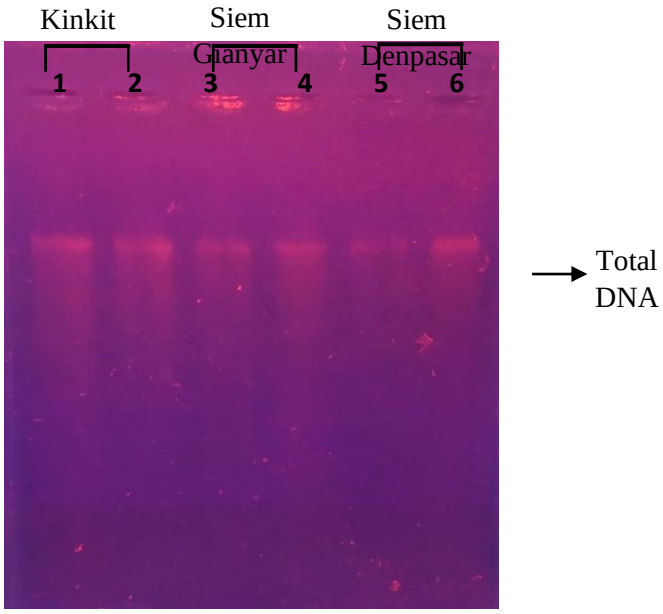

Fig. 6. Visualization of the total DNA isolation results of *T. trifolia, C. nobilis cul.* Gianyar, and *C. nobilis cul.* Denpasar

The PCR results were then electrophoresed on 1% agarose gel using a 1% TAE buffer solution. Warming is done for 20 minutes (100 volts). The agarose gel is then transferred to a UV transilluminator for PCR DNA bands. The presence or absence of DNA bands indicates the primer success in amplifying the target fragment needed. Visualization of PCR results using F-Kinkit and R-Kinkit primers can be seen in Fig. 7.

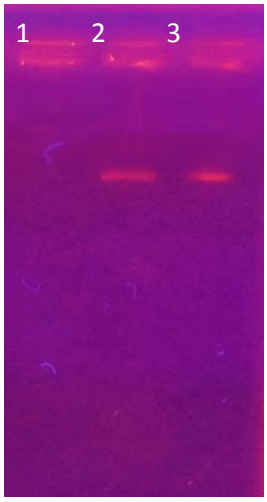

Fig. 7. The results of DNA amplification using F-Kinkit and R-Kinkit primers (1) *C. nobilis* cul. Gianyar, (2) *C. nobilis* cul. Denpasar, and (3) *T. trifolia*.

The primer pair F-Kinkit and R-Kinkit succeeded in amplifying the DNA of *C. nobilis* cul. Denpasar, and *T. trifolia*. DNA bands are seen in lines two and three which prove that DNA was successfully amplified by the primer pairs that had been designed. Lane one filled with *C. nobilis* DNA DNA solution. Gianyar did not show the DNA band (Fig. 7). DNA is not amplified according to *in silico* tests using the Ugene application.

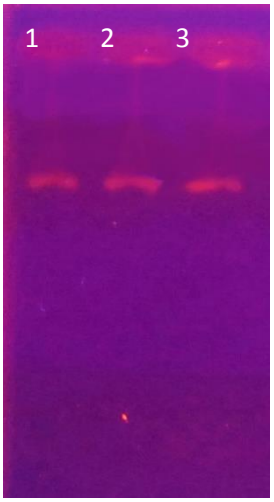

Fig. 8. The results of DNA amplification using F-Siem and R-Siem primers (1) *C. nobilis cul.* Gianyar, (2) *C. nobilis cul.* Denpasar, and (3) *T. trifolia.*

Primer F-Siem and R-Siem amplified all three DNA samples. The primer is expected not to apply *T. trifolia* DNA. The amplification of *T. trifolia* DNA is probably due to the high level of homology of *T. trifolia* and *C. nobilis* DNA. The high level

of homology between genes allows the primer to amplify several genes, although there are differences in several nucleotides (Kamel, 2003).

CONCLUSION

In this study it can be concluded that Primer design results for amplifying CVPDr DNA fragments in the plant of kinkit (*T. trifolia*) namely TCATCTGCATGGGATACC primer primer and reverse primer GCCTTGAGCTTGTAAGTG turned out to amplify the DNA of kinkit orange and also the siem orange cultivar Denpasar. The primer only succeeded in not amplifying the Gianyar cultivar siem orange. Primer design results to amplify CVPDr DNA fragments on the plant of siam (*C. nobilis*), namely GAATGGCTTAGCAGAAAGG forward primer and reverse primer GGTTGTAGATGGACATAGG are not able to only amplify *C. nobilis* but also amplify *T. trifolia.*

ACKNOWLEDGEMENTS

This study was supported by Udayana University Research Grant No. 383-5/UN 14.4.A/LT//2018

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