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Genetic characterization of diplodia resistant in somatic hybrids of *Citrus unshiu* and *Citrus nobilis* using resistance gene analogue (RGA)

D.P.P. Ragayatsu[1], Liliek Sulistyowati[2], Luqman Qurata Aini[2], C. Martasari[3]
[1]*Department of Plant Science, Faculty of Agriculture, University of Brawijaya, Indonesia*
[2]*Department of Plant Protection, Faculty of Agriculture,University of Brawijaya, Indonesia*
[3]*Research Institute of Citrus and Subtropic Fruit, Indonesia*

Abstract:*Information about disease resistant in somatic hybrids is essential in breeding program. RGA markers are useful tools for identification, tagging, and mapping genes for disease resistant. About 4 RGA primers were used to analyze genetic character among 20 somatic hybrids of Citrus unshiu and Citrus nobilis that vary in their resistance to diplodia disease. S1/AS1, Pt8 and 18P33 primers didn't result expected band in amplification products. PCR amplification of RPS2 primers resulted expected bands size 660 bp in SS 1, SS 2 and SS 10 and unexpected bands size 110 bp of all samples. Expected band in SS 1 as resistance sample to diplodia was potential as resistance gene candidate. Protein sequences of expected band and unexpected band in amplification products of RPS2 primers showed amino acid similarity in 70% and there were kinase 2 and kinase 3a as homolog conserved domain.18P33 and RPS2 primers resulted polymorphic bands were selected for dendogram. Dendogram showed clustering of genetic similarity in 91% and all samples has genetic similarity in 100%. It was indicated that 18P33 and RPS2 primers could not prove distinction genetic character of diplodia resistant.*

Keywords: *diplodia resistant, genetic characterization, resistance gene analogue, somatic hybrids of Citrus unshiu and Citrus nobilis*

I. Introduction

Citrus is one important crop in agroindustrial. Good quality is one important thing that required citrus to be competitive crop in agroindustrial. Research Institute of Citrus and Subtropic Fruit had efforts to increase quality of citrus in Indonesia. Protoplast fusion of *Citrus unshiu* and *Citrus nobilis* was done by Research Institute of Citrus and Subtropic Fruit for generating *Citrus nobilis* into seedless [1].

The success of *Citrus nobilis* in seedless usually requires resistance to biotic stress, in order to it has yield well. Biotic stress is one factor that causes decreasing seasonal yields. In Indonesia, the common disease of citrus, diplodia, caused by the fungus *Botryodiplodia theobromae* Pat. caused severe loss in yield, about 53,9% citrus died [2]. The use of resistant varieties is the most effective solution of prevention and controlling disease.

Evaluation is required in breeding program to obtain expected character. Evaluation of diplodia resistant in somatic hybrids of *Citrus unshiu* and *Citrus nobilis* had been done by phenotypic via observation diplodia symptom to obtain *Citrus nobilis* in seedless which resistance to diplodia [3]. However, evaluation disease resistant by phenotypic requires precise screening methodologies, accurating, and extensive knowledge on plant-pathogen interactions [4]. Recent advances in biomolecular have led to observe disease resistant in plants by genotypic via molecular markers. Molecular markers works in DNA level, identification genetic potential intra- and inter- species accurately, and able to works in early stage of plant growing [5] [6].

Resistance gene (R-gene) confers resistance to pathogen in natural defense pathways of plants. R-genes has been isolated from several studies showed similarities of amino acid sequences formed conserved domain. Conserved domain of R-gene has function as interactions resistance proteins and signal transduction in natural defense pathways [7]. Conserved domains of R-genes such as NBS (*nucleotide binding site*), LRR (*leuchine rich repeat*), STK (*serine/ threonine kinase*), TIR (toll/reseptor interleukin-1), and CC (c*oiled-coil*) or LZ (l*eucine zipper*) [8]. Conserved domains of R-genes has been developed as molecular markers known as RGA (*resistance gene analogue*) [9] [10] [11].

RGA markers can identify and approach resistance gene candidate (RGC) and resistance gene like (RGL) in diverse plants [12]. The most RGC sequences of soybean were amplified by degenerate primers designed based on NBS [10]. STK was designed as degenerate primers to approach RGL in other crops [13]. LRR of *Cf2* and *Cf9* genes were also designed as degenerate primers [14]. Degenerate primers designed based on NBS of *N* gene (tobacco) and *RPS2* gene (*Arabidopsis*) were used to approach R-genes to nematodes in

potato [11]. RGC sequences in citrus had been approached and mapped [15]. The most RGC sequences in citrus contains NBS-LRR. RGC NBS-LRR of citrus designed as 14 pair primers to approach R-genes of citrus.

In this study, we characterized diplodia resistant in somatic hybrids of *Citrus unshiu* and *Citrus nobilis* using RGA markers. However, R-gene to diplodia in citrus is not yet found specifically. We used degenerate primers from RGC NBS-LRR of Citrus and several known R-genes to approach RGC to diplodia. The advantage of this study was to find effective method of evaluation of diplodia resistant in somatic hybrids of *Citrus unshiu* and *Citrus nobilis*.

II. Materials And Method

2.1 Plant materials

The plant materials used *Citrus unshiu*, *Citrus nobilis*, *Citrus grandis* as control of susceptible variety to diplodia, *Citrus sinensis* as control of resistant variety to diplodia, and 20 somatic hybrids of *Citrus unshiu* and *Citrus nobilis* (SS) from Research Institute of Citrus and Subtropic Fruit [1] that vary in their resistance to diplodia disease [3] as shown in Table 1.

2.2 DNA extraction

DNA was extracted from 0,3 gram young leaves of samples using method of modified Doyle and Doyle. Testing of DNA quality was done using 1% agarose gel electrophoresis in 0,5x TBE buffer. Testing of DNA quantity was done using 5 ul lamda (2286 ng) and 10 ul lamda (4572 ng) as concentration standard of mass value analysis in Biodoc Analyze.

2.3 PCR amplification

PCR was performed in a Biometra Thermocycler using the following cyclic conditions initial denaturation at 94ºC for 4 min followed 45 cycles each consisting of DNA denaturation at 94ºC for 50 s, primer annealing at 51ºC for 1 min and primer extension at 72ºC for 1 min. Final extension followed 1 cycle at 72ºC for 7 min.

Table 1. List of samples were used for genetic characterization of diplodia resistant using RGA

Label	Samples	Information
1	Citrus grandis	Control
2	Citrus sinensis	Control
3	Citrus unshiu	Parental
4	Citrus nobilis	Parental
5	SS3	Susceptible
6	SS12	Susceptible
7	SS16	Susceptible
8	SS22	Susceptible
9	SS24	Susceptible
10	SS1	Resistant
11	SS5	Resistant
12	SS7	Resistant
13	SS11	Resistant
14	SS14	Resistant
15	SS15	Resistant
16	SS19	Resistant
17	SS2	Moderate
18	SS4	Moderate
19	SS6	Moderate
20	SS8	Moderate
21	SS9	Moderate
22	SS10	Moderate
23	SS13	Moderate
24	SS17	Moderate

PCR was carried in a 25 ul reaction mixture with 5 ul DNA template, 10 ul dream taq, 5 uM of each Forward and Reverse primer as shown in Table.2, and 4 ul aqua bidestilata. Amplification products were visualized at 100 volt for 60 min using 2% agarose gel electrophoresis in 0,5x TBE buffer. Amplification products were performed in Biodoc Analyze.

2.4 Dendogram analysis

Primers resulted polymorphic bands were selected to dendogram analysis. Amplification products were performed into biner profile. Biner profile was analyzed using NTSys, method of SAHN, matrix clustered by UPGMA.

2.5 Sequence analysis

Unique bands of amplification products were sequenced. Sequencing was done by BIOSAINS laboratory, University of Brawijaya. Nucleotide sequences were translated to protein sequences using http://www.ebi.ac.uk/tools/emboss/transeq. Multiple alignment of protein sequences of samples compared with known protein sequences from NCBI using BLASTP algoritm. Multiple alignment of protein sequences was analyzed using CLUSTAL W.

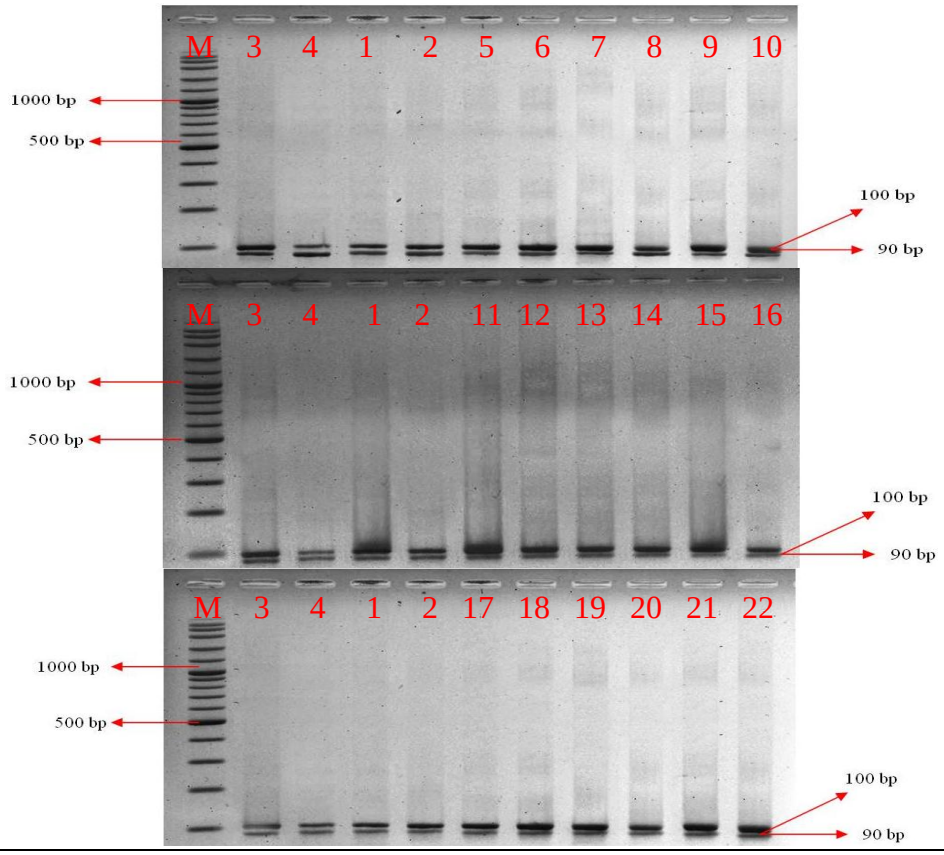
Table 2. RGA primers were used for genetic characterization of diplodia resistant

Primer	***Sequence 5'-3'***
S1 *Forward*	GGTGGGGTTGGGAAGACAACG [11]
AS1 *Reverse*	CAACGCTAGTGGCAATCC [11]
Pt8 *Forward*	ATTCGCGGAAAGATGATTTTGA [15]
Pt8 *Reverse*	ACACTCTTTCGTCACGGTTTCAG [15]
18P33 *Forward*	AAGTCAACAACAACTTCCGCTATCA [15]
18P33 *Reverse*	GGTTTCGGCTAGCTCTGGAATACT [15]
RPS2 *Forward*	ACCCATCAAGTCCGTTGTCG [16]
RPS2 *Reverse*	CAGTGCTTTCCACCATTTCTCC [16]

III. Results

3.1 PCR amplification

PCR amplification of S1/AS1 primers resulted bands size 100 bp and 90 bp of all samples as shown in Fig.1. PCR amplification of Pt8 primers resulted monomorphic bands size 430 bp of all samples as shown in Fig.2. PCR amplification of 18P33 primers showed SS3, SS12, SS16, SS22, SS24, SS4, SS6, SS13 and *Citrus nobilis* has bands size 440 bp and 360 bp; SS1, SS5, SS7, SS11, SS14, SS15, SS19, SS2, SS8, SS9, SS10, SS17, *Citrus sinensis* and *Citrus unshiu* has bands size 440 bp; *Citrus grandis* has bands size 440 bp and 350 bp as shown in Fig.3. PCR amplification of RPS2 primers resulted bands size 110 bp of all samples, except SS1, SS2 and SS10 has bands size 660 bp, 360 bp, 300 bp, and 110 bp as shown in Fig.4. PCR amplification of 18P33 primers resulted two polimorphic bands size 360 bp and 350 bp from total of three DNA fragments as shown in Fig.3. PCR amplification of RPS2 primers resulted three polimorphic bands size 660 bp, 360 bp, and 300 bp from total of four DNA fragments as shown in Fig.4.



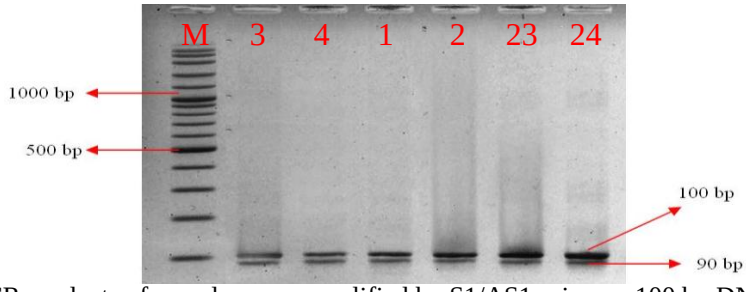


Fig. 1. PCR products of samples were amplified by S1/AS1 primers. 100 bp DNA ladder (Vivantis, USA) (Lane M), *Citrus grandis* (Lane 1), *Citrus sinensis* (Lane 2), *Citrus unshiu* (Lane 3), *Citrus nobilis* (Lane 4), susceptible samples to diplodia (Lane 5 to 9), resistance samples to diplodia (Lane 10 to 16), moderate resistance samples to diplodia (Lane 17 to 24).

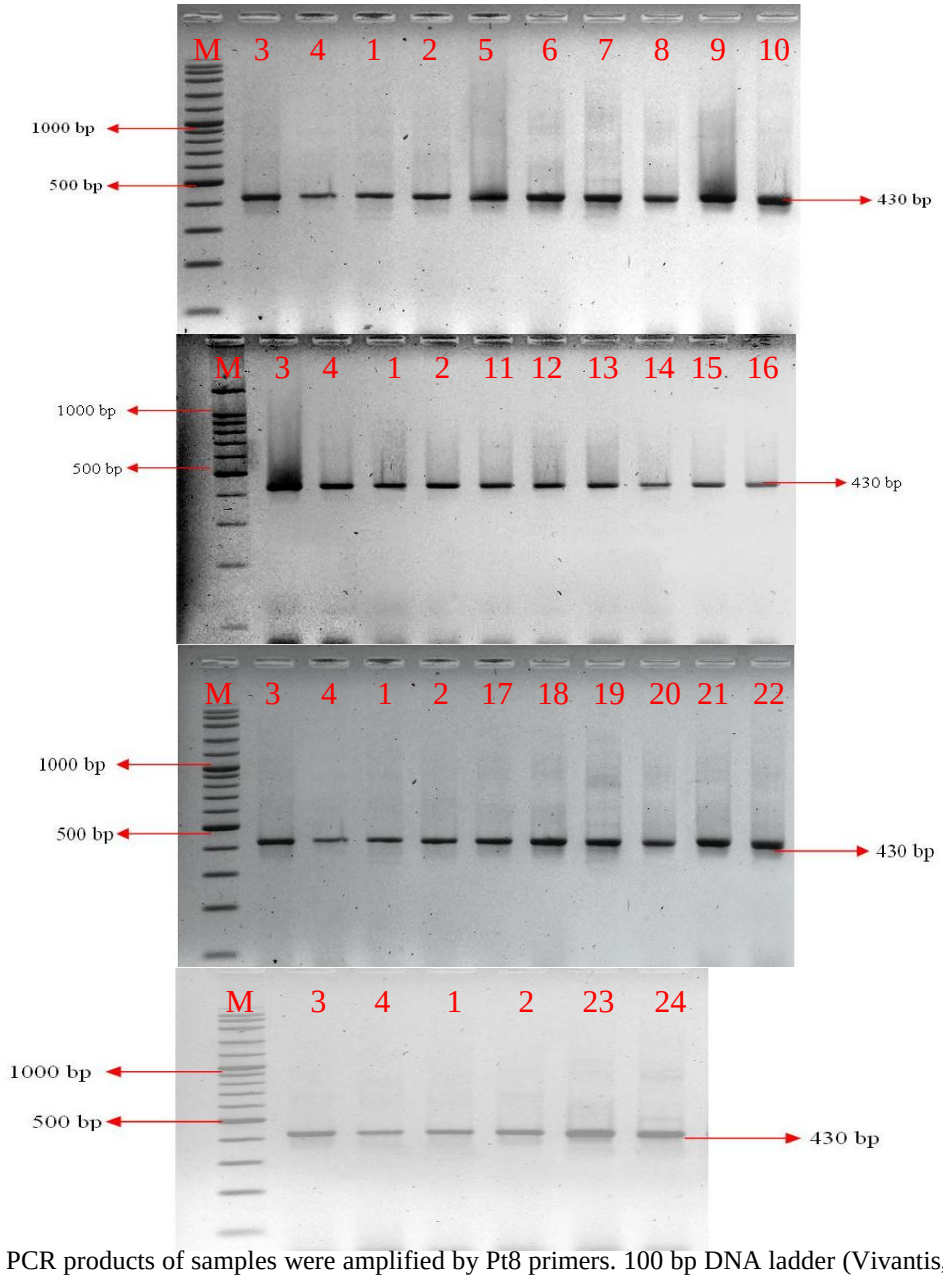


Fig. 2. PCR products of samples were amplified by Pt8 primers. 100 bp DNA ladder (Vivantis, USA) (Lane M), *Citrus grandis* (Lane 1), *Citrus sinensis* (Lane 2), *Citrus unshiu* (Lane 3), *Citrus nobilis* (Lane 4), susceptible samples to diplodia (Lane 5 to 9), resistance samples to diplodia (Lane 10 to 16), moderate resistance samples to diplodia (Lane 17 to 24).

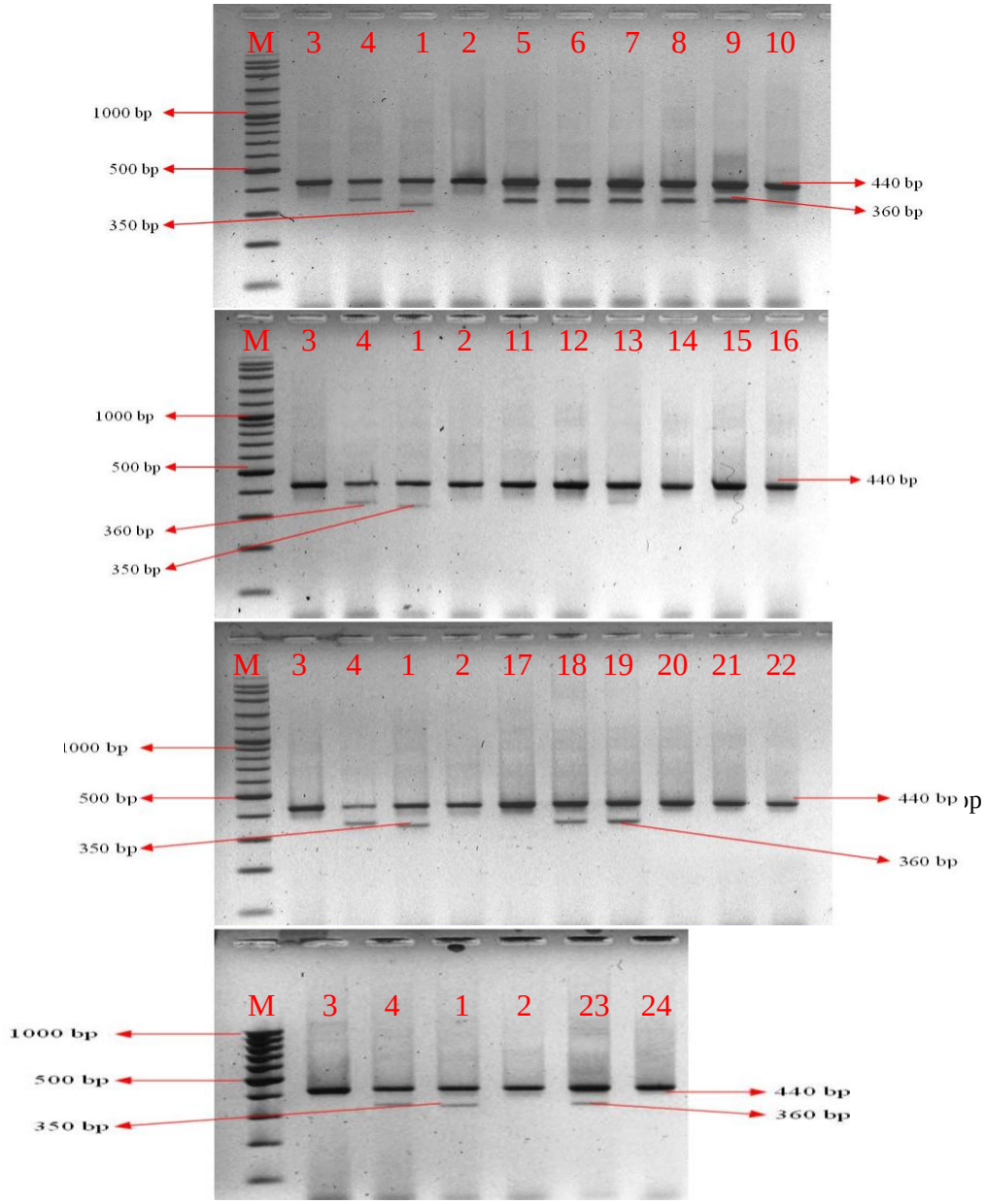
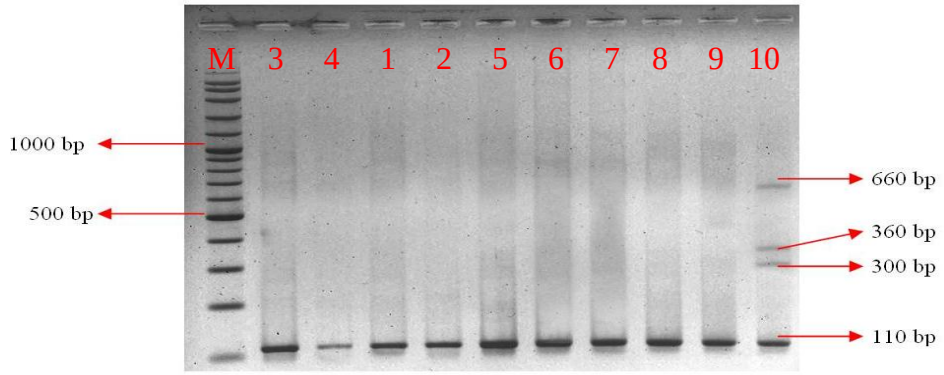


Fig. 3. PCR products of samples were amplified by 18P33 primers. 100 bp DNA ladder (Vivantis, USA) (Lane M), *Citrus grandis* (Lane 1), *Citrus sinensis* (Lane 2), *Citrus unshiu* (Lane 3), *Citrus nobilis* (Lane 4), susceptible samples to diplodia (Lane 5 to 9), resistance samples to diplodia (Lane 10 to 16), moderate resistance samples to diplodia (Lane 17 to 24).



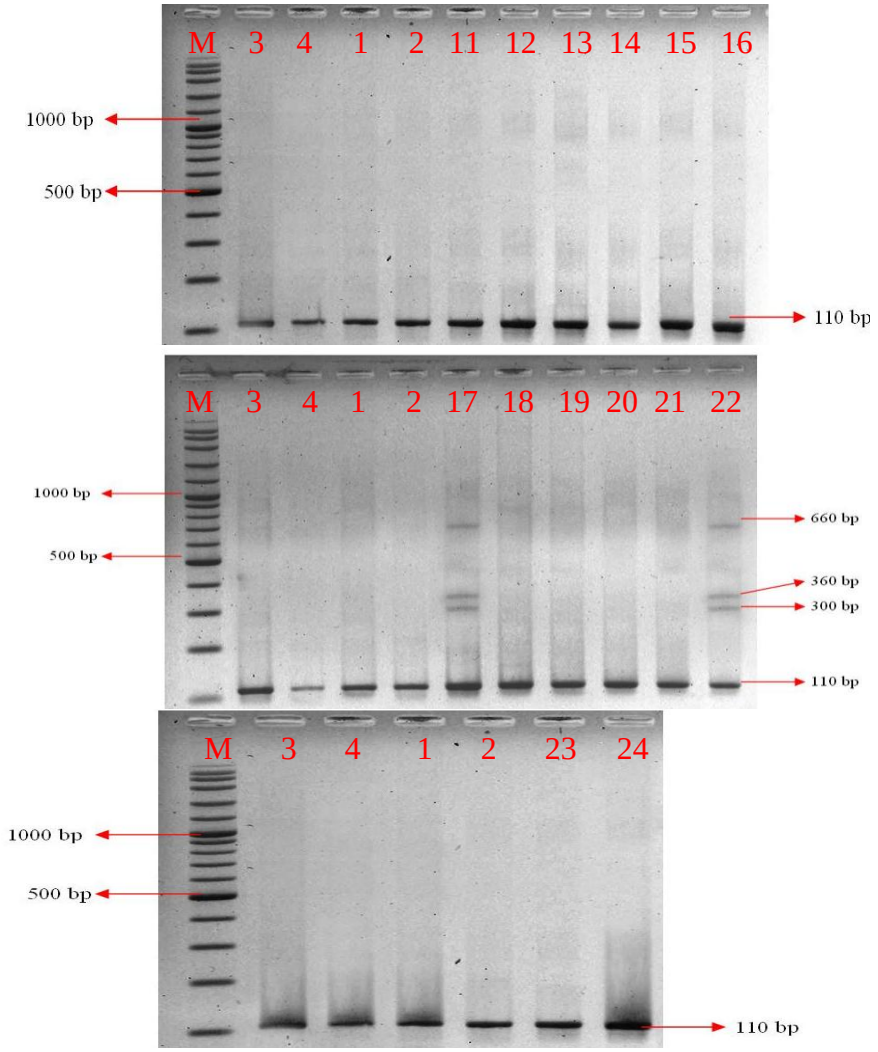


Fig. 4. PCR products of samples were amplified by RPS2 primers. 100 bp DNA ladder (Vivantis, USA) (Lane M), *Citrus grandis* (Lane 1), *Citrus sinensis* (Lane 2), *Citrus unshiu* (Lane 3), *Citrus nobilis* (Lane 4), susceptible samples to diplodia (Lane 5 to 9), resistance samples to diplodia (Lane 10 to 16), moderate resistance samples to diplodia (Lane 17 to 24).

3.2 Dendogram analysis

Dendogram was formed based on products of PCR amplification of 18P33 and RPS2 primers as shown in Fig.5. Dendogram has 2 main clusters with genetic similarity level in 91 %. Cluster 1 has 2 subclusters such as subcluster 1.1 and subcluster 1.2 with genetic similarity level in 92 %. Subcluster 1.1 showed *Citrus sinensis*, *Citrus unshiu*, SS5, SS17, SS9, SS8, SS7, SS19, SS15, SS14 and SS11 has genetic similarity level in 100 %. However, *Citrus grandis* has genetic similarity in 92,5%, it was dispersed of others in subcluster 1.1. Subcluster 1.2 showed *Citrus nobilis*, SS3, SS12, SS13, SS16, SS6, SS4, SS22 and SS24 has genetic similarity level in 100 %. Cluster 2 showed SS1, SS2, and SS10 has genetic similarity level in 100 %.

3.3Sequence analysis

RPS2 primers resulted expected bands size about 660 bp on SS1, SS2, and SS10. RPS2 primers was designed based on sequences of *N*, *Gro-4*, and *RPS2* genes which range 500 bp-600 bp [17] [18]. We took band size about 660 bp on SS1 which resistance to diplodia for sequencing. RPS2 primers also resulted bands size about 110 bp of all samples. We took unexpected band size about 110 bp on SS12 which susceptible to diplodia for sequencing.

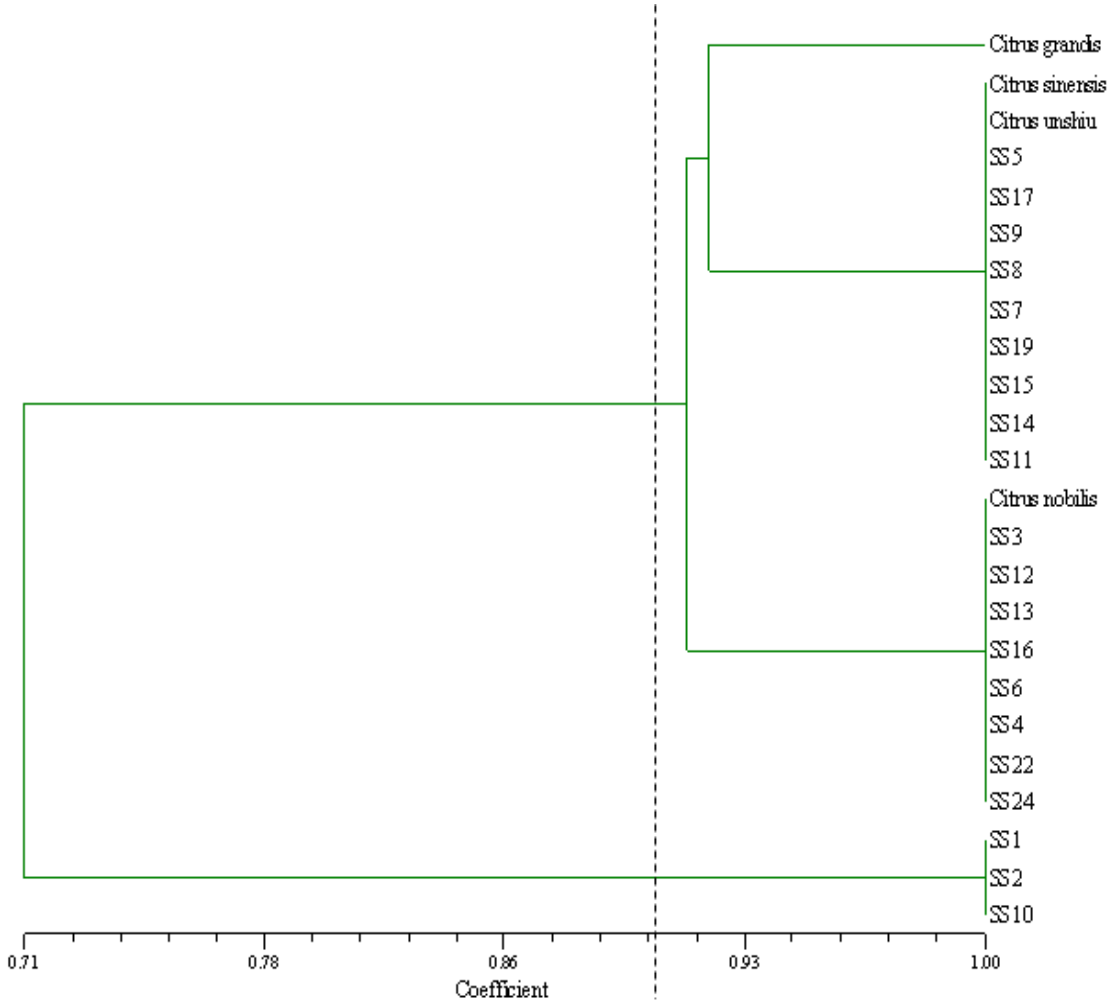


Fig. 5. Dendogram of genetic similarity of samples based on amplification products of 18P33 and RPS2 primers. Number 1 and 2 indicate the clustering detected in 91% genetic similarity using UPGMA.

Protein sequences of samples aligned with 2 accessions from NCBI which has amino acid similarities. Alignment of protein sequences of expected band and unexpected band has amino acid similarity in 70% and E-value in 1e-06 as shown in table 3 and 4. Alignment of protein sequences of unexpected band and 2 accessions has amino acid similarity in 66% and E-value in 1e-06 as shown in table 3. Alignment of protein sequences of expected band and 2 accessions has amino acid similarity in 83% and E-value in 3e-27 as shown in table 4.

Table 3. Similarities level of protein sequences of unexpected band compared by expected band,accession ADV31372 and accession ADV31381 using BLASTP algoritm

No	Description	E-value	Max Identity	Accession
1	Expected band	1e-06	70%	-
2	Nucleotide binding site protein (*Citrus reticulata*)	1e-06	66%	ADV31372.1
3	Nucleotide binding site protein (*Citrus reticulata* x *Citrus trifoliata*)	1e-06	66%	ADV31381.1

Table 4. Similarities level of protein sequences of expected band compared by unexpected band, accession ADV31372 and accession ADV31381 using BLASTP algoritm

No	Description	E-value	Max Identity	Accession
1	Unexpected band	1e-06	70%	-
2	Nucleotide binding site protein (*Citrus reticulata*)	3e-27	83%	ADV31372.1
3	Nucleotide binding site protein (*Citrus reticulata* x *Citrus trifoliata*)	3e-27	83%	ADV31381.1

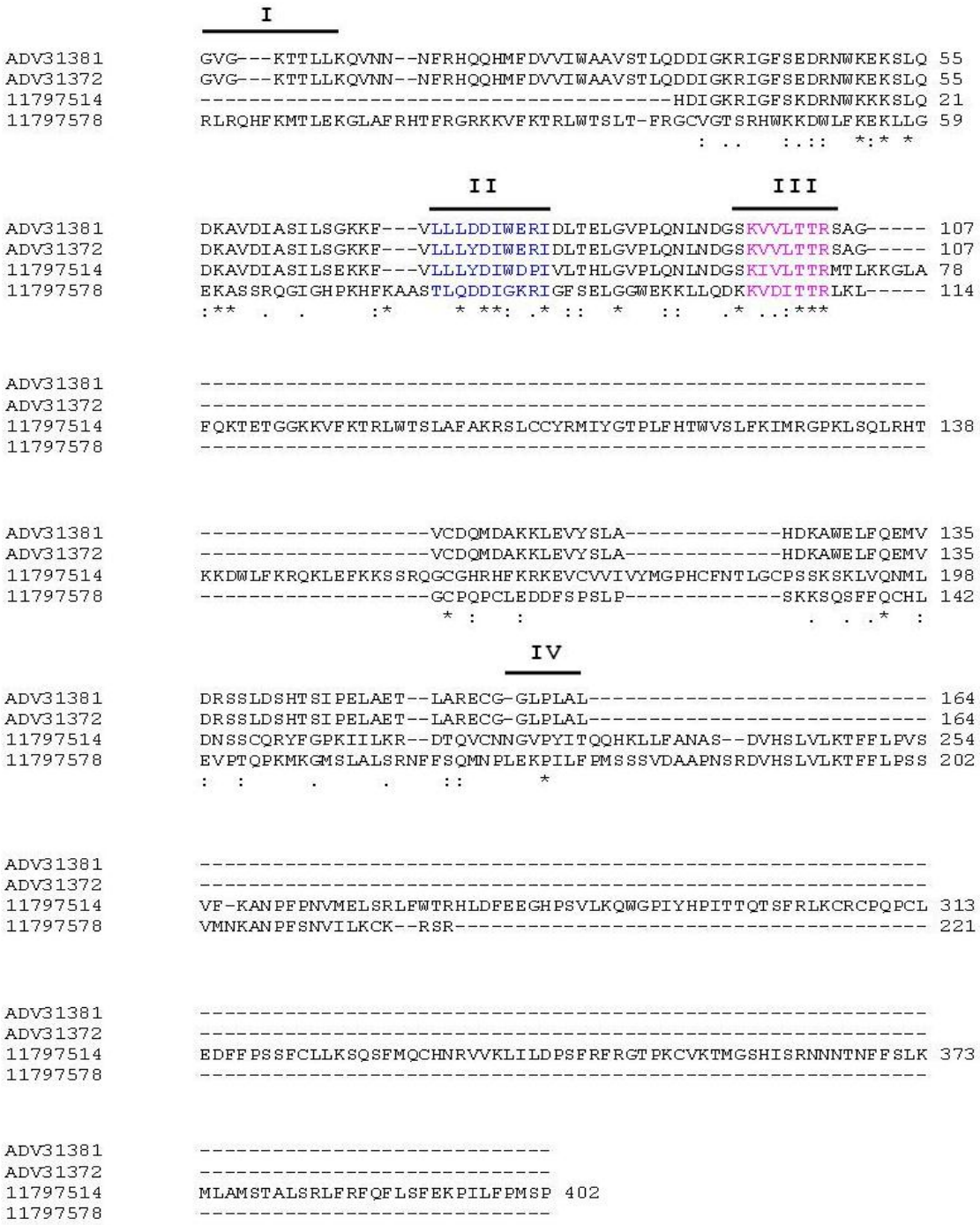


Fig. 6. Alignment of protein sequences of 11797514 (expected band), 11797578 (unexpected band), accession ADV31381, and accession ADV31372 using CLUSTAL W. I (P-loop), II (Kinase 2), III (Kinase 3a) and IV (Hidrofobik).

Alignment of protein sequences of expected band, unexpected band and 2 accessions from NCBI showed homolog conserved domains as shown in Fig.6. Number I showed p-loop domain with pattern formed GVG-KTLL in protein sequences of accession ADV31381 and accession ADV31372, but p-loop domain was not homolog significantly in protein sequences of expected and unexpected band. Number II showed homolog kinase 2 domain significantly in alignment of protein sequences. Number III showed homolog kinase 3a domain significantly in all protein sequences. Number IV showed hidrofobik domain with pattern formed GLPLAL in protein sequences of accession ADV31381 and accession ADV31372, but hidrofobik domain was not homolog significantly in protein sequences of expected and unexpected band.

IV. Discussion

Appearance of polimorphic bands on PCR amplification of 18P33 and RPS2 primers were selected for dendogram. Dendogram showed SS5, SS7, SS11, SS14, SS15, and SS19 as resistance samples to diplodia were clustered with *Citrus sinensis* and *Citrus unshiu* in subcluster 1.1. *Citrus nobilis* was clustered with SS3, SS12, SS16, SS22, and SS24 as susceptible samples to diplodia in subcluster 1.2. Dendogram showed some samples were not suitable based on clustering phenotypically such as : *Citrus grandis* was clustered with *Citrus unshiu* and *Citrus sinensis* in subcluster 1.1; SS1, SS2 and SS10 were clustered in cluster 2. SS8, SS9, and SS17 as moderate resistance samples to diplodia were clustered with *Citrus unshiu* and *Citrus sinensis* in subcluster 1.1. SS13, SS4, and SS6 as moderate resistance samples to diplodia were clustered with *Citrus nobilis* in subcluster 1.2.

We could not conclude that clustering of samples based on genetic similarity of resistant to diplodia because we used degenerate primers. Actually, RGA markers as known degenerate primers can detect sequences of R-gene in widely spectrum [10]. Genetic inheritance of protoplast fusion could influence clustering of samples. It was confirmed by genetic characterization of blast resistance rice varieties using RGA markers showed clustering based on genetic inheritance of breeding [19].

Dendogram has 2 main clusters with genetic similarity level in 91 %. Cluster 1 has 2 subclusters such as subcluster 1.1 and subcluster 1.2 with genetic similarity level in 92 %. All samples in dendogram has genetic similarity in 100%. High percentage of genetic similarity in clustering was caused numbers of primers which used less to confirm distinction of genetic character [20]. It was confirmed by genetic characterization of citrus germplasm using less numbers of primers showed clustering of genetic similarity in 96% [21].

Degenerate primers were used to approach R-gene to diplodia in samples. RGA primers as degenerate primers can approach and detect resistance gene like (RGL) or resistance gene candidate (RGC) in diverse plants [22]. S1/AS1 primers was designed based on NBS of rice [11]. RPS2 primers was designed based on NBS of *N, L6,* and *RPS2* genes contains non-TIR-NBS-LRR [10]. Pt8 and 18P33 primers were designed based on sequences of RGC NBS-LRR in citrus which similar with known R-genes such as *N, L6, I2C-2, RPM1*, and *RPS2* [15]. RGA primers which has NBS-LRR were used cause the most RGC sequences in citrus contains NBS-LRR domain [15].

RPS2 primers resulted expected band size 660 bp in SS 1 as resistance sample to diplodia, SS2 and SS10 as moderate resistance samples to diplodia. RPS2 primers was designed based on sequences of *N, L6* and *RPS2* genes which has size 500 bp – 600 bp [17] [18]. RPS2 primers also resulted unexpected band size 110 bp of all samples. We took unexpected band in SS12 as susceptible sample to diplodia and SS1 for sequencing. Alignment of sequences of SS1 and SS12 showed similarity level of amino acid in 70%. Protein sequence of SS1 showed higher homology level with known accessions from NCBI than protein sequence of SS12. Comparison of amino acid of SS1 and accessions was 83% as shown in table 4. Comparison of amino acid of SS 12 and accessions was 66% as shown in table 3.

RPS2 primers resulted unexpected band size 110 bp of all sample. This condition indicated that these bands was not potential as resistance gene candidate to diplodia. RPS2 primer also resulted expected bands size 660 bp resulted in SS1, SS2 and SS10. Expected band in SS 1 indicated as potential resistance gene candidate to diplodia. Kinase 2 and kinase 3a were found as homolog conserved domain on sequences of SS1 and SS12 as shown in Fig.4. Kinase 2 and kinase 3a were domains of NBS. Domains of NBS such as p-loop and hidrofobik were not found as homolog conserved domain in sequences of SS1 and SS12. Kinase 2 as homolog conserved domain in sequences of SS1 and SS12 showed tryptophan amino acid (W) which conserved significantly. Tryptophan (W) of kinase 2 is character of LZ/CC-NBS-LRR sequence [15]. This condition indicated that sequences of RGC NBS-LRR in SS1 and SS12 similar with known R-genes which contain LZ/CC-NBS-LRR.

V. Conclusion

The present study had proven that 18P33 and RPS2 primers could not show distinction genetic character of diplodia resistant based on clustering of dendogram showed genetic similarity level in 91 % and somatic hybrids of *Citrus unshiu* and *Citrus nobilis* has genetic similarity in 100 %. PCR amplification of RPS2 primers resulted expected band size 660 bp in SS1, SS2 and SS10. SS1 as resistance sample to diplodia was potential as resistance gene candidate to diplodia. PCR amplification of RPS2 primers resulted unexpected band size 110 bp of all sample. Alignment of protein sequences of expected band and unexpected band in amplification products of RPS2 primers showed amino acid similarity in 70% and there were kinase 2 and kinase 3a as homolog conserved domain.

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