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Development of SCAR Marker Linked to *Begomovirus* Resistance in Melon (*Cucumis melo* L.)

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Abstract

Begomovirus is one of the most devastating diseases of melon causing high economic losses for farmers. The most eco-friendly strategy to overcome *Begomovirus* infection is to develop resistant or tolerant cultivar. However, this strategy is time consuming, expensive and complex, especially for hybrid selection process. A pair of Sequence Characterized Amplified Region (SCAR) marker was successfully developed from OPA-4 Random Amplified Polymorphisms DNA (RAPD) marker to discriminate potential *Begomovirus* resistant genotypes from the susceptible ones. Leaf samples were amplified using OPA-4 primer and produced specific band at 1198 bp. The band was purified from gel then sequenced. The sequence was analyzed and extended until 20 bp so it becomes a more specific SCAR marker. BLAST analysis showed that amplified sequence had 95.8% identity with *Cucumis melo* L. mitochondrial DNA. To ensure the consistency of marker, SCAR marker was applied in bulk F_2 plants. The results showed that the marker was consistent amplified same DNA fragment only in *Begomovirus*-uninfected samples. Chi-square test determined that the F_2 bulk segregated following 3:1 pattern for resistant. It was likely indicated that *Begomovirus* resistance trait was possibly controlled by single dominant genes. Furthermore, this marker has been validated using eight different melon cultivars and confirmed not to be affected by environmental conditions.

Keywords: *Begomovirus*; PCR; *Cucumis melo*, L.; SCAR; RAPD

1. Introduction

Melon (*Cucumis melo* L.) is a fruit that has a high economical value. In Indonesia, melon export volume has increased every year since 2008. It also has high nutrition value such as ascorbic acid, beta-caroten, folate acid, and potassium (Park and Crosby, 2004). However, virus infection is a serious threat in melon production either in Indonesia or in the world. *Begomovirus* is one of the most devastating diseases of melon causing high economic losses for the farmer. It was transmitted by *Bemisia tabaci* and caused yellowing leaves, curling leaves, and stunted growth of plants and fruit (Chang *et al*. 2010). Julijantono *et al.* (2010) reported that *Begomovirus* infected melon in East Java and Yogyakarta at 2008 with the infection ranged between 5-100% in the first and 14.3-100% in the second cultivation. The infection causes significant quality and yield loss when high temperature and humidity increased vector population (Alemandri *et al*., 2012; Khrisnareddy, 2013).

Begomovirus control management that is widely used in Indonesia is still dependent on intensive use of insecticides. Some farmers have used greenhouses to reduce the incidence of *Begomovirus* infections, but the development of *Begomovirus*-resistant melon cultivars has not been intensified. The development of resistant or tolerant cultivar is the most eco-friendly and efficient strategy to overcome *Begomovirus* infection, especially for farmers with limited resources (Seal *et al*., 2006, Meija *et al*., 2005). Some melon accession has been confirmed resistant to *Begomovirus*, such as Sudan Accession HSD 2445-005, African accession PI 282448, three Indian accession; 90625, PI 124112, and PI 414723, and Wm-7 (Yousif *et al*, 2007; Saez *et al.*, 2017) but no one reported from Indonesia.

However, the development of resistant cultivar through conventional breeding is time consuming, expensive and complex. Field screening is necessary to select the hybrids that have resistance to *Begomovirus* from some big populations based on visual symptoms. The results also must be confirmed many years at least using two generations (Eathington *et al*., 2007; Nevame *et al*, 2018). The advance of molecular markers is very helpful in accelerating hybrid selection process. The marker helps to distinguish a character among hybrids in early stages, so it can also minimize the costs (Hayward *et al*, 2015).

Molecular marker can be divided into non-PCR based techniques (Restriction Fragment Length Polymorphisms (RFLP)) and PCR-based techniques (RAPD, Amplified Fragment Length Polymorphims (AFLP), Simple Sequence Repeat (SSR), Single Nucleotide Polymorphisms (SNP), etc.). Each method has its advantages and disadvantages (Kumar *et al*., 2009). This study focused on RAPD and SCAR. RAPD is a random method using a short primer to find the molecular marker, whereas SCAR is developed by extending primer based on a specific sequence of DNA. The main advantage of

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RAPD is easy and fast to determine. But it has low reproducibility, which is its main drawback (Bhagyawant, 2016). A marker resulted from SCAR is relatively more stable than those from RAPD. One significant advantage of SCAR compared to RAPD is more specific and reproducible (Lu *et al*., 2010; Yadav *et al*., 2012, Bhagyawant, 2016). Development of SCAR marker linked to plant resistance gene has been widely reported previously such as, powdery mildew in melon (Daryono *et al*., 2011; Daryono *et al*., 2009), powdery mildew in pea (Srivasthava *et al*., 2012), *Fusarium oxysporum* in Banana (Cunha *et al*., 2015), *Puccinia psidii* in *Eucalyptus grandis* (Laia *et al*, 2015), Mungbean yellow mosaic virus in mungbean (Dhole and Reddy, 2012), and *Begomovirus* in tomato (Garcia-Andrez *et al*., 2007). However, no SCAR marker linked to *Begomovirus* resistance in melon has been reported.

MG3 cultivar was used as sample in this study. MG3 was resulted from crossing Melodi Gama-1 (MG1) and Lad-3 cultivar. MG1 was the result of crossing cultivars, the Andes and PI 414723. PI 414723 has been confirmed to be resistant to *Begomovirus* (Yousif *et al*., 2007). Hence, genetically, MG3 may inherit the resistance characters of PI 414723. T Considering the urgency of development *Begomovirus* resistant melon cultivars, this study was conducted to develop SCAR marker linked to *Begomovirus* resistant based on RAPD marker and to reveal inheritance pattern of bulk F_2 through the application of SCAR marker in the population. Furthermore, SCAR marker was also applied to 8 othercultivars for further validation.

2. Materials And Methods

2.1. Plant materials and symptoms observation

A total of 1000 ‘MG3’ melon plants was observed in the university field located in Berbah, Sleman, Yogyakarta. The incidence of *Begomovirus* in this location has been reported by Julijantono *et al.*, (2010) and Subiastuti *et al.*, (2019). *Begomovirus* infection occurred naturally in the field. The symptoms observation was conducted according to Lopez *et al* (2015). For bulk segregation analysis, 100 ‘MG3’ melon plants were planted in open field where *Begomovirus* infections were naturally found. Additionally, 8 cultivars were used for validation developed SCAR marker. *Begomovirus* symptoms were also evaluated under natural conditions.

2.2. DNA extraction

A total of 21 of the 1000 plants observed were used for DNA extraction, consisting of 10 uninfected samples and 11 infected samples. As much as 0.3- 0.5 g of melon leaves was used for DNA extraction using Phytopure (Healthcare) according to Daryono and Natsuaki (2002) with little modification on samples weight and the volume of some reagent. We used 0.3 g leaf samples and 25 µL resin. Then extracted DNAs were analyzed quantitatively using Nanovue Plus Nanodrop spectrophotometer at absorbance 260/280 nm.

2.3. DNA Amplification using CP Primer and RAPD Marker

Molecular detection of *Begomovirus* particle was done to confirm the result of symptoms evaluation. This method was to ensure whether DNA *Begomovirus* in plants or not. A total of 11 DNA melon samples were amplified using CP primer according to Julijantono *et al.* (2010). The reaction was carried out in Bio-Rad T 100TM Thermal Cycler PCR which began with 95 ˚C for 5 minutes, followed by 35 cycles of 95 ˚C for 1 minutes, 52.5 ˚C for 1 minutes, and 72 ˚C for 1 minutes, then ended with 72 ˚C for 3 minutes. Infected samples were marked with 770 bp DNA band.s Amplified DNA were sequenced and analyzed using BLAST. PCR reaction was also conducted using RAPD marker. In this study, OPA-4 primer (5’-AATCGGGCTG-3’) was used to identify specific band correlated with *Begomovirus* resistance in ‘MG3’ melon (Julijantono*et al*., 2012). A total of 25 microliters of PCR mixture containing 12.5 µL PCR kit ready mix (Fastart), 3 µL of 70µg/ml DNA template, 2.2 µL of 3 pmol OPA-4 primers, and 7.3 µL distilled water (ddH_2O) were prepared for PCR. The PCR was done with the following programs: an initial denaturation at 94 ˚C for 3 minutes, 45 cycles of denaturation at 94 ˚C for 3 minutes, annealing at 36 ˚C for 3 minutes and elongation at 72 ˚C for 2 minutes, followed by final extension at 72 ˚C for 5 minutes. PCR results were confirmed by electrophoresis on 1 % agarose gel at 50 V for 60 minutes. The gel was stained using etidium bromide for 30 minutes. A 100 bp DNA ladder (Vivantiz) was used as standar for estimation of DNA band size. After 60 minutes, the gels were visualized using UV transilluminator then analyzed.

2.4. Markers Screening and Sequence Analysis of Polymorphic Fragments

Markers screening was done by selecting bands showing polymorphism between infected and non-infected samples. Those bands were excised from gels and purified according to Daryono *et al*., (2011) using Agarose Gel Extraction Kit (Roche). The purified DNA samples then sent for sequencing that was performed using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) and BigDye® Terminator v3.1 sequencing kit.

2.5. Development SCAR Markers

SCAR primer design was initiated by aligned sequence of selected DNA fragment to identify the locations of OPA-4 primer sequences. SCAR markers were developed by extending forward and reverse primer up to 20 bp. Each primer consisted of first 10 bases of OPA-4 primer. Subsequently, both were tested using the primer Oligo calculator to determine the temperature of melting (Tm) of primers, GC content, and the possibility of a hairpin loop.

2.6. Linkage analysis and validation of SCAR Markers

Linkage analysis was performed to analyze segregation pattern of the SCAR marker linked to resistance to *Begomovirus* in the F_2 progenies of ‘MG3’ melon. A total of 100 plants were analyzed using chi square test. Meanwhile, validation of SCAR marker was performed on 9 different cultivars (Tacapa GB’, ‘Tacapa Silver’, ‘Tacapa Gold’, ‘Hikapel’, ‘Meloni’, ‘Melona’, ‘Gracia’, ‘Luna’, and ‘Madesta’). A total of 13 melon samples, consisted of uninfected samples from all cultivars and 4 infected samples from cultivars ‘Tacapa GB’, ‘Tacapa Silver’, ‘Hikapel’, and ‘Meloni’, were used. DNA samples were amplified using designed SCAR marker in 25 µL PCR reaction consisting of 12.5 µL PCR kit ready mix (Fastart), 3 µL of 250 µg/ml DNA template, 2.5 µL of 100 nM of

each SCAR primer, and 4.5 µL distilled water (ddH_2O). The PCR was performed on 5 minutes initial denaturation at 95 ˚C followed by 35 cycles of 1 minute at 95 ˚C, 1 minutes at 60 ˚C, and 2 minutes at 72 ˚C, then followed with a final extension for 10 minutes at 72 ˚C. PCR products were separated by electrophoresis on 1.5% agarose gel stained with ethidium bromide. Electrophoresis was carried out at 50 V for 45 minutes. Then gels were visualized using UV trans-illuminator.

3. Results

3.1. Begomovirus Infection Assesment

Disease assessment was conducted by visual observations and PCR detection. Visual observations revealed that 10 from 1000 observed plants exhibit common *Begomovirus* symptoms i.e. mosaic, leaf curling, and stunting (Fig.1). *Bemisia tabaci* populations, vector of *Begomovirus*, were also found in bottom surface of melon leaf. Furthermore, all infected leaves and one uninfected leaf (as control) were tested using PCR. Infected samples amplified ~770 bp DNA fragment which not found in uninfected samples (Sawangjit *et al*., 2004). Three out from 10 infected samples did not show targeted DNA band (Fig.2) but amplified untargeted band at 550 bp. However, untargeted band was found either at infected samples or uninfected samples.

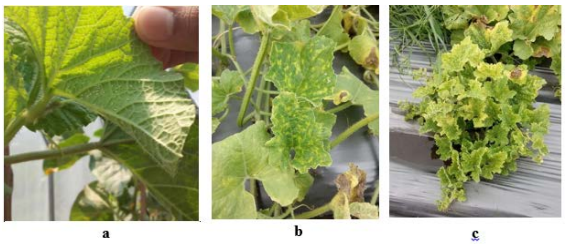

Figure1.*Begomovirus* infected melon leaves with observed *Begomovirus* symptoms, *Bemisia* tabaci population found in bottom surface of the leaf (a), mosaic and leaf curling (b), and stunting growth (c).

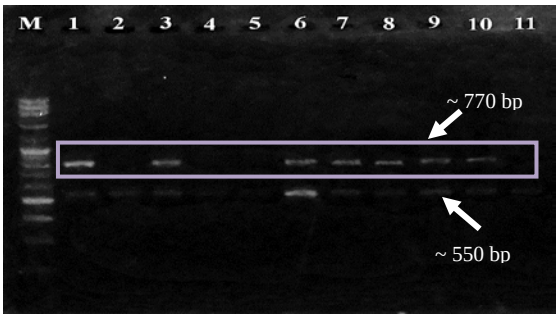

Figure 2. PCR assessment of *Begomovirus* infected melon using CPA2 and CPA5 primer. Infected samples showed targeted band at 770 bp (M: Marker; 1-10: Infected melon samples; 11: Uninfected sample).

3.2. Development of SCAR Markers linked to Begomovirus resistance

'MG3', a hybrid from 'MG1' and 'La-3', were used to detect polymorphisms by RAPD Markers. A total of 13 samples consisting of 9 uninfected plants and 4 *Begomovirus* infected plants were used to detect specific markers linked to *Begomovirus* resistance. OPA-4 primer generated 7 bands consisting of 6 monomorphic bands and 1 polymorphic band (Fig.3). An approximately 1198 bp was consistently present in uninfected plants while absent in infected plants. To construct SCAR marker, the specific band was excised from the gels, purified, and then sequenced. The obtained sequence matched with the amplified band size (i.e. 1198 bp) and showed 95.88 % identity with *Cucumis melo* subsp. melo mitochondrial sequence (Gene Bank Accession No:JF412792). Based on this sequence, a set of SCAR primer, named SCOPA4-1 (5'-TCTCGGGCTGCTAACTGCAG-3') and SCOPA4-2 (5'-TACGAGAACCGTCAGGCCTG-3') were designed containing 60% GC content. Each primer was overlapped with 6-8 first base of OPA-4 primer sequence in both ends (Fig 4). The annealing temperature (Tm) of developed SCAR primer was 55.9 ˚C, calculated by the program OligoCalc-Oligonucleotide Properties Calculator (Kibbe, 2007). However, an optimum temperature for obtained clear bands was 49.7 ˚C.

3.3. Segregation Analysis and Marker Validation

To confirm the linkage of newly developed SCAR marker with *Begomovirus* resistance, 100 F_2 progenies were tested. Phenotypic evaluation revealed that 70 plants showed *Begomovirus* symptoms while 30 do not showed. Then DNA of observed plants was amplified using developed SCAR marker. Each plant was scored for the presence of specific amplicon. An 1198 bp specific band was consistently amplified in 82 uninfected plants (Fig. 5). The segregation pattern of marker locus was expected as Mendelian segregation ratio of 3 (resistant): 1 (susceptible) and confirmed using Chi square test. The results confirmed that segregation pattern was equivalent with expected ratio either on phenotypic evaluation or PCR validation (X^2= 2,25; df=1; P= 0.05). Meanwhile, developed SCAR markers are known to differentiate individuals which potentially resistance to *Begomovirus* in a population. However, further analysis is needed to determine the accuracy of this primer in other cultivars. Nine cultivars from different breeding lines, named 'Tacapa GB', 'Tacapa Silver', 'Tacapa Gold', 'Hikapel', 'Meloni', 'Melona', 'Gracia', and 'Madesta', were used for this analysis. Gracia and Madesta were commercial cultivars that were confirmed as resistant cultivars. There were 9 unifected samples from each cultivar and 4 infected samples which have confirmed by PCR using CP gene primer for *Begomovirus*. SCOPA4-1 and SCOPA4-2 were consistently amplified 1198 bp band only in uninfected samples for all cultivars (Fig 6). This result was in agreement with symptom observation data and PCR identification. So, it can be confirmed that newly developed marker was consistent and reliable.

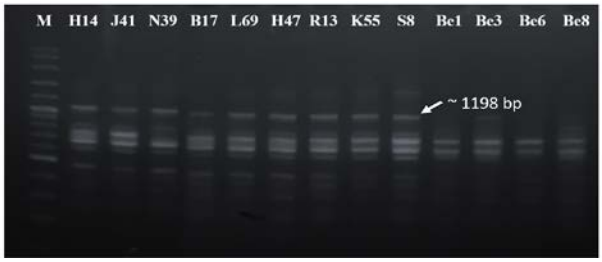

Figure 3. Identification of RAPD DNA fragment related to *Begomovirus* resistance was performed with DNA from 9 unifected samples (from H-14 to S8) and 4 infected samples (from Bel to Be8) Arrow indicates the band that discriminated infected and uninfected melon. (M= 100 bp molecular weight DNA marker (Vivantiz)

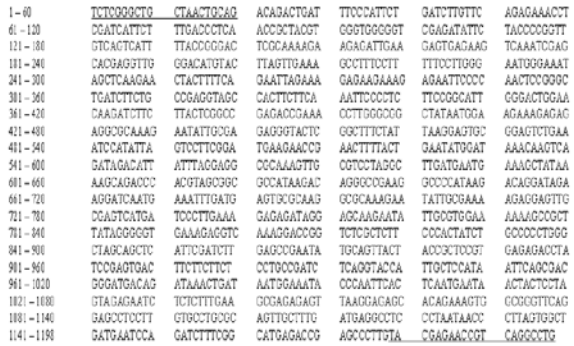

Figure 4. The complete sequence of RAPD fragment related to *Begomovirus* resistance (1198 bp). Underlined sequences indicate sequences of newly developed SCAR primers.

4. Discussion

Begomovirus is one of the most devastating diseases in melon crops. *Begomovirus* infected melon was initially reported in America in 1981 while in Indonesia in the early 2010 (Cohen*et al*., 1983; Julijantono *et al*., 2010). After almost a decade, Begomovirus in Indonesia has developed rapidly. There were three *Begomovirus* species that have been reported to infect melon plants, namely PepYLCIV, ToLCNDV, and TYLCV (Subiastuti et al., 2019). In order to overcome *Begomovirus* outbreaks, development resistant cultivar is a promising solution. However, the selection of *Begomovirus*-resistant genotypes will be very tricky if it only relies on symptom observation. This method is often ambiguous in discriminating tolerant and susceptible genotypes because appearing symptoms usually look similar (Maiti *et al*., 2010, Nevame *et al*., 2018). Sometimes, *Begomovirus-infected* plant yield as good as uninfected plants and showed different level of symptoms. This may be due to virus strain, time of infection, plant of genotype, biotype of vector, and environmental factor (Polston and Anderson, 1997; Delatte *et al*., 2006; Azizi *et al*., 2008).

Another method that might be used is molecular detection using universal CP gene primers to detect viral genomes in plants. This method was accurate, less time-consuming, and only required little amount of leaf tissue (Sharma *et al*., 2005). PCR based detection has been successfully used to detect *Begomovirus* in tomato (Yang *et al*., 2011), cucurbit (Mizutani *et al*., 2011), okra (Venkataravanappa *et al*., 2013), sweetpotato (Wasswa *et al*., 2011), legume (Tsai *et al*., 2013), melon (Subiastuti *et al*., 2019), chili and eggplant (Maruthi *et al*. 2007). But this method has a disadvantage if the viral DNA concentration in plants is low, the obtained DNA bands will be not clear (Swanson *et al*., 1992; Ghosh *et al*., 2009). Moreover, this method also cannot be used to identify whether an individual was resistant or susceptible. Since the concentration of *Begomovirus* genome in plants was found to be very low, rapid detection by amplifying plant genome was more promising for early identification of resistant genotype as well as *Begomovirus* infection.

As *Begomovirus* resistance gene in melon has to be found yet, many studies try to develop molecular markers linked to resistance against *Begomovirus* (Diaz-Pendon *et al*., 2004; Saez *et al*., 2017). This study developed a SCAR marker from RAPD for early identification of *Begomovirus*-resistant individuals in a population as a resistant genotype source in breeding melon resistant cultivar. This method provides an alternative screening method to avoid long-term phenotypic and to characterize genetic resources with potential role in resistant plant development. Several reports explicated that RAPD is often used to distinguish individuals resistant to disease among susceptible individuals. Luongo *et al*., (2012) managed to identify RAPD, with OP-F15 primer, which is able to recognize melon cultivars resistant to *Fusarium oxiporum* infection. The OP-F15 primer is able to bring the base length of polymorphic band of 420 bp and has been developed into a SCAR marker. In addition, primers OPE - 14 and APB-05 are capable of distinguishing between melon cultivars resistant and susceptible to Cucumber mosaic virus (CMV) (Daryono *et al*., 2009). Similar to this RAPD, pUBC411 has been proven to be able to distinguish the melon cultivars resistant to powdery mildew by generating the 1061 bp polymorphic band (Daryono *et al*., 2011). By using MG3 melon DNA, the SCAR marker developed was able to distinguish resistant/tolerant individuals from susceptible ones. Primer OPA-4 was used to develop SCAR marker in this study because it had reported could differentiate between infected and uninfected samples in melon populations infected by *Begomovirus* (Julijantono *et al*., 2012). However, it is relatively unstable and influenced by the conditions of PCR reaction. SCOPA4-1 and SCOPA4-2, newly developed SCAR marker constructed from OPA-4 primer, were able to distinguish uninfected and infected individuals of 9 Indonesian melon cultivars through the appearance of 1198 bp DNA band. SCAR marker was codominant marker, more accurate, and more reliable as marker linked-trait than RAPD. Converting RAPD to SCAR marker by adding nucleotide of primer sequence will increase primer specificity to targeted locus. Even though, SCAR marker construction was not easy because it is still possible to produced non-targeted fragment (Ardiel *et al*., 2002; Bhagyawant, 2016).

Furthermore, similarity analysis using BLAST revealed that amplified sequence matched relatively with DNA mitochondria sequence of melon. Mitochondria was cellular organelles of eukaryotic cells and has important role in provide energy for cells, apoptosis, regulation of cell metabolisms, antiviral responses, signal transduction, stress responses, and cell-cycle control (Anand and Tikoo, 2013). Some study reported that mitochondria has important roles in biotic stress, including in viral infection. Virus infection triggered reactive oxygen species (ROS) accumulation and induces alternative oxidase (AOX) in plants. The induction of AOX would increase plant resistance against virus as reported in *Arabidopsis thaliana* and *Solanum tuberosum* infected by Tobacco Mosaic Virus (TMV) (Amirsagedhi *et al*., 2007; Fu *et al*., 2010). These results informed that developed SCAR marker was related with plant resistance trait yet further study was needed to analyze the distance between developed SCAR marker with resistance gene locus. It usually happened in development of linked-trait marker, some advance studies were needed to improve previous results. However, molecular marker linked to *Begomovirus* resistance was first reported in melons. Andersen and Lubberstedt (2003) showed that functional gene markers are polymorphic DNA sequences that are likely to be involved in phenotypic variation, in this case discriminating *Begomovirus*-infected and uninfected individuals.

Furthermore, segregation analysis showed that newly developed SCAR marker was segregated to F_2 progeny with ratio 3:1 for resistant. This indicated that *Begomovirus* resistance trait was regulated by single dominant gene. Many studies revealed that molecular marker linked to disease resistance trait segregated in ratio 3:1, such as Daryono *et al*.,(2010) reported that SCAR Marker linked to CMV-B2 resistance gene in melon also segregated in expected ratio 3:1. Other studies in different plant also reported that SCAR marker linked to *Xca1Bo* conferring resistance to black rot disease in cauliflower and SCAR marker linked to *Rpf1* gene that control resistance to red stele disease also segregated with ratio 3:1 (Kalia *et al*., 2017; Ruguenius *et al*., 2006). Furthermore, such studies which have contributed to identification of resistance gene explained that resistance gene for plant viruses appear to be predominantly inherited as monogenic dominant characters (Diaz-Pendon *et al*., 2004).

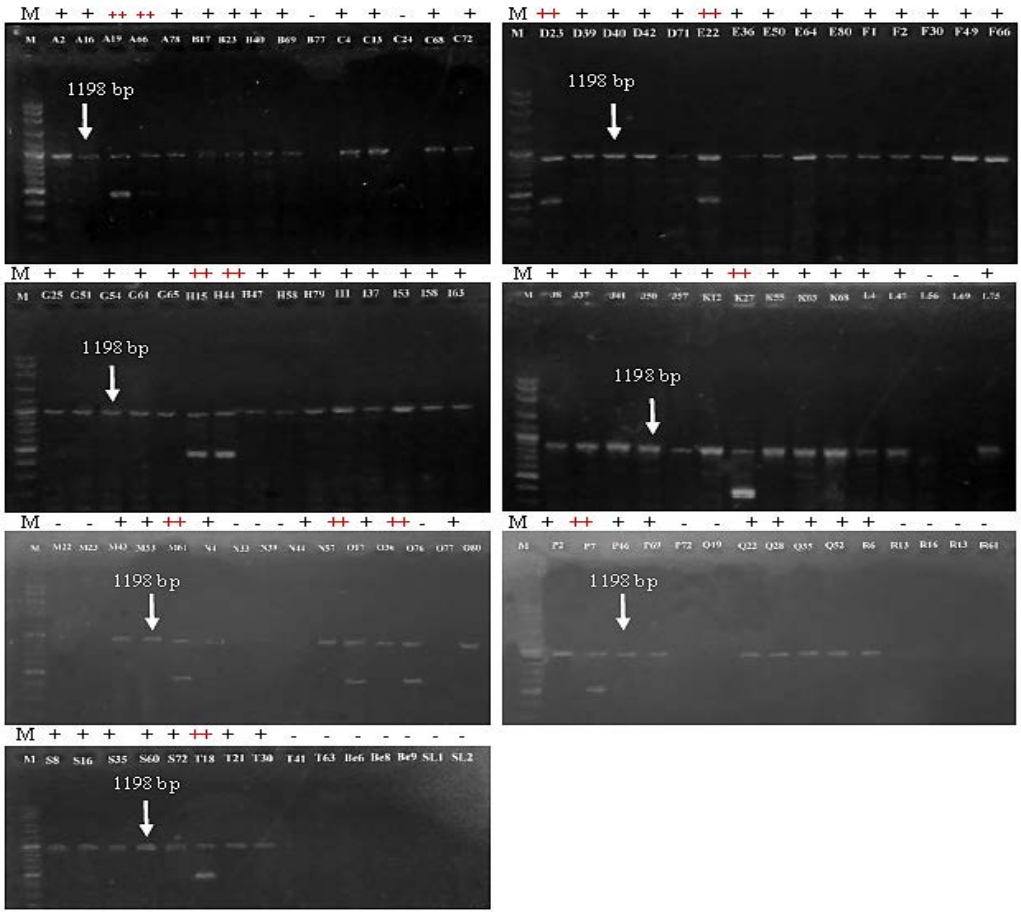

Figure 5. Validation of SCOPA4-1 and SCOPA4-2 primers in F_2 genotypes.

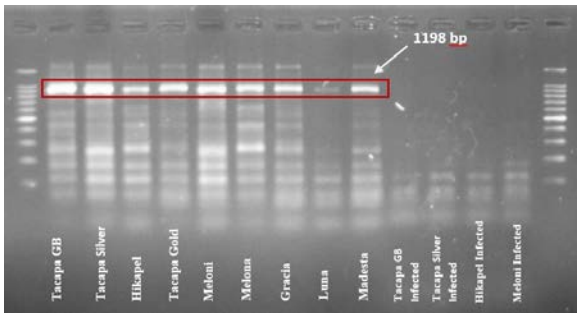

Figure 6. Validation of developed SCAR Marker in 9 melon cultivars. Amplified bands discriminated *Begomovirus* infected samples from uninfected one (M= 100 bp molecular weight DNA marker (Promega).

One of essential properties for molecular marker is havinghigh repeatability and independence from any environmental conditions (Kesawat and Das, 2009). It means the molecular marker can not only be used in certain object of the study but also apply to other objects in similar studies. In the present study, obtained SCAR marker was validated in different genetic backgrounds. An eight different melon cultivars was tested and revealed that this marker was reproducible and able to be used in other melon species. Because of limited information about *Begomovirus*-resistant genotype in melon, this marker only discriminate *Begomovirus* infected and uninfected individuals. The results also indicated that this marker was neutral from any genetic background with any doubt.

Development of SCAR marker linked to *Begomovirus* resistance to identify potential resistant melons genotype was firstly reported. An 1198 bp band was confirmed can consistently differentiate resistant and susceptible melon hybrid against *Begomovirus* infection. There was still limited report about molecular marker linked to *Begomovirus* resistant in melon. Therefore, this marker make a potential contribution for melon resistant breeding programs by reducing the time, labor, and production cost as well as increasing the efficiency of plant screening. Furthermore, this study only uses melons that develop in the tropics, especially Indonesia. Validation of non-tropical cultivars needs to be done to further confirm this

primary reliability. Moreover, experimental investigations are required, particularly to find out the specific gene that has a role to play in plant resistance mechanisms against *Begomovirus*.

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