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Identification of molecular difference of papaya hermaphrodite with males using SNP (single nucleotide polymorphism) and development of SNAP markers

[1]Noflindawati,[2]Aswaldi Anwar,[3]Agus Sutanto,[4]Yusniwati

ABSTRACT--- *This study aims to obtain a SNAP marker that can differentiate male crops with hermaphrodite ones. This research has been conducted in the Quality Testing Laboratory of the Indonesian Tropical Fruit Research Institute, Solok West Sumatera from January to December 2019. The research used local papaya crops and papaya Merah Delima, DNA isolation derived from plants that have been fruitful as a reference plant, perform sequencing DNA genome papaya and data processing sequencing result in alignment and Identify single nucleotide polymorphisms (SNP) to further design the primary single nucleotide cation polymorphisms (SNAP) over the web. SNAPPER and perform primary optimization SNAP.The results of the second primary optimization of SNAP showed different amplification patterns where the sample of the male plant with hermaphrodite, in the sample of the male crop amplification pattern band appears on the primary reference, the female plant does not appear pattern Amplification, while the band's hermaphrodite crop appears on the alternate primer.Both primary SNAP can be used as a marking that distinguishes the female, male and hermaphrodite plants.*

Keywords--- *hermaphrodite; SNP; female; male; papaya*

I. INTRODUCTION

Papaya plants have three types of flower (basic flower type) namely: female Flower (pistillate), Male flower (staminate) and hermaphrodite flower(Storey 1976; Samson 1980; Nakasone 1986; Villegas 1997).Based on these types of flowers, there are three kinds of papaya trees, namely: the female, the male and the hermaphrodite tree (Nakasone 1986).

Papaya multiplication through generative seeds was often used because it was easier and faster. But the weakness of this way is that generative seed will experiencesegregation in the sex expression of plants (Marin et al. 2006; Ming et al 2007),which causes difficulty to determine the seed that will be the male, female and perfect plant (Parasnis et al. 1999; Costa et al. 2011;Suketi et al. 2010: Suketi et al 2011; Vashisthaet al. 2016).

Identifying the sex of papaya plants in the seedling stage is a extremely importance step, as it will allow earlier fruit production, of lower cost and natural resources as well such as water and nutrients. As sex identification and sexing are performed only after flowering, at approximately 90 days post transplanting, three to four seedlings

[1] *Andalas University, Padang West Sumatera, Indonesia*

[2] *Andalas University, Padang West Sumatera, Indonesia*

[3] *Indonesian Tropical Fruit Research Institute, Solok West Sumatera Indonesia*

[4] *Andalas University, Padang West Sumatera, Indonesia*

must be planted per hole to attain a higher number possibility of hermaphrodite plants and consequently higher yields per planted area (Pirovani et al,2018)

A type of sex affects the form in resulting fruit (Liao et al. 2017).Papaya fruit that was generally preferred by consumers is that a fruit oval-shaped (elongata) derived from hermaphrodite, while plants with male flowers are not productive in producing fruit,female flowers produce rounded shaped fruit and in some subtropical countries they areused as a source of rubber (Magdalita and Mercado 2003; Ming et al. 2007; Yu et al. 2007; Suggestion et al. 2015; Liaoet al. 2017).

It is morphological and physiological markers allow to be performed however environmental factors greatly affect the emerging characters.As reported by papaya hermaphrodite, abnormal flower formation was associated with several environmental factors, such as humidity and soil fertilizer (Awada & Ikeda 1957).It is also affected by temperature and plant growth and average daily temperature as well (Awada 1958; Lange 1961), relative humidity (Singh et al. 1963), cultivars (Chan 1994) and season (Hofmeyr 1939;Storey 1941, 1953, 1969; Nakasone & Paull 1999;Ray 2002).

The determination of sex in papaya is very unique and complex. According to Storey (1953) that sex in papaya was controlled by a complex gene that is a single gene and complex gene on a small region in a sex chromosome.Hofmeyr (1939) and Storey (1953) separately revealed that the gender determination in papaya has been controlled by a single gene with three alels, namely: M1, M2, and M and according to Hofmeyr they are marked as M, Mh, and M.According to Liu (2004),papaya sex was determined by a pair of newly evolved sex chromosomes with the XX genotype in female plants, XY genotype in male plants, and XYh genotype in hermaphrodite plants (Liu et al. 2004).

Single nucleotide polymorphisms (SNP) is a polymorphisms which is caused by one base substitution process on nucleotides in the plant genome (Syvanen 2001). SNP has been known to spread throughout all parts of the plant genome, thus potentially it isused as a molecular markings (Gupta et al. 2001).The strength of the SNP technique was highly depend on availability sequence information and cannot be replaced with other information (Jamsari, 2007; 2013).

The development of SNAP markers modified by (Sutanto et al. 2013) in the RGA gene-based banana plant for disease resistance with Fuasrium. SNAP markers are based on variations of one base change (A, T, G, or C) on particularsites of DNA base in the genome of an organism (Ganal et al. 2009).This study aims to identify single nucleotide polymorphic (SNP) related to male and hermaphrodite as well as SNAP markers design, so that it can be used as a SNAP marking to distinguish between papaya and hermaphrodits papaya.

II. MATERIALS AND METHOD

Plant Materials

Two cultivars of papaya isused as a source of genome in this study,They they are local papaya Indonesian cultivars and Merah Delima cv.The leaves of the plant sample takenfrom plants that have been already flowering or fruitful as a plant reference. DNAisisoloatedfrompapaya leaves by using modified CTAB method (Doyle and Doyle 1987).

DNA extraction DNA extracted from papaya leaves byusing modified CTAB method (Doyle and Doyle 1987). 100 mg Papaya leavescrushed with 1.5 ml extraction buffers, 1% β-mercaptoethanol and 10 mg of PVP-10 to form pasta. Then the sample is transferred to a tube 2 ml and incubated at 65 ^{0} C for 60 minutes, then centrifuged at 12,000 rpm for 10 minutes.The DNA is precipated with the addition of 500 ml of cold ISO-propanol, while the RNA is degraded with 2 mg/ml RNAse, then centrifuged at 12,000 rpm for 10 minutes.The pellet DNA is madeby air dry, rinsed with ethanol 70%, and dissolved in 50 ml TE buffer.

Sequencing of male and hermaphrodite PCR products

Products of PCR (male and hermaphrodite plant samples) by using selected primary pair (forword and reverse) are sent for sequencing. Six tube samples consisted of 3 male samples and 3 hermaphrodite plants. For the purpose of sequencingit is used 5 µl of PCR products for one-time reaction with a concentration of 30 ng/µl, while the primer used as much as 5 µl T 12 (forword and reverse) with a concentration of 5pmol/µl. The PCR results are sent to the sequencing service company.

Data analysis and SNP identification

The analysis was conducted to identify the sequence of DNA fragments, thesequencing resulted by comparing the male DNA sequences and hermaphroditeone that had been deposited in the NCBI GenBank database by using an online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).The BLAST olgaritma results are selected with the highest similarity (Iquery) value.

Male specific primary design and hermaphrodite SNAP markers

Primer was designed at thebeginning with access to SNP sequence data from W11 (comparator) and SNP T12 sequence at NCBI Data Bank (http://www.ncbi.nlm.nih.gov).The selected sequences are sequenced with high levels of similarity in a single gene group.Each DNA are sequenced in a gene group is arranged in a text file according to the pasta format.Sequences are aligned using the multiple alignment facility in order to identify the location of a different nucleotide base byusing Geneious Pro 5.6.6 software Trial version (Biomatters, USA).
Nucleotides with a point that distinguishes male and hermaphrodits are submitted to the Web snapper http://ausubellab.mgh.harvard.edu/) to produce SNAP's primary pair. Based on SNAP Primary selected two sets (four primary) consisting of a set (primary pair) for the reference Allel (R) and a set for alternate allele (A).

SNAP's Primary Effectiveness Evaluation

The resulting SNAP Primer was then tested to see a flat resulting in a mold amplification product for DNA genome ofpapaya. The Reference plant samples are local and pomegranate varieties that have been determinated in the expression of male, female and hermaphrodite. The PCR reaction was carried out with a total volume of 12.5 µl using the Bioline PCR Kit consisting of 5.0 µ L 5x buffer PCR (which contained 1.5 mM MG2 +), 0.5 µl MgCl2 25 mM, 0.5 µl dNTPs 10 mM, 1.0 µl, each SNAP primer W11 with a concentration of 10 Mm primary (forward and reverse), 25 ng genome DNA, 0.1 µl of Taq DNA polymerase (5 U µl-1) and 15.4 µl ddH2O.

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The PCR process uses the Eppendorf Mastercyler engine. Denaturation of DNA prints at the beginning of the reaction on the initial denaturation of 94 ° C for 2 minutes, during 35 cycles with 95 for 15 minutes, annneling 58 °c for 15 seconds and extention 72 °c for 5 seconds, followed by a final extension at 72 °c for 10 minutes. The PCR product is viewed by electrophoresis agarose Gel 1.2%, with electrophoresis machine at a voltage of 80 V for 30 minutes and visualized with the coloration of the etidium bromide 1% seen using the Biometra BDA Digital Geldoc.

III. DISCUSSION

Sequencesare aligned by using multiple alignment to identify the location of a different nucleotide base.Two different nucleotide bases are selected according to the bi-alel principle, which is a SNP-point estimation that may occur in two homologous chromosomes of a diploid individual genome. Point of example of a sequence shown in Figure 1.

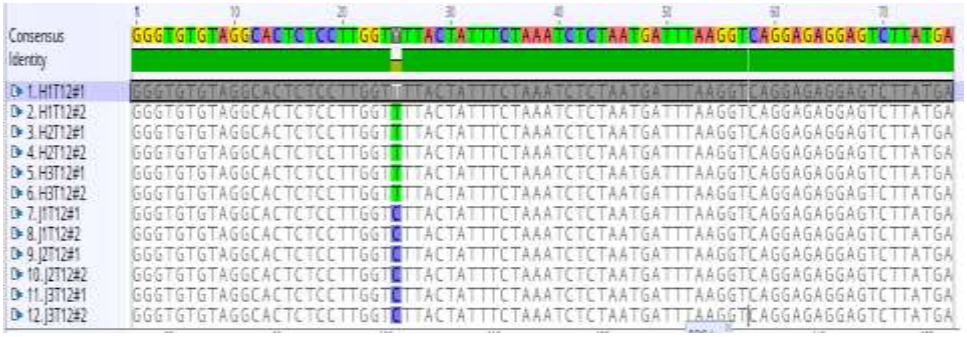

Figure 1: The multiple sequence alignment of the hermaphrodite and male DNA indicates the position of Single Nucleotide Polymorphisms (SNP) in the base size to the point of 25 of the sequence.

Table 1: Result BLAST ofhermaphrodite plant with Primer T12

No	Accesi no	Description	Query Converage	Identical Site	E value
1.	AY428940-1	Carica papaya isolate T12-Herm sex herma prodite chromosom Y male specific sequence	100 %	100 %	0, 0
2.	AY861345-1	Carica papaya isolate T12 (TW)-Herm chromosom Y male specific sequence	100 %	100 %	0,
3.	AY428941-1	Carica papaya isolate T12-Male 1 sex male chromosom Y male specific sequence	100 %	99,88 %	0
4.	AY861346-1	Carica papaya isolate T12 (TW) Male 1 sex male chromosom Y specific sequence	100 %	99,88 %	0, 0
					0, 0

The result of a multiple aligment unidentified single nucleotide polymorphisms (SNP) on the base position to 25, withthe length of the fragment 838 bp.SNP sites are found by using the nucleotide variant (T/C). In the DNA fragment of hermaphrodits encountered Thymine (T) whereas in the DNA of alles alkaline cytosine (C).The difference between a single base between the male and hermaphrodites is very small as reported by the order of HSY (spsific hermaphrodites Y) papaya and X specific region of sex reveals a difference of 4.6 Mb between both genders.The difference in genome size has been detected among the genders in species with sex chromosomes that are heteromorphic using citometric.

BLAST results suggest the similarity of nucleotide sequences of DNA of hermaphrodite plants with the NCBI database, it is demonstrated by the query value converage 100% and e value 0.0.According to Hall (2001) the value of expectation-value (e-value) is a statistical value representing the signification of BLAST search results, so that the E value of close to zero indicates that the trust level lose up to 100 %.

Table 2: Result BLAST hermaphrodite plant with Primer T 12

No	Accesion no	Description	Query Converage	Identical Site	E value
1	AY428941-1	Carica papaya isolate T12-Male 1 sex male	100 %	100 %	0,0
	AY861346-1	chromosom Y male specific sequence	100%	100 99,88 %	0,0
2.	AY428940-1	Carica papaya isolate T12 (TW)-Male 1 chromosom Y	100 %	99,88 %	0,0
3.	AY861345-1	male specific sequence	100 %		0,0
4.		Carica papaya isolate T12-Herm sexherma prodite chromosom Y male specific sequenceCarica papaya isolate T12 (TW)-Herm chromosom Y male specific sequence			

The results of the analysis of WebSNAPER will obtained as SNAP primer candidates should be designed asshown on Table 3.In the primary design of SNAP by using WebSNAPER, from one SNP site can be obtained a number of alternatives of the primary pair to produce SNAP markers. The primer that will be designed need consideration, criteria for designing primer SNAP, around the SNP site there are at least 25 bases.Thus, from eight SNP sites, it is generated 16 pairs of primary selected SNAP.

Table 3: Alternative primers as the output of primer design using SNAPPER for SNP

No	Primer Id	Primer Sequence	Tm	Primer Length	Produt Size	Warnings
	T12SNP-H-Fw	TGTAGGCACTCTCCTTGGCT	56.237	20		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	431	
	T12SNP-H-Fw2	TGTGTAGGCACTCTCCTTGTTT	55.326	22		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	433	
	T12SNP-H-Fw3	TGTGTAGGCACTCTCCTTGATT	55.249	22		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	433	
	T12SNP-J-Fw1	TGTAGGCACTCTCCTTGGCA	58.068	20		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	431	
	T12SNP-J-Fw2	TGTAGGCACTCTCCTTGGAA	54.594	20		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	431	
	T12SNP-J-Fw3	GCACTCTCCTTGTTA	54.956	23		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	434	
	T12SNP-J-Fw	TGTAGGCACTCTCCTTGGGA	56.941	20		
	T12SNP-Rev	AGGATTCCCCTGCAAAATAA	55.023	20	431	

It is to meet the length of oligonucleotide as the appropriate primer for PCR, especially on the site SNP at the end of the 5 ′ and 3 ' DNA fragment is runing (Shahinnia dan Sayed Tabatabaei, 2009)and each SNP site is required two primary pairs, primary first spouse forward and reverse for allel reference while the second pair is primary forward and reverse for alternate allele.

According to Sutanto *et al.* (2013) the selection of primary pairs is done by observing several things, temperature is not much different and the mismatch position is not far from the SNP position or is from one to four nucleotides of the SNP site, then the selected SNAP primer is those that have mismatch positions at the far distance from the end.

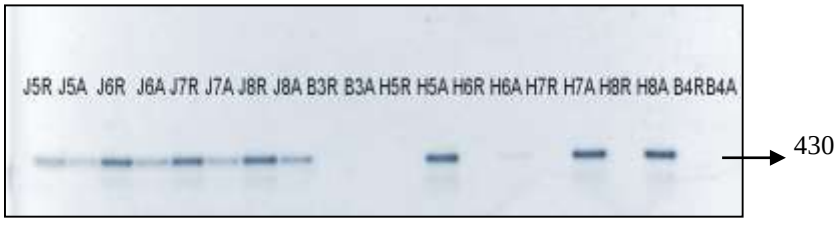

Figure 2: Primary amplification pattern SNAP T12 (J5-J8 sample of the male plant, H5-H8 plant samples hermaprodite, B3 and B4) sample of the female plant anneling temperature 58 ° C

The result of two primary PCR of SNAP amplification is tested to produce products of around 400-435 bp (Fig. 2 and 3) both primary reference and alternate. Primary SNAP T 12-reference produces a single band of DNA (J5R, J6R, J7R, J8R) thicker, whereas on the primary SNP-J-Alt (J5A, J6A, J7A and J8A) is more pricopic, while on females samples do not appear DNA tapes.On the sample plant hermaphrodite DNA band is on the primary SNP T12 Alternate, the difference pattern of emergence of DNA bands can be a marker of differences between males, females and hermaphrodites.

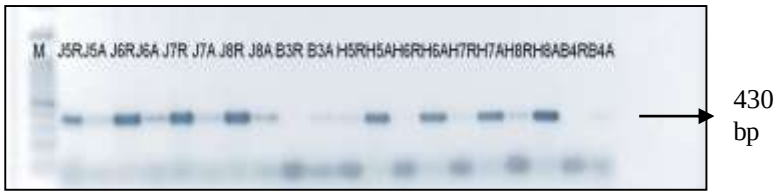

Figure 3: Primary amplification pattern of SNAP W11 (J5-J8 male plant samples, H5-H8 hermaprodite plant samples, B3 and Bfemale plant samples) anneling temperature 57.5° C

The result of primary PCR amplification SNAP W11 shown on Figure 3, The male pattern sample tape on the primer reference is thicker than that of the thinner alternate primer and smir. In the hermaprodite plant sample the band pattern produces alternate primary whereas the female sample did not produce band amplification.

IV. CONCLUSION

SNP succeed in the identification of the male DNA fragment length and hermaphrodite at a size of 25 bp of the fragment length of 830 BP, the difference of a base between the hermaphrodites and males (T/C).Primary design stages obtained by 2 primary pairs that can be tested to produce SNAP markers (1 pair for reference allele and 1 pair for alternate allele).The primary effectiveness test of the resulting SNAP can be used to distinguish male and hermaphrodite plants.

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