Optimization of Annealing Cycle and Temperature SNAP T12 Primer Distinguishing Markers for Male, Female and Hermaphrodite Plants in Papaya (*Carica papaya* L)

Noflindawati¹,²,*, A Anwar², A Sutanto¹, Yusniwati²

¹) Indonesian Tropical Fruit Research Institute, Solok
²) Faculty of Agriculture, Andalas University, Padang

*Corresponding author: noflindawatiacik@gmail.com

Abstract. Papaya has three types of efflorescence which are male flowers, female flowers, hermaphrodite. That was difficult to determine in advance for the type of flower that appear. The molecular technology approach is an effort to quickly predict the types of flowers that will appear. SNAP primers were designed based on SNP on genomic sequences of male papaya and hermaphrodite DNA. This research aimed to found the specific cycle and annealing temperature for PCR. The research was conducted at the in Indonesia Tropical Fruit Research Institute from January to March 2020. The SNAP primer used was T12, The plants used were papaya varieties of Pomegranate and local cultivar from male and hermaphrodite aged 8 months trees. The results showed that an annealing temperature of 59ºC with a cycle of 28 times as effective in producing male DNA amplification on SNAP T2 reverse (R) primers and hermaphrodite DNA amplification plants appeared on alternate SNAP T12 primers (A) with a product size is 430 bp, while female plants did not occur. Amplification according to the SNAP T12 primer.

Key words: papaya, optimization, annealing temperature, cycle, snap primer

1. Introduction

Molecular research related to the sex determination of the papaya plant has been carried out by many researchers in the world, starting from the research of Sondur [1] who create a model by developing a genetic relationship map using Random Amplified Polymorphic DNA (RAPD) markers. The results showed that the Sex 1-M allele for male code (male) elements involved transacting in the induction of male flower organs and reduction and suppression of capel development (female flowers). For the advance studies, RAPD and microsatellite markers associated with sex were reported [2][3][4].

Determination of the sex of papaya plants using 6 DNA markers, namely, W-11, T12, SDP, Napf-76, PKBT4, PKBT5 showed the presence of sex-related alleles, respectively 98%, 96% and 93% of male plants, meanwhile for female plants these markers did not show up the presence of any sex-related alleles[5]. Furthermore, [6] reported the results of validation on seven SCAR primers related to papaya sex, where NaPf and T1 primers failed to produce related PCR products, PKBT 4, PKBT 5, W11, SCARPm, SDP and T12 were able to detect male or bisexual plants (hermaphrodites) with a reliability range of 82.4% to 92.9%.

SCAR primers for sex detection of papaya plants were not able to distinguish between male and hermaphrodite plants. Those primers can only distinguish between females and males and hermaphrodites, so it is necessary to trace the differences in DNA sequences of male and hermaphrodite
genomes by tracking Single nucleotide polymorphism (SNP) on genomic DNA. Single nucleotide polymorphism (SNP) is a polymorphism caused by a one-base substitution process for nucleotides in the plant genome [7]. The presence of SNPs is known to spread throughout all parts of the plant genome, so that it has the potential to be used as molecular markers [8]. The strength of the SNP technique is very dependent on the availability of sequence information and cannot be replaced with other information [9].

The research of [10] showed the results of the multiple alignment identified a single nucleotide polymorphism (SNP) site at the base position of 25, from a fragment length of 838 bp. SNP sites founded with nucleotide variants (T/C). Thymine (T) was found in hermaphrodite plant DNA fragments, while male DNA was cytosine (C). Primary design was done by accessing SNP sequence data from W11 (comparison) and SNP T12 sequences on NCBI data bank (http://www.ncbi.nlm.nih.gov) to design SNAP T12 primers. Each SNP site required two primary pairs, the first pair of forward and reverse primers for the reference allele while the second pair of forward and reverse primers for alternative alleles [11][12]. SNAP markers also have been shown to produce better data quality than a large number of samples in genetic and evolutionary research [13].

In PCR process, there are three temperatures or incubation stages that was repeated 20-50 times. One repetition of these three stages is called a cycle. The first stage is called denaturation, where the two strands of the target DNA molecule are separated (denatured) by heating the DNA at 94°C-96°C to break the hydrogen bonds between the bases, resulting in two separate DNA strands. The second stage is annealing, where the two primers hybridize into a complementary sequence on the single strand of DNA [14]. The primers are synthetic single-stranded DNA sequences and short in size (20-30 bases long). The primers that was selected is similar for the primers and complementary to one end of the desired gene on one of the strands [15]. Meanwhile, the second primer is complementary to the other end on the other DNA strand. Primers will form hydrogen bonds (stick) with the complementary sequence so that a stable double-stranded molecule is formed. The attachment temperature ranges from 45-60°C. The third stage is extension or elongation, namely the elongation of the primer into a new DNA thread by the DNA polymerase enzyme.

The primers used in the analysis process need to find the optimum temperature in order to obtain the optimum PCR conditions so that a specific PCR product is produced. Temperature at the annealing stage is an important [16]. Primers was attached to DNA strands to amplify target genes [17]. The annealing temperature is the temperature where the primer will stick to the DNA template, the amount of temperature can be calculated based on the melting temperature (Tm) value of each primer. Finding the optimal conditions for the annealing temperature is very important, because that was relates to the specificity and sensitivity of PCR products [18].

The design of primer is one of the parameters that determine the success of a PCR process. The primer functions as a barrier for the target DNA fragment to be amplified as well as providing a hydroxyl group (-OH) at the 3' end which is needed for the process of DNA existence [19]. Usually the optimal annealing temperature is 5°C lower than the melting temperature (Tm) of the duplex DNA primer-template. Incubation for 0.5-2 minutes is usually sufficient. However, if an unspecified PCR product was obtained, the annealing temperature can be increased at this stage by 1-2°C. The number of PCR cycles depends on the number of DNA templates in the reaction mixture and the number of PCR products desired. For less than 10 copies of the DNA template, 40 cycles can be performed. If the initial number of DNA templates is high enough, then 25-35 cycles are sufficient [20].

Band quality is determined by proper annealing temperature. Lower annealing temperature results the primary anneals on incorrect site whereas the higher annealing temperature can cause failure in primary annealing [21]. This study aimed to obtained the correct number of PCR cycles and annealing temperature until SNAP T12 primers can produces specific amplifiers male and hermaphrodite are match to target.
2. **Research Methodology**

2.1. Plant materials and DNA extraction

Two papaya genotypes from Indonesia Tropical Fruit Research Institute namely MerahDelima and young leaf were used in this study. Young leaf were collected from the plants grown at experimental field. The laboratory experiment were completed at Indonesia Tropical Fruit Research Institute Solok, West Sumatra. Genomic DNA was isolated from young leaf of male, female and hermaphrodite papaya plants, according to the procedure described by [22] with some modification. DNA concentration was determined in a *EppendorfMastercyler*, spectrophotometer and quality was verified on a 1.2% agarose gel.

2.2. DNA Extraction and Isolation

DNA was extracted from papaya leaf following CTAB method [22] with some modifications. As much as 100 mg of papaya leaf were crushed with 1.5 ml of extraction buffer, 1% β-mercaptoethanol and 10 mg PVP-10 to form a paste state. The samples were transferred to 2 ml centrifuge tubes and incubated at 65°C for 60 minutes. The protein was degraded three times with 500 ml of chloroform: isoamyl-alcohol (24: 1), and then centrifuged at 12,000 rpm for 10 minutes. The DNA was precipitated with the addition of 500 ml of cold iso-propanol, and the RNA was degraded with 2 mg/ml of RNAse, then centrifuged at 12,000 rpm for 10 minutes. The DNA pellets form air dry and rinsed with 70% ethanol, and dissolved in 50 ml of TE buffer.

2.3. PCR

After obtaining the DNA samples, PCR was performed with a composition of 1 µg the genome DNA, 1 µ for each primer (primary pair according to Table 1). The first pair of forward and reverse primers for the reference allele while the second pair of primers forward and reverse for alternative alleles (6,5 µl Ex Taq DNA (KAPPA) and 4.25 µl ddH2O). PCR was performed with several combinations of cycles and annealing temperature: 35 cycles with an annealing temperature of 59ºC; 35 cycles with an annealing temperature of 57.5º C; 32 cycles with an annealing temperature of 59ºC; 32 cycles with an annealing temperature of 59ºC; 30 cycles with an annealing temperature of 59ºC; 28 cycles with an annealing temperature of 59ºC at an initial denaturation of 94ºC for 2 minutes, denaturation of 95 for 15 minutes, and extention of 72 ° C for 1 minute, and followed by final extension at 72°C for 5 minutes. PCR product was seen by electrophoresis of 1.2% agarose gel and visualized by ethidium bromide staining.

| Primer Id     | Primer Sequence   | Tm (°C) | Primer Length | Product Size | GC count (%) |
|---------------|-------------------|---------|---------------|--------------|--------------|
| SNAP T12-H-Fw | TGTAGGCACTCTCCTGGCT | 60,5    | 20            | 55           |
| SNAP T12-Rev  | AGGATTCCCTGCAAAAATA | 54,3    | 20            | 431          | 40           |
| SNAPT12-J-Fw  | TGTAGGCACTCTCCTGGGA | 60,5    | 20            | 55           |

3. **Results and Discussion**

The amplification pattern of male, female and hermaphrodite genomic DNA with SNAP T12 primer at 35 cycles with an annealing temperature of 59°C is showed in Figure 1, the J11R sample produces DNA band pattern on the reference allele while in J11A there are smer band on the alternative allele, then in sample J13 formed bands on both alleles, both reference and alternate alleles. The female genomic DNA samples did not produce an amplicon pattern (B1R and B1A), while in the H1A and H2A hermaphrodite genomic DNA samples with clear amplification was formed, where DNA bands were formed on the
alternate alleles, while the reference alleles (H1R and H2R) did not form DNA bands. The SNAP T12 primer was successfully amplified the papaya genomic DNA sample, but in the J13 sample the PCR results were not in target because of the appearance of bands in both alleles.

The SNAP T12 primer was designed to distinguish between male and hermaphrodite genomic DNA, where the SNP difference between male and hermaphrodite lies in the T/C base, so that the SNAP T12 primer will specifically produce amplicons in the allele reference for male genomic DNA, and hermaphrodite genomic DNA in alternate while female genomic DNA does not produce PCR products. The design of primer is one of the parameters that determine the success of a PCR process. The primer functions is as a barrier for the target DNA fragment to be amplified as well as providing a hydroxy group (\(-\text{OH}\)) at the 3'end which is needed for the process of DNA existence [15]. In the hermaphrodite DNA genome, it was expected that SNAPT12 primer will produce PCR products on alternate alleles. Each SNP site required two primary pairs, the first pair of primary forward and reverse for the reference allele while the second pair of primary forward and reverse for the alternative allele.

The results of optimization were decreases in the annealing temperature of 57.5 °C while remaining 35 cycles are shown in Figure 1b, the amplification results of the male plant genomic DNA samples (J1 and J13) on a pair of SNAP T12 primers on the reference and alternate alleles produce products in the range of 430 bp, whereas The target female genomic DNA is not amplified. PCR results with 35 cycles and an annealing temperature of 57.5 °C. The hermaphrodite genomic DNA according to the primary target will produce PCR products on alternate alleles.

Furthermore, the optimization with the cycle was shortened to 32 times and, the annealing temperature was 59°C and 57.5°C. Figures 2. The PCR results in Fig. 2a show that male DNA samples still showed shadows of DNA bands on alternate alleles, while for female DNA samples there are not in PCR products. H1 and H2 samples matched the target DNA band appearance on alternate alleles at 430 bp. The cycle settings at the three stages of PCR are repeated so that the area bounded by the two primers are amplified exponentially (called amplicons, which are double strands). Amplification like this will cause the number of copies which can be formulated with \((2^n)\) x, where n is the number of cycles and x is the initial number of DNA molecules. So, if there is a copy of DNA before the cycle takes place, after one cycle there will be 2 copies, after 2 cycles there will be 4 copies, and so on [23]. Figure 2b shows the results optimization of SNAP T12 primer with 32 cycles and an annealing temperature of 57.5° C. The results show that the H1R and H2R plant DNA samples appear according to the primary target, namely the alternate alleles, but the PCR results on samples J11 and J12 are not amplified in specific alleles, so that the annealing cycle and temperature not amplified according to the target sequence. Tm is used as the basis for determining the annealing temperature. Too high annealing temperature high will cause the primer attached to the printed DNA to be released so that PCR products
will not be formed, too low temperatures will cause non-specific primer attachment to the printed DNA [24].

**Figure 2.** Optimization of SNAP T12 PCR 32 cycles and 59º C and 57.5 º C annealing temperature.

SNP primary design was based on bi-alleles, SNP sites consist of 2 base polymorphisms at one point. Then at the 3’ end of the forward primary, the first base was determined as the reference allele (the first allele is in Primary Ref), and the second base is determined as the allele alternative (the second allele is in Primary Alt). Reverse primer plays a dual role in the amplification of target fragments, both to pair with Primary Ref and Alt Primer.

**Figure 3.** Optimization of SNAP T12 PCR 30 and 28 with cycles and 59º C annealing temperature

The results of the annealing temperature optimization of 59ºC and cycles of 30 times and 28 times are shown in Figures 3. In Figure 3a., DNA samples J11 and J12 still showed band shadows on alternate alleles, but for samples H1 and H2 was already on target for the appearance of PCR products in alternative alleles. To make sure that the difference between male and hermaphrodite DNA, another optimization was carried out by reducing the cycle to 28 times with a constant annealing temperature of 59ºC. DNA bands in reference alleles and hermaphrodite plant samples H1 and H2 DNA bands appear on alternate alleles with a product size of 430 bp whereas female DNA B1 R and B1A do not produce amplicons. The number of cycles is adjusted, it is essential to select the temperature and duration of each step in the cycles [25].
4. Conclusion
The results of cycle optimization and annealing temperature for the pair SNAP T12-H Fw primer was match to the primers target were obtained at the number of cycles of 28 times and the annealing temperature of 59°C with the PCR product size of 430 bp according to the target when designing the SNAP T12 primer. Henceforth, the number of cycles and the annealing temperature were validated with a larger number osf samples.

5. References
[1] Sondur, S. N, Manshardt, R. M., Stiles, J. I., 1996: A Genetic Linkage Map of Papaya Based on Randomly Amplified Polymorphic Markers. *Theoretical and Applied Genetics*. 93:547–553.
[2] Parasnis AS et al (1999) Microsatellite (GATA)n reveals sex specific differences in papaya. *TheorAppl Genet* 99:1047–1052.
[3] Lemos Eliana Gertrudes Macedo, Silva Cristina Lacerda Soares Petrarolha, Zaidan Humberto Actis (2002) Identification of sex in *Carica papaya L.* using RPAD markers. *Euphytica* (127):179-184.
[4] Urasaki, N., Tarora, K., Uehara, T., Chinen, I., Terauchi, R. Tokumoto, M.2002: Rapid and highly reliable sex diagnostic PCR assay for *papaya Caricapapaya L.*) *Breeding Science* 52, 333—335.
[5] Ejaz Mahwish, Iqbal Muhammad, NaeemullahMuhammad, Ahmed Itikhar Shahzad, Armghan, Masood M Shahid & Ali Ghulam M 2015. Validation and use of DNA markers for sex determination in papaya (*Carica papaya*), *Pak Journal Botany*, no. 47, no. 3, pp. 1051–1059.
[6] Leela Manoharan & Chinnasamy, K 2018, Validation of sex expression in papaya using molecular markers, *Research Journal of Agricultural Sciences*, vol. 9, no. 6, pp. 1219–1222.
[7] Gupta, PK, Roy, JK & Prasad, M 2001, ‘Single nucleotide polymorphisms: a new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants’, *Current Sci.*, vol. 80, pp. 524-35.
[8] Jamsari. 2013. RekayasaGenetikauntukAnalisisGenomdanProduksiOrganismeTransgenik. UR Press : Riau
[9] Pesik Anneke, Efendi Darda, Novarianto Hengky, Dinarti Diny, Sudarsono. 2017. Development of SNAP markers based on nucleotide variability of WRKY genes in coconut and their validation using multiplex PCR. *Biodivesita* ; 18, Number 2 (18), Pages: 465-47
[17] Ludyasari, Ayu. 2014. The Annealing Temperature Effect on the Success of the Programme Against PCR Amplification of DNA Finger Shrimp (Metapenaeus elegans De Man, 1907)
[18] Asy’ari Mukhammad dan Noer A. Saifuddin. Optimasi Konversi MgCl2 Dan Suhu Annealing Pada Proses Amplifikasi Multi-fragments Mtdnadengan Metoda PCR. 2005. J. Kim. Sains&Apl. Vol. VIII. No.1
[19] Handoyo, D dan A. Rudiretna. 2001. Prinsip Umum dan Pelaksanaan Polimerase Chain Reaction (PCR). Unitas. Vol. 9. No. 1. Halaman: 17-29.
[20] Borah, P. 2011. Primer Designing for PCR. Science Vision. Vol. 11 (3): P. 134 -136.
[21] Renganathan P, Ruiz-Alvarado C, Hernández-Montiel L G, Durasamy P and Rueda-Puente EO 2017. Evaluation of genetic diversity in germplasm of paprika (Capsicum spp.) using random amplified polymorphic DNA (RAPD) markers J. Plant Sci. Phytopathol 080-086
[22] Stefanova Petya, Taseva Marieta, Georgieva Tzveta, Gotcheva Velitchka & Angelov Angel 2013, ‘A modified CTAB method for DNA extraction from soybean and meat products’, Biotechnology and Biotechnological Equipment, vol. 27, no. 3, pp. 3803–3810, doi: 10.5504/BBEQ.2013.0026.
[23] Hasibuan, E. 2015. Peranan Teknik Polymerase Chain Reaction (PCR) Terhadap Perkembangan Ilmu Pengetahuan. Fakultas Kedokteran. Universitas Sumatera Utara, Medan
[24] Roberdi, Ramadhan Ogi Ajitiyo. 2019. Optimasi Primer Single Nucleotide Amplified Polymorphism (SNAP) pada Gen brassinosteroid (bri) Kelapa Sawit. Jurnal Sains Natural Universitas Nusa Bangsa. Vol. 9, No.2, 80 – 89.
[25] Mubarak Shaden M. H.; Al-Koofee Dhafer A. F.; Radhi Ohood A; Ismael Jawad Mohammad; Al-Zubaidi Zubaida Falih. 2020. An Optimization and Common Troubleshooting Solving in Polymerase Chain Reaction Technique. Sys Rev Pharm; 11(2): 427 436