Developing Detection Methods for Tomato Diseases
Innovation to Support Food Availability

Muslihah Nur Hidayati[1,2(✉)], Suranto Suranto[1], and Ari Susilowati[1]

[1] Bioscience Program, Sebelas Maret University (UNS), Surakarta, Central Java, Indonesia
muslihahnh@gmail.com
[2] Applied Research Institute of Agricultural Quarantine, Ministry of Agriculture Republic Indonesia, Bekasi, West Java, Indonesia

Abstract. Plant mortality and reductions in yields caused by plant pathogens can affect food availability and food security. One of the most important bacterial diseases of tomatoes can be caused by *Clavibacter michiganensis* subsp. *Michiganensis (Cmm).* This pathogenic bacterium induced cancer and bacterial wilt that caused heavy losses of crops. Based on genomic analysis, the largest *Cmm* region contains a gene related to pathogenicity known as t*omA*. In tomato plants and other members of the Solanaceae, the *tomA* gene acts as a regulator of enzymes associated with the mechanism of disease infection. This study aims to optimize the detection and identification of *Cmm* by the polymerase chain reaction (PCR) method. Specific primers based on the *tomA* gene have been designed. Design and potential analysis of primer pairs were done using the Primer3Plus program. The program resulted in ten primer candidates. Analysis of primer parameters selected the best candidates for specific primers. Two specific primers for PCR were successfully synthesized and used in the *tomA* gene amplification process. Both primers generate 232 bp of PCR product in electrophoresis visualization. As an innovation in the accurate detection of tomato diseases involving *Cmm*, this pair of primers was highly recommended to be used.

Keywords: Food availability · Food security · Primer · TomA gene · Tomato

1 Introduction

Global food security issues involving a balance between global food availability and consumption recently attracted public attention. Food security includes four basic things: 1) availability, 2) access (from an economic and socio-cultural perspective), 3) utilization, including food security and the process of preparation, and 4) stability of the three factors. Many things can cause a decline in agricultural productivity, but the causal factors originating from pests and pathogens are a significant concern worldwide. Pests and plant diseases significantly affect the availability and security of agricultural products, reduce the volume and quality of the produce, and subsequently cause losses [1].

The discovery of new variants of pests and pathogens and their wider distribution will significantly disrupt food production and the global economy. Reduced production

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of staple food products consumed by humans due to pests and pathogens can cause losses estimated at billions of dollars [2]. These losses focus on plant pests and diseases originating from disease, insects, and weeds groups, excluding other abiotic factors such as poor soil quality, drought, or excess water. When this staple food disease problem occurs in areas with low incomes or minimal resources, it will undoubtedly threaten food availability, further creating new issues in terms of malnutrition and hunger. Commercially, the decline in revenue from the agricultural sector can also have a long-term effect on exacerbating poverty, especially in populations who depend on agriculture for their economy [3].

Tomato is one type of vegetable and fruit crop produced in large quantities. The success rate of tomato production worldwide in 2019 was relatively high, marked by achieving a production volume that reached 180 million metric tons. In Indonesia, tomatoes are one type of plant that is in great demand by farmers to be cultivated. The market potential of tomatoes is promising at the local, national and export levels. The need for consumption of tomatoes is increasing today. But on the other hand, this creates new problems because there is insufficient stock, both in quantity and quality [4].

The finding of the presence of *Clavibacter michiganensis* subsp. *Michiganensis* (*Cmm*) will encourage the formation of bacterial cancer structures in tomatoes. This should be a warning to the industrial sector, especially those focusing on fresh product processing and marketing. This disease has the status of an epidemic that has a broad impact on tomato plantations spread throughout the world. Symptoms of the disease that will appear on plants infected with *Cmm* are not the same and will be affected by the virulence of *Cmm* itself, the age of the plant being attacked and the resistance level of the host cultivar. Temperature and humidity, which are environmental factors, will also influence the severity of infection. Infection may occur in the early stages of life (seeds or young seedlings). In that case, systemic infection (also known as primary infection) will affect the quantity and quality of the resulting fruit of the plant. Infection at more severe levels can cause plant death. On the other hand, if the infected plant is older, the infection that develops is called a secondary infection and causes chlorosis or leaf drop. This secondary infection also affects production yields, although in small amounts [3].

The damage caused by diseases to plants is currently getting attention to be minimized. The damage includes the growth phase, harvest period and post-harvest processing system. Meanwhile, advanced disease detection must support productivity and ensure food availability. Inmust addition to effective pest and disease management, the methods used to detect pathogens must also be appropriate. One direct disease identification method currently used in agriculture is polymerase chain reaction (PCR). At the beginning of its use, PCR was used for the specific detection of diseases caused by bacteria and viruses. But now, its use has expanded to detect a wide variety of other plant pathogens [5].

Primer efficiency plays an essential role in the efficacy and sensitivity of PCR. The primer design that will amplify DNA using the polymerase chain reaction must be efficient [10]. Primer is a vital component that determines the success of the PCR process. A good primer must recognize a specific sequence in the genome or template used as a target. Primers with inappropriate criteria will incorrectly amplify other regions of the genome that are not targeted. On the other hand, mismatched primers may also be unable

to amplify the targeted genomic region [6]. One of the specific genes closely related to the virulence mechanism found in *Cmm* is the *tomA* gene. This gene is responsible for the synthesis of tomatinase compounds used in glycoalkaloid tomatin degradation and influences self-defense mechanisms in the plant body [7]. This study aimed to analyze the primer pairs used in the PCR process to amplify the *tomA* gene that is part of *Cmm*.

2 Materials and Methods

2.1 Designing Primers

The process steps in designing primers were initiated by tracing the six *tomA* gene sequences from *Cmm* contained in the gene bank database on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The traced sequences are then aligned in the Bioedit program, and the conserved regions resulting from the running program are used as specific primer templates. Design of specific primer was performed with Primer3Plus software. The sequence used as a template in FASTA format is entered into the column provided in the application and then clicked on the pick primer button. The program will display results in the form of ten specific primer candidates. The primer is composed of 20 nucleotide bases with different characteristics.

Several analytical parameters, such as the number of nucleotides, amplicon length, melting temperature (Tm), and GC content, were used to analyze all candidate primers. The best specific primer candidates must meet several requirements, including the length of each primer ranging from 17 bases to 25 bases, the GC percentage between 40 to 60%, and a melting temperature difference that does not exceed 4 °C. The possibility of finding secondary structures in dimers, hairpins and self annealing was analyzed with a DNA calculator olygoanalyzer. To further ensure that the selected primers match the target species, the Genbank BLAST program is presented.

2.2 Validation of Primers Specificity

The best primer pairs that met all the analysis criteria were then synthesized with the help of Integrated DNA Technologies (IDT). The amplification process for the *tomA* gene was then carried out using the specific primer. At a total volume of 25 μl containing 1 μl DNA genome of *Cmm* (DNA undiluted, 5x, 10x, 20x and 50x dilution), 1 μl for each primer forward and reverse, 12.5 μl PCR master mix (Qiagen), and 9.5 μl ddH2O, the complete PCR reaction was carried out. A total of 35 cycles performed in the gene amplification process using PCR method. Each cycle has three stages known denaturation, attachment, and elongation. The first stage, pre-denaturation, was carried out for 5 min at 94 °C, the denaturation stage for 1 min, and the primer attachment stage at 55 °C for 1 min. The elongation step takes 30 s and is carried out at a temperature of 72 °C. In the last cycle, the chain was extended longer at 72 °C for 5 min. After completion, electrophoresis was carried out to transfer the PCR product obtained into 1% agarose gel stained with ethidium bromide (EtBr). This stage requires a voltage of 70 V and takes 60 min. The final results were analyzed through the visualization of the gel documentation.

3 Results and Discussion

3.1 Primer Design

The *tomA* gene sequence of *Cmm* was traced from the gene bank with accession numbers KJ724012.1, KJ724011.1, KJ724010.1, KJ724009.1, KJ724008.1, and KJ724007.1 consisted of 509 bp. Based on the alignment results using the Bioedit program, it is known that the conserved region is in sequence numbers 54 to 509 with a segment length of 456. This sequence measuring 456 bp is then used for specific primer templates.

The Primer3Plus program was then used to design primers. The specific primer template part of the *tomA* gene sequence included in the conserved region is inputted into the program. After running, several primer candidates will be accepted. Five combinations of forward (F) and reverse (R) primer candidate pairs obtained from this program are shown in Fig. 1. The primer design results using this program also performed primer parameter analysis, including a number of nucleotides, GC content, melting temperature, and product length, as shown in Table 1. Based on olygoanalyzer DNA calculator, four pairs of primer candidates have potential hairpin formation. Only the fifth candidates have no potential for hairpin, 3'complementarity and self annealing. This candidates with the sequence CACAGTGGTGAAGTGCTCTG (F) and ACAGTTCAATGGCCTTTCTC (R) selected as the best specific primers, namely Primer 5.

Secondary analysis, including hairpin formation, is critical when designing a primer. The structure of the hairpin is closely related to the success of the amplification process. Both secondary structures (hairpin loops and primer dimers) will occur when complementary sequences are found in length. Hairpin loops with less than three complementary nucleotides had no effect, whereas primer length was directly proportional to hairpin size. Hairpin loops can decrease the efficiency of the reaction because they can limit its ability to bind to the target site. The characteristic of the hairpin structure that has the

Table 1. Primer parameter analysis from Primer3plus program

Primer		Parameter Analysis				
		Nucleotide Length	*Product Length*	*GC (%)*	*Tm (°C)*	*Hairpin*
1	F R	20	235 bp	50 45	57.2 56.8	Yes[a]
2	F R	20	236 bp	50 40	57.2 56.4	Yes[a]
3	F R	20	181 bp	50 55	57.2 57.8	Yes[a]
4	F R	20	234 bp	50 45	56.1 56.8	Yes[a]
5	F R	20	232 bp	55 45	58.0 56.8	None[b]

F: forward; R: reverse

[a] not recommended primers candidates; [b] recommended primers candidates.

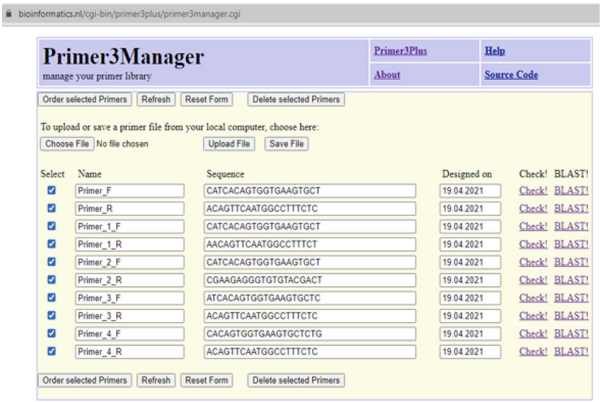

Fig. 1. Result of primer design from Primer3Plus program.

most significant influence on smooth amplification is the size of the loop itself. There should be no complementarity between the primers at their 3' ends because if this were to happen, a counterfeit product would be created by reinforcing themselves. Of course, this condition can reduce the overall amplification efficiency [8].

The specific primer, Primer 5 (Table 1), was selected for synthesis after seeing the results of the analysis of the excellent primer characteristics for amplification using PCR. The characteristics that must be met include the primer length must be between 17–25 nucleotides, the primary GC composition range (%) is between 40 to 60% and the proper melting temperature. All of these criteria will be displayed on the output of the Primer3Plus program. The nucleotide primer that is too long will cause a decrease in the efficiency of the annealing temperature. On the other hand, nucleotide primers that are too short will cause miss-priming (priming primers elsewhere that are not targeted) [9]. Based on the results of this study, the nucleotide length of the forward and reverse primer pairs selected for synthesis was 20 bp. Primer 5 has a GC content of 55% and 45%, respectively. The percentage of GC elements (%) is also a focal point in designing primers. The right GC content will result in a more specific and stable bond [9].

The presence of base GC in Primer 5 designed in this study is not more than 5 GC for forward and reverse primers. This condition will support the stability of the bond between the specific primer designed and the DNA template when tested using the PCR method. In PCR testing, improper primer selection will fail in the amplification process. Areas not used as targets are amplified and are known as miss-priming. Therefore, a specific primer pair must match the desired target sequence [10]. Primer 5 has been successfully designed and also does not have a GC base repeat sequence more than four times (e.g., AGCTGGGGGATCGGG). This composition is needed to prevent miss-priming earlier. Primer 5 respectively has melting temperatures (Tm) at 58 °C and 56.8 °C. Tm owned by a primary pair should be the same, or if there is a difference, the value is not too significant.

Melting temperature (Tm) is essential to determine or optimize the annealing temperature (Ta). The high Ta will affect the hybridization process between the primer and the target DNA, resulting in a low PCR product. On the other hand, low Ta will lead

to nonspecific DNA amplification due to errors in primer attachment to the target DNA [9]. A suitable Tm primer is required for gives dominance to the interaction between the primer and the target DNA. That way, the PCR amplification process will run efficiently [11]. Based on the analysis in Table 1, it can be seen that Primer 5 has met the criteria for a good primer for PCR.

3.2 Primer Specifity Validation

The primer specificity test was carried out using the PCR method on the *Cmm* DNA genome isolated from a pure bacterial culture with sample variation in DNA undiluted, 5x, 10x, 20x, and 50x dilution. Sample testing using the PCR method used three stages. Starting with an initial denaturation at a temperature of 94 °C for 5 min. Followed by 35 cycles with changes in the denaturation step. Denaturation was carried out at 94 °C for 1 min. The annealing process followed at a temperature of 55 °C for 1 min, and the extension step was carried out at 72 °C for 30 s. The cycle ended with a final extension at 72 °C for 5 min. Electrophoresis testing was conducted to obtain DNA visualization results from PCR products amplified using Primer 5. Visualization in agarose gel showed the formation of DNA bands with a size of approximately 232 bp, as shown in Fig. 2.

Primer is an essential component of any PCR test performed. Primers play a significant role in determining the specificity and sensitivity of a PCR-based detection method [12]. The PCR testing process is susceptible to mismatches between primers and templates. This mismatch can cause amplification in areas that are not DNA targets so that the testing process becomes inefficient [13]. Primer design is the first phase to determine the success of DNA amplification using the PCR method [14]. A pair of primers are said to be specific if it only succeeds in amplifying one fragment of size from the entire target DNA area. The selection of primers must be sure to recognize the unique

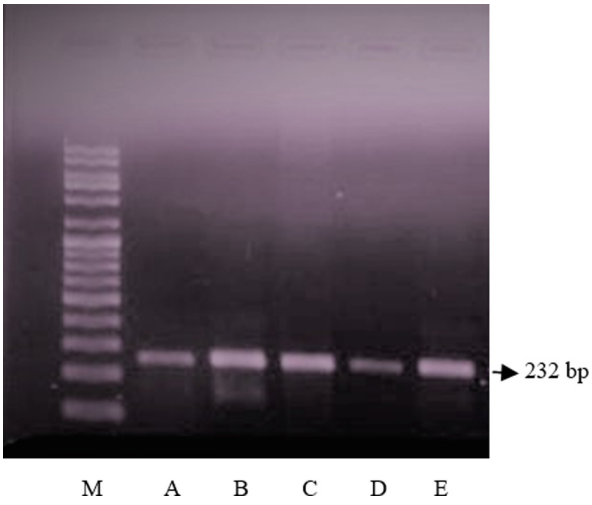

Fig. 2. Detection results of PCR products. M is a marker benchtop ladder 100 bp; A is DNA undiluted; B is 5x dilution; C is 10x dilution; D is 20x dilution; E is 50x dilution.

sequence in the DNA template to be amplified. Primers designed with sequences with many base repeats will produce interfering stains when genomic DNA is amplified. On the other hand, primers with the correct sequence variation will provide a single band that is strong and clean, making it easier to identify the detection results [9].

The results of this study indicate that the Primer 5 primer pair successfully amplified the specific target site contained in the *tomA* gene from *Cmm*. The success of amplification is characterized by the formation of DNA bands measuring 232 bp. However, there are differences in the thickness of the DNA bands that appear in each sample. The thin bands can be caused by the low quality of the DNA so that the template has little homogeneity with the extracted DNA [6, 15].

4 Conclusion

From this research we can conclude that specificity of Primer 5 set can be applied for further specific detection of tomato diseases caused by *Cmm*. On the other hand, the need for an accurate and timely diagnosis of plant diseases is the main determining factor in maintaining the productivity of all agricultural products to support food availability.

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References

1. S. Savary, S. Bregaglio, L. Willocquet, D. Gustafson, "Crop health and its global impacts on the components of food security," Food Security 9, p. 11–27, 2017.
2. S. Savary, L. Willocquet, S.J. Pethybridge, P. Esker, N.M. Roberts, A. Nelson, "The global burden of pathogens and pests on major food crops," Nat. Ecol. E. vol. 3 (3), p. 430–9, 2019.
3. M. Nandi, J. Macdonald, P. Liu, B. Weselowski, Z.C. Yuan, "*Clavibacter michiganensis* ssp. *michiganensis*: bacterial canker of tomato, molecular interactions and disease management," Molecular Plant Pathology 19 (8), p. 2036–2050, Aug 2018.
4. T. Khoirinisah, A. Fadhila, T. Wibowo, L.R. Kartikasari, M Cahyadi, "Specific primer design of COI gene and its potential application for species identification of meats," Earth and Environmental Science 478, 2020.
5. Y. Fang, R.P. Ramasamy, "Current and prospective methods for plant disease detection," Biosensors 5 (3), p. 537–561, Sep 2015.
6. F.D. Wahyuni, H. Saraswati, K.S. Dewi, "In-silico analysis for *cryI* gene amplification from *Bacillus thuringiensis*," Bioedukasi XVIII, April 2020.
7. J. Antonio, V. Hernandez, N. S. Alvarez, M. Angel, R. Rodriguez, A. R. Ontiveros, "Eustressic Dose of Cadmium in Soil Induces Defense Mechanisms and Protection Against Clavibacter michiganensis in Tomato (Solanum lycopersicum L.)," Journal of Plant Growth Regulation, 2022.
8. V.K. Singh, R Govindarajan, S. Naik, A. Kumar, "The effect of hairpin structure on PCR amplification efficiency," Molecular Biology Today 1 (3), p. 67–69, 2000.
9. K.A.A. Elsalam, "Bioinformatic tools and guideline for PCR primer design," African Journal of Biotechnology 2 (5), p. 91–95, 2003.

10. J. Piriyapongsa, C. Ngamphiw, A. Assawamakin, P. Wangkumhang, P. Suwannasri, U. Ruangrit, G. Agavatpanitch, S. Tongsima, "RExPrimer: an integrated primer designing tool increases PCR effectiveness by avoiding 3'SNP-in-primer and mis-priming from structural variation," BMC Genomics 10 Suppl 3 (Suppl 3): S4, 2009.
11. A. Naqib, T. Jeon, K. Kunstman, W. Wang, Y. Shen, D. Sweeney, M. Hyde, S.J. Green, "PCR effects of melting temperature adjustment of individual primers in degenerate primer pools," PeerJ, p. 1–17, 2019.
12. S.A. Bustin, R. Mueller, T. Nolan, "Parameters for successful PCR primer design," Methods in Molecular Biology (Clifton, N.J.) 2065, p. 5–22, 2020.
13. S.J. Green, R. Venkatramanan, A. Naqib, "Deconstructing the polymerase chain reaction: understanding and correcting bias associated with primer degeneracies and primer-template mismatches," Plos One 10 (5), p. 1–21, 2015.
14. R. Yunita, S. Isnaeni, "Study of growth and production of tomato cultivars in response to fruit thinning at Tamansari, Tasikmalaya, West Java, Indonesia," Journal of Tropical Crop Science 7 (02), 2020.
15. H. Wahyuningsih, F.K. Cayami, U. Bahrudin, M.A. Sobirin, F.E.P. Mundhofir, S.M.H. Faradz, I. Hisatome, "Optimization of PCR condition: the first study of high-resolution melting technique for screening of APOA1 variance," Yonago Acta Medica 60, p. 24–30, 2017.