Quantitative polymerase chain reaction (Q-PCR) for detection of sugarcane streak mosaic virus

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Abstract. Conventional PCR is a reliable method for detecting SCSMV. The availability of quantitative PCR (qPCR) can increase the power of PCR as detection method due to its high sensitivity and ability to quantify the template DNA in the reaction mixture. The study was aimed to validate the qPCR method for detection of SCSMV, and to develop detection methods for virus indexing program in seed canes production. qPCR method developed in this study was relative-qPCR with the regression equation of Y = -7.255 * log X + 11.752 for the SCSMV standard curve, so the value of X = 10 (-0.138 * Ct + 1,620). Ct value is inversely proportional to the concentration of cDNA. The greater the Ct value, the lower the concentration of cDNA in the sample. It was proved that qPCR method is more sensitive compared to conventional PCR, because it was able to detect SCSMV in samples not detected by conventional PCR methods. The relation between the Ct value and the incidence and severity of the disease on the field was assessed using field samples for qPCR. It was shown that Ct value and virus concentration did not have relation to the incidence and severity of the disease in the fields.

Keywords: conventional PCR, Ct value, regression equation, seed cane indexing, standard curve

1. Introduction

Sugarcane streak mosaic virus (SCSMV) is one of major virus that cause important disease in sugarcane plants. Detection and identification of SCSMV is usually done by conventional polymerase chain reaction (PCR) methods [1, 3]. In some conditions the sensitivity level of PCR method will decrease, for instance when the concentration of virus in the samples is very low or the samples contain inhibitor compounds; in addition, contamination in the reaction may cause false positive to occurred [9]. The availability of quantitative PCR (qPCR) method can increase the power of PCR as detection method due to its high sensitivity and ability to quantify the template DNA in the reaction mixture. This method has been widely applied for virus detection, for instance Wheat yellow mosaic virus [5], Tomato leaf curl virus and Tomato yellow leaf curl virus [8].

This study was conducted to validate the qPCR method for detection of SCSMV, and calculated virus concentration in sugarcane. It is important to develop detection methods for virus indexing program in seed canes production in order to support national government programs for sugar self-sufficiency. In this article we will discuss the results of qPCR method optimization for detecting SCSMV from infected sugarcane samples.
2. Materials and methods

2.1. Time and location
Research was conducted from March 2016 to April 2019 at Plant Virology Laboratory, Department of Plant Protection, Faculty of Agriculture, IPB University and Biomolecular Laboratory, Center for Diagnostic Standards of Agricultural Quarantine, Ministry of Agriculture.

2.2. Primer design
Specific qPCR primers for SCSMV was constructed using PrimerQuest Tool from Integrated DNA Technology, Inc. and successfully obtained five pairs of primers (Table 1), then one pair of primer was selected, namely SCSMV RT120F (5’GTCTATTTCTGCTGGTAGG3’) and SCSMV RT120R (5’CGTAGCGGGAACCCAATAT3’) which will amplify 120 bp DNA target. The primers were designed based on the sequence of SCSMV isolate from Gowa (LC469714).

| Primer | Sequen (5’ – 3’) | Start Tm (°C) | GC Presentase (%) | Amplicon PCR (pb) |
|--------|------------------|---------------|------------------|------------------|
| Primer 1 Forward (SCSMV RT120F) | GTCTATTTCTGCTGGTAGG | 282 | 62 | 50 | 120 |
| Reverse (SCSMV RT120R) | CGTAGCGGGAACCCAATAT | 401 | 62 | 50 |
| Primer 2 Forward | CCTTGGTTACGCACGTTATTT | 269 | 62 | 47.6 | 91 |
| Reverse | GGATTGACCACACCAGGTTT | 359 | 62 | 47.6 |
| Primer 3 Forward | GCAGAAGTCGCTTCCTCAGATTTT | 249 | 62 | 50 |
| Reverse | CCGGATGCGGAAGCTTTAT | 457 | 62 | 50 |
| Primer 4 Forward | GGGCCAGCCACCACAGATTT | 457 | 62 | 47.6 |
| Reverse | CCGGATGCGGAAGCTTTAT | 457 | 62 | 50 |
| Primer 5 Forward | CCATCAGCCTATCCAAGGAAAC | 46 | 62 | 47.6 |
| Reverse | CAAACTATGGAAGGACGCAGA | 169 | 62 | 47.6 |

2.3. Standard sample preparation
Recombinant plasmid containing insert DNA of CP-SCSMV isolate Gowa (pSCSMV-Gowa#30) was used as standard sample to develop a standard curve (Fig.1). Plasmids were serially diluted from $10^{-1}$ to $10^{-10}$ and DNA concentration on each dilution factors was measured using Thermoscientific Nanodrop 2000c.

**Figure 1.** Recombinant plasmid pSCSMV-Gowa#30
2.4. cDNA synthesis and amplification

cDNA synthesis was carried out by reverse transcription (RT) reaction. Each RT reaction consists of 2 μl RT 5x buffer, 0.5 mM dNTP (Thermo Scientific), 1.75 mM DTT (Thermo Scientific), 14 units RNase inhibitor (Thermo Scientific), 70 units M-MuLV (Thermo Scientific), 3.7 μl nuclease-free water, 0.75 μM oligo d(T) (Thermo Scientific), and 2 μl RNA. RT reaction was performed following this consecutive incubation periods: 65 °C for 5 min, 37 °C for 60 min, and 70 °C for 5 min.

Once the cDNA was ready it will be used as templates for amplification. The amplification reaction consists of 10 µl Bioline SensiFAST™ SYBR® Non-ROX Kit, 7.4 µl nuclease-free water, 0.4 µM of the SCSMV RT120R/ F Primers, and 1 µl cDNA. The reaction mixture was then inserted into the Qiagen Rotor-Gene™ 3000 PCR machine. qPCR program consisted of activation phase at 95 ºC for 2 min, denaturation at 95 ºC for 5 sec, and annealing/ extension at 60ºC for 30 sec. Denaturation and annealing/ extension stages were repeated for 40 times.

2.5. Standard curve formation

Two components for formatting standard curve are initial concentration of nucleic acid and Ct value (Y). The value of initial concentration of nucleic acid was obtained from the previous nanodrop measurement of standard samples. Ct value was obtained by setting the threshold value of standard samples. Based on DNA concentration and Ct value, a regression equation Y = M * log X + B will be obtained, which can be used to calculate the number of SCSMV DNA (X) found in samples.

2.6. SCSMV concentration in sugarcane

Testing was carried out SCSMV infected sugarcane leaves at position -2 until -6 by running qPCR with protocol as above.

2.7. Feasibility assessment of qPCR

Feasibility assessment was done by running 2 methods, i.e. qPCR and conventional PCR together by side using the same samples. Two samples used in this assessment were SCSMV-infected sugarcane leaves from the greenhouse and field samples showing symptoms of SCSMV infection, i.e. streak mosaic. Conventional PCR was performed using specific primers AP3/547F as described by [1, 3].

In addition, 2 field samples known for their disease intensity were selected for SCSMV detection by qPCR. The assessment may explain the relation of virus concentration in leaf samples with disease intensity in the field.

3. Result and discussion

qPCR is a direct method of nucleic acid amplification and uses fluorescence reporter molecules to monitor amplification process. Fluorescence will be adjusted according to PCR products accumulation in each amplification cycles. This method can be used to determine DNA amount in viral genome. There are 2 types of qPCR, i.e. qPCR-absolute and qPCR-relative quantification. Absolute quantification needs a standard curve of reference gene and network, whereas relative qPCR require standard curve from standard sample only. Recombinant plasmid pSCSMV-Gowa#30 was used as standard sample in this research. The qPCR standard curve has been found for SCSMV with regression equation Y = -7,255 * log X + 11,752, so the value of X = 10 (-0,138 * CT + 1,620) (Fig 2). The standard curve was obtained from the amplification of standard sample with serial dilutions (Table 2). Ct value of the standard sample ranges from 3.46 to 20.715. Ct value is a reflection of the parameter which reflects template quantity in the reaction and the number is inversely proportional to DNA and amplicon concentration.
Table 2. Result of qPCR from standard sample pSCSMV-Gowa#30 with serial dilution

| Dilution factor | DNA concentration | Ct value | DNA amplicon concentration |
|-----------------|-------------------|----------|---------------------------|
| $10^{-1}$       | 56.60             | 3.46     | 13.88                     |
| $10^{-2}$       | 4.30              | 3.735    | 12.72                     |
| $10^{-3}$       | 1.13              | 5.765    | 6.685                     |
| $10^{-4}$       | 1.07              | 8.18     | 3.115                     |
| $10^{-5}$       | 0.60              | 12       | 0.91                      |
| $10^{-6}$       | 0.57              | 15.59    | 0.295                     |
| $10^{-7}$       | 0.23              | 15.5     | 0.3                       |
| $10^{-8}$       | 0.23              | 17.695   | 0.15                      |
| $10^{-9}$       | 0.20              | 20.715   | 0.6                       |
| $10^{-10}$      | 0.20              | 19.66    | 0.8                       |
| H$_2$O (negative check) | -       | 27.99    | 0.01                      |

Figure 2. qPCR detection curves of standard sample pSCSMV-Gowa#30 with serial dilution, (a) linear curve; (b) logarithm curve with threshold 0.0816; (c) standard curve for SCSMV.

Table 3. Ct value and SCSMV DNA concentration on infected sugarcane leaf

| Sample       | Average of Ct value (Y) | Average of SCSMV DNA (X= 10$^{0.138Y+1.620}$) (ng µl$^{-1}$) |
|--------------|-------------------------|---------------------------------------------------------------|
| Positive control | 2.973                   | 16.226                                                        |
| Water        | 28.483                  | 0.005                                                         |
| Leaf -2      | 9.52                    | 2.037                                                         |
| Leaf -3      | 9.653                   | 1.950                                                         |
| Leaf -4      | 12.43                   | 0.808                                                         |
| Leaf -5      | 11.787                  | 0.990                                                         |
| Leaf -6      | 12.45                   | 0.803                                                         |

Among other advantages of quantitative PCR are real time data, on screen observation, do not wasting time for post-amplification processing (e.g. electrophoresis, colorimetric reaction or hybridization), short DNA fragment amplification, and higher level of efficiency and sensitivity [2]. Furthermore, the main benefit of real-time PCR involved high sensitivity and ability to perform quantitative assessment so that making it suitable for studying biology, epidemiology, and ecology of pathogen [6, 7, 10].
The result of qPCR-based assay to measure concentration of SCSMV on sugarcane leaf samples indicated that the lowest and the highest virus concentration was found on leaf no. -6 and no. -2, respectively, i.e. 0.803 ng.µl^{-1} and 2.037 ng.µl^{-1} (Table 3). It means that accumulation of SCSMV is higher in old leaves than on young leaves. Furthermore, method for field sampling aimed at detecting viruses can be recommended based on this result. Leaves no. -2 should be selected as field sampling in a survey with the objective to detect virus and determine frequency of virus infection.

Comparison of conventional PCR and qPCR indicated that qPCR resulted more sensitive reaction for SCSMV detection from leaf samples. Specific DNA fragments was amplified from samples no. 1 and 2, but no amplification was observed from samples no. 3, 4, nor 5 when using conventional PCR. However, 4 out of 5 samples gave positive reaction with qPCR. Sample no. 4 showed negative reaction for both methods (Table 4). Ct value of samples no. 1 and 2 is smaller than those of samples no. 3 and 5. Therefore, DNA concentration of samples no. 1 and 2 is higher than those of samples no. 3 and 5.

| Samples          | Size of DNA amplicon (bp) | Conventional PCR | qPCR |
|------------------|---------------------------|------------------|------|
| Sample 1         | 500                       | No amplification | 11.46 | 1.10  |
| Sample 2         | 500                       | No amplification | 9.90  | 1.80  |
| Sample 3         | No amplification          | 21.78            | 0.04  |
| Sample 4         | No amplification          | 26.99            | 0.01  |
| Sample 5         | No amplification          | 22.50            | 0.03  |
| SCSMV-infected leaf | 500                       | 8.72             | 2.61  |
| pSCSMV-Gowa#30   | 500                       | 3.75             | 12.69 |
| H2O              | No amplification          | 28.15            | 0.01  |

Notes: *conventional PCR and qPCR was conducted using SCSMV specific primers, AP3/547F and RT120R/F, respectively

| Field location | Disease incidence (%) | Disease severity (%) | Range of Ct value* |
|----------------|-----------------------|----------------------|--------------------|
| Bone 1         | 90                    | 43.75                | 25.96 - 26.14      |
| Bone 2         | 80                    | 36.67                | 19.55 - 25.13      |
| Bone 3         | 90                    | 47.5                 | 23.36 - 24.55      |
| Bone 4         | 95                    | 57.5                 | 24.07 - 25.61      |
| Bone 5         | 65                    | 28.75                | 25.37 – 25.91      |
| Bone 6         | 70                    | 36.25                | 25.11 – 27.17      |
| Takalar 1      | 70                    | 42.5                 | 21.66 – 26.33      |
| Takalar 2      | 5                     | 5                    | 23.83 – 26.02      |
| Takalar 3      | 65                    | 45                   | 25.03 – 26.30      |
| Gowa 1         | 100                   | 60                   | 22.46 – 24.15      |
| Gowa 2         | 100                   | 67                   | 25.08 – 26.60      |
| Lampung 1      | 100                   | 66.67                | 24.73 – 25.65      |
| Lampung 2      | 100                   | 71.67                | 24.14 – 25.82      |
| Lampung 3      | 100                   | 61.67                | 24.53 – 26.65      |

Notes: *qPCR was conducted using SCSMV specific primers (RT120R/F)

Based on detection of sugarcane samples from 14 locations in South Sulawesi (Bone, Takalar, and Gowa) and Lampung with qPCR method, there is no relation between Ct value with disease incidence.
and severity caused by SCSMV in sugarcane (Table 5). Similarly, SCSMV concentration alone did not determine the progression of disease in the field. As it is explained by disease triangle concept, disease intensity is influenced by interaction of virus, host and environment [4].

4. Conclusion
Based on qPCR method it is determined that disease incidence and severity does not relate to Ct value and virus concentration. Quantitative PCR or qPCR is recommended as a method to detect SCSMV from field samples and probably could be used also for disease indexing in seed canes. Sugarcane leaf at position -2 is the best leaf for survey sample due to high virus concentration.

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