Regeneration and protein profile analysis of callus transformant with geminivirus β-satellite fragment

F R M Bagus¹, S Sihotang¹, B Nova², and J Jamsari¹,³*

¹Postgraduate Biotechnology Program, Andalas University, Padang, West Sumatra, 25163, Indonesia
²Doctoral Program of Agriculture Science, Departement of Agriculture, Andalas University, Padang, West Sumatra, 25163, Indonesia
³Departement of Agrotechnology, Andalas University, Padang, West Sumatra, 25163, Indonesia

E-mail: ajamsari@yahoo.com

Abstract. The β-satellite fragment is a single-strand DNA that has a size of 1.4 kb. This fragment plays role in pathogen-derived resistance [PDR]. The genetic transformation of the β-Satellite fragment carried out by the previous researcher is limited by the regeneration of transformant callus. Therefore, this research was aimed to find some factors affecting the regeneration of transformant and non-transformant calli. Furthermore, protein analysis was performed on both calli to identify any specific fragment differentiating both proteins. SDS-PAGE analysis showed 5 and 8 fragments from transformant and transformant respectively. One of the 5 fragments has a size of 13.32 kDa which was predicted as C1 protein. These results proved that the β-satellite fragment has been successfully inserted and expressed in the transformant calli. Subsequent research is suggested to identify some differential fragments produced by both transformant and non-transformant.

1. Introduction

Geminivirus is one of the viruses that cause curly yellow disease in chili plants. The field survey showed that there was an increase in the distribution and intensity of geminivirus attacks [1]. Generally, virus infection is characterized by stunted growth in young plants. The next symptoms will show pale and yellowing leaves following by whitening of leaf bones. The symptoms continue until the leaves become curly and yellow and the thickened leaf bone.

Geminivirus is part of the Geminiviridae family which belongs to the genus Begomovirus. Based on the components of the genome, geminiviruses are divided into two groups namely monopartite and bipartite. The bipartite group consists of DNA-A and DNA-B and the monopartite group consists of DNA-A like and DNA β-Satellite instead of DNA-B [2].

Jamsari and Pedri [2] reported that geminivirus isolates from West Sumatra have several important genes. These genes are related to the pathogenicity of viruses to plants, including coat protein [CP/V1], movement [V2], replication protein [C1] and β-satellite DNA. The existence of a β-satellite in its genome has led to the conclusion of the monopartite type.

The β-Satellite DNA is a single-stranded DNA that has a size of 1.3 kb and has a structure similar to DNA-B. The β-Satellite plays a role in the mechanism of virus infection in its host. According to [2], β-satellite DNA is considered to have an important role in the process of increasing viral nucleic acid in its host cell. In some cases, the Begomovirus β-satellite DNA also plays a role in the process of
encapsulation and replication. Besides, this DNA also plays a role in causing symptoms and increasing the accumulation of viruses in the body of its host [3].

Jamsari and Pedri [2] successfully identified β-satellite DNA sequences derived from TD-21 isolates with a size of 1351 bp using specific primers β01 and β02. The sequence data have been deposited in the NCBI database with accession number GU382667. Zhou et al [4] identified a satellite DNA molecule [Y47β] from the Y47 Malvastrum isolate with a size of 1348 bp. The size is 3 bp shorter than the results of Jamsari and Pedri [2].

Although the function of β-satellite DNA is related to the mechanism of virus infection to plants, β-satellite DNA can also be utilized to develop plant resistance to plant viruses. This strategy is known as PDR [Pathogen Derived Resistance] where certain genes of pathogens are used to induce plant resistance to viral infections [5].

One step in developing the strategy is through genetic transformation. Transformation of β-satellite DNA into chili plants has been carried out by Renfiyeni [6] via Agrobacterium tumefaciens. However, the result is limited by the regeneration of transformant calli. For that reason, this study was carried out. This study was aimed to investigate proteins expressed in a callus during its regeneration stage. A comparison between transformant and non-transformant calli should provide data proteins differentiating during the regeneration process.

2. Materials and method

2.1. Preparation of transformant and non-transformant calli
Callus was prepared from seedling on MS0 media. Fourteen days after planting [DAP], the hypocotyl was transplanted onto callus induction media [CIM]. Fourteen days after induction calli were chosen based on their compact structure and yellowish-green color.

2.2. Agrobacterium tumefaciens infection
The selected calli were infected in the suspension of A. tumefaciens for 20 minutes in a shaking incubator. The infected calli were then co-cultivated for 2 days in a dark room with room temperature.

2.3. Transformant selection
Transformants selection was carried out using kanamycin and cefotaxime. Following selection, calli were washed using distilled water containing cefotaxime 500 mg/l for 3 times. After that step, calli were planted on selection media for 5 days at room temperature.

2.4. Transformant and non-transformant calli verification
Verification of calli transformant was started by conducting DNA isolation. The isolated DNA was then checked using the electrophoresis technique while the concentration was measured using nanodrop. PCR analysis was then performed using specific primers and T-DNA in-out primers.

2.5. Callus protein analysis
Total protein concentration was measured using the Bradford method [7] with minor modification. Standardization of protein concentration was performed before SDS-PAGE analysis.

3. Results and discussion

3.1. DNA of transformant and non-transformant
The concentration of callus DNA was checked using the electrophoresis technique as well as nanodrop. The result is presented in Figure 1.
Figure 1 shows that the DNA isolation of transformant and non-transformant calli were successfully carried out. Non-transformant callus has a concentration of about 80 ng/µl while the transformant callus has a lower concentration [Table 1].

Table 1. The DNA concentration of transformant and non-transformant callus

| No. | ID Sample | Nucleic Acid Con. | Unit  | 260/280 | 260/230 |
|-----|-----------|------------------|-------|---------|---------|
| 1   | NT        | 80.20            | ng/µl | 1.84    | 1.44    |
| 2   | T1        | 71.40            | ng/µl | 1.98    | 0.70    |
| 3   | T2        | 70.60            | ng/µl | 1.96    | 1.23    |
| 4   | T3        | 57.80            | ng/µl | 2.05    | 1.37    |

The data above shows the ratio of 260/280 ranging from 1.98-2.05. The ratio indicates that the purity of isolated DNA is acceptable. However, the ratio of 260/230 exhibit range from 0.70-1.44, indicating that the isolated DNA is not free from polysaccharide contaminant [8]. Plant cell walls, in general, has rigid cell walls that are difficult to purify during the DNA extraction process. This contamination could also be caused by improper pipeting. Jena et al [9] also stated that the quality and quantity of DNA isolation are influenced by several factors, one of which is the presence of polysaccharide compounds, polyphenols, and other secondary metabolites produced by used material.

3.2. Transformant and non-transformant callus verification

To verify transformation successfulness amplification using primer β0ITD BamHI-A and β02TD SmaI-A was carried out. The result is presented in Figure 2. Figure 2 shows that samples 1 and 2 could not produce the expected PCR product. Sample callus 3 produced a single fragment of 1351 bp in size, while the non-transformant callus also could not produce any fragment at all. Based on these result it can be concluded that the T-DNA was successfully inserted into the callus genome of sample 3. Failure in producing fragments from sample 1-2 could be caused by the contaminant. Polysaccharides and tannins are the main problems in the isolation of plant DNA. Both contaminants can inhibit the activity of DNA polymerase and interfere with the amplification process [10].
3.3. PCR of in-out and out-out primer

Further verification of transformation was done using an *in-out* and *out-out* primer [Figure 3].

![Figure 2. Visualization of the results of transformant and non-transformant callus DNA amplification using specific primers β0ITD BamHI-A and β02TD SmaI-A. 1KB = marker, T1-T3 [replicate] = transformant callus DNA and NT = Non-Transformant callus DNA](image)

![Figure 3. PCR product using in-out/out-T-DNA primers, 1KB = size marker, T1-T3 = transformant amplification results](image)

Figure 2. Visualization of the results of transformant and non-transformant callus DNA amplification using specific primers β0ITD BamHI-A and β02TD SmaI-A. 1KB = marker, T1-T3 [replicate] = transformant callus DNA and NT = Non-Transformant callus DNA

Figure 3. PCR product using in-out/out-T-DNA primers, 1KB = size marker, T1-T3 = transformant amplification results

Figure 3 obviously shows that there is no PCR product using an in-out and out-out primer. This data, prove that the T-DNA was successfully inserted into the callus genome. The in-out primer will amplify the region of in-T forward DNA to the reverse T-DNA out and the out-primary primer will amplify the T-DNA forward region to the reverse T-DNA outs. Estimation of the PCR product using the 2 both primers should be 319 bp and 595 bp respectively. Further explanation of the primer position is presented in Figure 4. The forward primer of T-DNA is located between the right border [RB] and the left border [LB] or within the T-DNA region, while the forward and reverse of out T-DNA are located outside the T-DNA region.
Figure 4. Primer position of in and out T-DNA in the pBI121 plasmid after [6]

*A. tumefaciens* can transfer parts of T-DNA into the plant genome [11]. During transformation only the T-DNA part will be moved along with the T-DNA. So when amplification using T-DNA in-out primer, no results will be obtained. This is because the reverse T-DNA part is not in the T-DNA region. Furthermore, amplification using the T-DNA out-out primer aims to confirm the results of the amplification using the T-DNA in-out primer because the forward and reverse T-DNA parts are outside the T-DNA region.

3.4. Protein analysis of transformant and non-transformant

Protein analysis was aimed to differentiate protein profiles between transformant and non-transformant calli. This analysis could provide data, whether the inserted gene is expressed or not, or to identify some changes in gene expression profile. The data is presented in Table 2.

| Sample    | Absorbance | Protein concentration [ng/µl] |
|-----------|------------|-------------------------------|
| Non-Transformant |
| 1         | 0.085      | 140.83                        |
| 2         | 0.075      | 124.17                        |
| 3         | 0.081      | 134.17                        |
| Transformant |
| 1         | 0.083      | 138.33                        |
| 2         | 0.073      | 120.83                        |
| 3         | 0.080      | 132.50                        |

Table 2 showed that the obtained protein concentrations ranged from 120 to 140 ng/µl for non-transformant and 120 to 138 for transformant. No significant differentiation in total protein is seen. Before the SDS-PAGE analysis, the concentration of each sample was set to 100 ng/µl. SDS-PAGE analysis is presented in Figure 5.
Figure 5. SDS-PAGE visualization of transformant and non-transformant callus. P = protein ladder, T1-T3 = transformant, and NT1-NT3 = non-transformant.

Figure 5 shows that the non-transformant callus produced 8 protein fragments, while the transformant callus produced 5 protein fragments. Seven fragments show different molecular weight and only 1 fragment shows similar molecular weight with different concentrations. The summary of molecular weight fragment between transformant and non-transformant is presented in Table 3.

Table 3. Fragment molecular weight of transformant and non-transformant

| No. | Molecular Weight [kDa] | Sample                      |
|-----|------------------------|-----------------------------|
| 1   | 59.43                  | Non-Transformant            |
| 2   | 56.42                  | Non-Transformant            |
| 3   | 48.78                  | Non-Transformant & Transformant |
| 4   | 33.33                  | Non-Transformant & Transformant |
| 5   | 31.54                  | Non-Transformant            |
| 6   | 25.53                  | Non-Transformant & Transformant |
| 7   | 20.67                  | Transformant                |
| 8   | 20.11                  | Non-Transformant            |
| 9   | 13.69                  | Non-Transformant            |
| 10  | 13.32                  | Transformant                |

Differential profile of fragments and their concentration between the transformant and non-transformant seemed caused by the transformation effect. Some protein fragments appearing in the non-transformant callus do not appear in the transformant and vice versa. Furthermore, some fragments appear in both transformant and non-transformant but show different intensity, indicating a different level of expression. Fragment of 33.33 kDa shows a difference concentration between the transformant and non-transformant. The non-transformant produced a higher concentration than the transformant callus. Seemed that incorporating the β-satellite fragment suppresses the expression of that fragment [down-regulated] representing a lower concentration or low intensity in SDS-PAGE. The thicker the protein band obtained, the greater the concentration of protein, and vice versa [13].

Figure 4 also shows a fragment of 13.32 kDa in size produced by the transformant. This could be a C1 protein fragment expressed by the β-satellite gene since the Extasy tool prediction exhibited a molecular weight of 13.33149 kDa. However, this assumption should be proved empirically further.
4. Conclusion

Based on the data presented above, the β-satellite fragment seemed to be successfully transformed into a chili genome. The incorporation of the β-satellite fragment seemed to cause some differential expressions of some host genes.

References

[1] Trisno J, S H Hidayat, J Jamsari, T Habazar and I Manti 2012 Identifikasi Molekuler Begomovirus Penyebab Penyakit Kuning Keriting pada Tanaman Cabai (Capsicum annum L.) di Sumatera Barat J. Nat. Indonesia 13 41-46

[2] Jamsari J and J Pedri 2013 Complete Nucleotide Sequence of DNA A-like Genome and DNA-β of Monopartite Pepper Yellow Leaf Curl Virus, A Dominant Begomovirus Infecting Capsicum annuum in West Sumatera Indonesia Asian J. Plant. Pathol. 5 1-14

[3] Saeed M 2006 The Role of A Geminiviral DNA β Satellite in Viral Pathogenicity and Movement (Dissertation ) Australia Faculty of Sciences University of Adelaide 6-14 pages

[4] Zhou X, Y Xie, Y Peng, and Z Zhang 2003 Malvastrum Yellow Cein Virus, A New Begomovirus Species Associated with Satellite DNA Molecule Chinese Sci. Bull. 48 2206-10

[5] Sanford JC and S A Johnston 1985 The Concept of Parasite-Derived Resistance Deriving Resistance Genes from The Parasite’s Own Genome J. Theor. Biol. 113 395-405

[6] Renfiyeni R 2015 Studi Regenerasi In Vitro dan Transformasi Genetik Gen Coat Protein dan Beta Component Geminivirus melalui Agrobacterium tumefaciens pada Tiga Genotipe Cabai Merah (Capsicum annum L.) (Dissertation) Padang Program Pascasarjana Universitas Andalas 181 pp

[7] Jamsari J and S N Aisyah 2018 Modul Praktekum Dasar-Dasar Bioteknologi Tanaman Padang Penerbit Erka 84 pp

[8] Sambrook J, E F Fritsch, and T Maniatis 1989 Molecular Cloning A Laboratory Manual New York Cold Spring Harbor Laboratory Press 1546 pp

[9] Jena R C, KC Samal, and B K Das 2010 Optimization of DNA Isolation and PCR Protocol for RAPD Analysis of Mangifera indica L J. Agric. Technol. 6 559-71

[10] Murray M G, and WF Thompson, 1980 Rapid Isolation of High Molecular Weight Plant DNA Nucleic Acids Res. 8 4321-26

[11] Rahmawati S 2006 Status Perkembangan Perbaikan Sifat Genetik Padi Menggunakan Transformasi Agrobacterium J. AgroBiogen 2 36-44

[12] Sudarmadji S 1996 Teknik Analisa Biokimiawi Yogyakarta Liberty 307 pp

Acknowledgment

This research was partially funded by the General Directorate Higher Education of the Republic of Indonesia through Professorship Cluster Research Grants through contract number 43/UN.16.17/PP.HGB/LPPM/2017, 18/UN.16.17/PP.RGB/LPPM/2018 and T/22/UN.16.17/PP.KP-KRP1GB/LPPM/2019.