Transformation and Transient Ekspresion Analysis of L-HBsAg DNA in Fruits of *Musa acuminata* Colla cultivar 'Ambon Lumut" and 'Mas' using *Agrobacterium tumefaciens*

Sony Suhandono*, **Lailatul B,** Ernawati Giri R, Putri DU, Dina RR, Ima MZ

School of Life and Sciences, Bandung Institute of Technology, Bandung, Indonesia
** Corresponding author: Bandung Institute of Technology. Jl. Ganesa No 10, Bandung 40132, West Java, Indonesia.*
Tel./fax.:+62-22-2534107, +62-22-2511575.
sony@sith.itb.ac.id

Abstract

Conventional HBsAg is still expensive for most Indonesia citizens. Recently, scientists developed plant-based vaccine which will give easy delivery, flexible production scale up, and produce cheaper vaccine. *Agrobacterium* transformation has been widely used in genetic engineering. This research was conducted to transform and analyze the L-HBsAg DNA expression in banana fruit using *Agrobacterium tumefaciens* and vacuum infiltration method. Unripe *Musa acuminata* Colla cultivars 'Ambon Lumut' (AAA) and 'Mas' (AA) were used in this transformation. The slices of banana were co-cultivated with *A. tumefaciens* strain AGL1 for Ambon lumut and GV3101 for Mas banana in three days at 25˚C±1˚C in the dark. Both strains contain a plasmid pCAMBIA 1390 with MeEF-1α promotor and L-HBsAg gene. Expression of L-HBsAg was confirmed by using RT-PCR with gene-specific primers of L-HBsAg. A 1,323 bp band of L-HBsAg was observed in gel electrophoresis as a result of RT-PCR. The sequence of recombinant plasmid was read with specific primers for L-HBsAg. These steps were performed using MACROGEN services. These results indicated that the cDNA sequences of samples and protein-coding DNA sequences of L-HBsAg were identical. Therefore, it can be concluded that the L-HBsAg DNA was inserted and expressed in unripe fruits of Ambon lumut and Mas banana.

Keywords : transformation, Agrobacterium tumefaciens*, L-HBsAg gene,* Musa acuminata*, ambon lumut banana, mas banana*

Introduction

Hepatitis B is infectious disease caused by Hepatitis B virus (HBV). It was estimated that more than 400 million people worldwide suffer from this disease (Piramanayagam *et al*., 2008). Effort to tackle this problem like vaccination has been available since 1980 (European Centre for Disease Prevention and Control, 2008). However, the vaccination program does not run well in developing countries like Indonesia because of the high price of the vaccine and syringe and inadequate facility for the storage and distribution of vaccines (Streatfield and Howard, 2003). One of the solutions developed to overcome these problems is the production of vaccine in a plant.

Research on the production of Hepatitis B vaccine in a plant is constantly being developed. Large hepatitis B surface antigen or L-HBsAg is the latest variant of HBV vaccine. It consists of pre-S2, pre-S1, and S antigen (Lou *et al*., 2007). It is expected that vaccination using the L-HBsAg may trigger immune responses effectively in preventing HBV infection.

Agrobacterium-mediated transformation is a method to be used to transform plant with foreign DNA fragments encoding L-HBsAg into the plant genome. A segment of *Agrobacterium tumefacines* Ti-plasmid called T-DNA is moved from a bacterium cell into the host plant chromosomal genome. The T-DNA region is bordered by two 25 bp direct repeat sequences, called left border and right border. After entering the plant host cells, the T-DNA sequence will express genes that incorporated into it. For this reason, the targeted recombinant gene usually is inserted in the T-DNA region (Taiz and Zeiger, 2002).

One of the plants that can be used to express the hepatitis B vaccine is banana. Banana trees can be grown in tropical regions and many people from various backgrounds and ages consumed the fruit. Bananas can be directly consumed so that the degradation process of the vaccine (protein) due to heating can be avoided (Radji, 2004). In this study expression analysis of the gene encoding L-HBsAg proteins in Ambon lumut and Mas banana fruit was made transiently because it requires less time and the result in transgene expression levels could be higher than stable expression in transgenic plant (Sheludko, 2008).

Materials and Methods

Plant material

Unripe banana used in this experiment was Ambon lumut and Mas fruits. The banana was specially ordered from a farmer. Age of the fruit was about three months from flower formation. The fruit skin color is green and gummy when peeled. While the fruit flesh is white and hard. The fruit was cut with a thickness of about 2-3 cm. Then the skin of the fruit was peeled off. The pieces were immersed in 1% sodium hypochloride for 10 min. After that, it was rinsed twice using sterile distilled water. The soaked pieces were cut again with a thickness of 2 mm. Each of the banana slices as thick as 2 mm was further divided into 8 sections.

Agrobacterium tumefaciens

Strain *A. tumefaciens* that used in transformation were AGL1 for Ambon lumut and GV3101 for Mas banana. Both strains contain a plasmid pCAMBIA 1390 with MeEF-1α promotor and L-HBsAg gene. The *Agrobacterium* strain with no binary plasmid was used as a control for the experiment.

Bacteria were activated on solid YEP medium with antibiotic for three days at 25˚C±1˚C, 250 rpm, in dark condition. Antibiotics for strain GV3101 were rifampicin at 50 ppm and kanamycin at 50 ppm. Antibiotics for AGL1 were carbenicilin at 100 ppm, rifampicin at 50 ppm and kanamycin at 50 ppm. While the bacteria lacking-plasmids were inoculated into the same medium, but without kanamycin.

Transferred of L-HBsAg gene into banana fruit

The vacuum infiltration method used in this experiment was modified from Matsumoto *et al* (2009). Single colony of bacteria was inoculated into 10 ml of liquid YEP medium containing antibiotics until the cell density has reached an OD_{600} of 0.5. An aliquot of 10% of the *Agrobacterium* cell suspension was again subcultured in 20 ml of same medium at same condition until the OD_{600} of 0.8. After that, the cells were collected by centrifugation at 2000 g for 5 min and suspended in infiltration medium on ½ MS (Murashige and Skoog) containing 2% of sucrose, 200 µM of acetosyringone, and 0.01% of *Silwet L-408*.

The banana slices cut and divided into eigth pieces, were put into 1.5 ml microtube containing 1 ml of the *Agrobacterium* cell suspension (OD_{600} = 0.8) in the infiltration medium. Then those were subjected to vacuum for 10 min, followed by co-cultivation for three days at 25˚C±1˚C in dark condition.

Expression analysis of L-HBsAg gene in banana fruit

To analyze the gene expression of L-HBsAg, the RNA isolation was performed using the method from Kansas *et al* (2008). The presence of RNA was confirmed by the protocol on agarose gel electrophoresis of RNA concentration of 2% (w/v) containing EtBr in 1x TAE buffer. DNase was added to the RNA preparation to eliminate genomic DNA isolation. Then the reverse transcriptase-PCR (RT-PCR) was made to convert total RNA into spesific cDNA of L-HBsAg gene in the banana fruit.

The iScript cDNA Synthesis Kit from Bio Rad Laboratories, Inc. were used to reverse transcript RNA into cDNA. PCR using specific primers of MeEF-1α promotor in 25 cycles was done to ensure the absence of genomic DNA from the total RNA. PCR was performed with Applied Biosystem 2720 Thermal Cycler. Primer sequences used were Me-EF1α forward *(*5' AAGCTTCCAGTGAATGGTCA 3') and Me-EF1α reverse (5'TGTGAACCTTCTCTCTAGACATTGTTAGT 3'). The PCR cycles consisted of 5 min at 94^{o}C, 30 sec at 94^{o}C, 30 sec at 50^{o}C, 1 min at 72^{o}C, and 7 min at 72^{o}C.

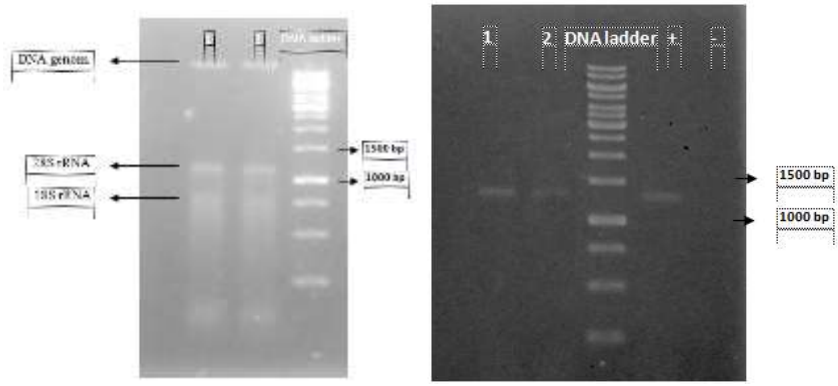
An order to amplify the expression of L-HBsAg from cDNA , the primers LHB-*forward* (5'GGATCCTGATGAAAATGAAGGTCCTTGTTTTCTTCGTTGCTACAATTTTGGTAGCATGGCAATGCCATGC GATGGGAGGTTGGTCCTCAAAACC-3') and LHB-*reverse* (5'GGTCACCTTAAATGTATACCCAAAGAC-3') were used. The series of cycles used were five min at 94^{o}C, 30 sec at 94^{o}C, 45 sec at 55^{o}C, 1 min and 30 sec at 72^{o}C, and 7 min at 72^{o}C.The PCR reaction composition were dNTP mix at 0,2 mM, Taq buffer 1x, $MgCl_2$ 1,5 mM, of primer forward at 1 µM, primer reverse at 1 µM, sample cDNA at 1 µg, DNA *polymerase* at 1 unit, and deion.

cDNA from the RT-PCR was cloned using the vector pGEM ®-T Easy vector system from Promega. Restriction analysis with EcoRI was made to confirm the clone in the plasmid. Restriction results were visualized on agarose gel electrophoresis. A nitrogen base sequence of cDNA was read as the last stage using specific forward and reverse primer L-HBsAg from MACROGEN.

Results and Discussion

Transformation of L-HBsAg gene into banana fruit

RNA isolation results indicated the presence of two RNA bands that dominant in the mid-agarose gel, which was ribosomal RNA (rRNA) 28S and 18S (Figure 1a). Quantification of total RNA with a spectrophotometer showed that the ratio λ A260/λ A280 in samples isolated RNA was 1.36 for Ambon lumut and 1.35 for Mas banana. The ratio obtained indicated that the purity of the isolated RNA was low because there may contain the impurities, such as genomic DNA and proteins. Electrophoresis results were also seen on the tape at the top indicating the presence of genomic DNA. Therefore, the isolation of total RNA were added DNase to eliminate genomic DNA so it was not interfere the next process.



a. b.

Figure 1. (a) Electrophoresis result of mRNA and (b) RT-PCR product from Ambon lumut (a) and Mas (b) banana

Confirmation the presence of L-HBsAg gene was made using PCR spesific primer of L-HBsAg. Electrophoresis results of RT-PCR products is shown in Figure 1b obtained after three consecutive PCR. This suggests that the gene encoding L-HBsAg was successfully inserted and expressed in unripe Ambon lumut and Mas banana.

Confirmation the presence of L-HBsAg gene was also made by restriction analysis and reading the sequence of nucleotide bases from cloned RT-PCR result. The results of restriction (Figure 2b) indicate that the presence of two bands of DNA size at 1300 and 3000 bp in Ambon lumut and Mas banana.

Final stages was reading the sequence of recombinant plasmid with specific primers for L-HBsAg. These steps were performed using MACROGEN services. The results from MACROGEN shows that the cDNA sequences of samples and protein-coding DNA sequences of L-HBsAg are identical. Therefore, it can be concluded that the cDNA sample was a cDNA of L-HBsAg. This means the L-HBsAg transgene successfully expressed at the mRNA level (transcription) in unripe ambon lumut and mas banana in transient system.

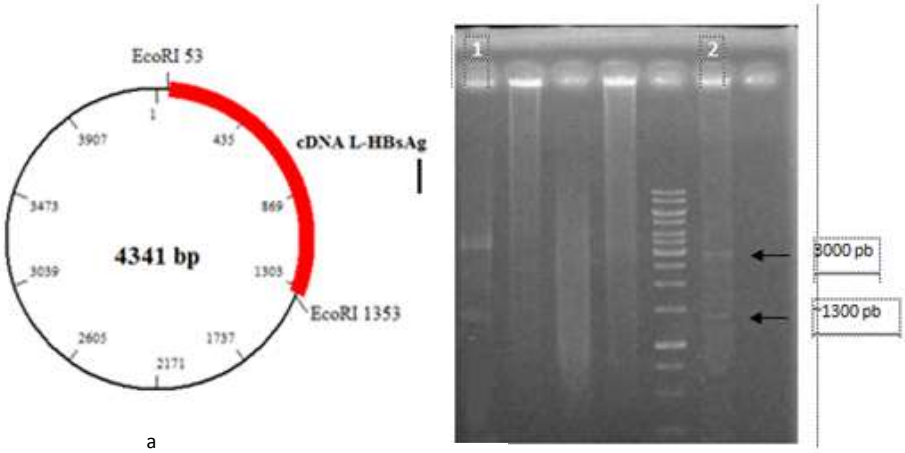


Figure 2. (a) restriction map of clone vector, (b) electrophoresis result from white colony that restricted using *Eco*RI. Sample 1 was ambon lumut while sample 2 was mas banana

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