Agrobacterium tumefaciens-MEDIATED IN-PLANTA TRANSFORMATION OF INDONESIAN MAIZE USING pIG121Hm-Cs PLASMID CONTAINING nptII AND hpt GENES

Transformasi Tanaman Jagung Indonesia dengan Plasmid pIG121Hm-Cs yang Mengandung Gen nptII dan hpt melalui Agrobacterium tumefaciens secara In-Planta

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ABSTRACT

Maize (Zea mays L.) productivity in Indonesia is challenged to be increased using genetic engineering. Recent advances in Agrobacterium tumefaciens-mediated in-planta transformation makes it possible to transform maize with low cost, and simple method. This study aimed to confirm pIG121Hm-Cs plasmid in A. tumefaciens, and to estimate the efficiency level of A. tumefaciens-mediated in-planta transformation of Indonesian maize by using pIG121Hm-Cs plasmid containing nptII and hpt genes. A series of studies were conducted including confirmation of gene construct of pIG121Hm-Cs plasmid in A. tumefaciens, transformation of four maize lines through A. tumefaciens-mediated in-planta technique, acclimatization of transformant plants and molecular analysis of selected plants using polymerase chain reaction (PCR). The pIG121Hm-Cs plasmid was confirmed via PCR analysis using specific primers for nptII and hpt genes and resulted 700 bp and 500 bp for fragments of nptII and hpt, respectively. After selection, acclimatization and molecular analysis steps, the efficiency levels of transformation of four maize lines were low, ranging from 3.8% to 12.8%. The level of transformation efficiency of ST-27 line was the highest accounting for 12.8% of 45 planted embryos on selection medium based on PCR analysis using specific primer for nptII gene. Overall, A. tumefaciens-mediated in planta transformation on maize floral pistil in this study proved to be successful and rapid. Therefore, this enhanced transformation method will be beneficial for Indonesian maize genetic engineering.

Keywords: Maize, Agrobacterium tumefaciens, in-planta transformation

INTRODUCTION

Maize (Zea mays L.) is a cereal crop that is widely grown and has the highest productivity in the world. This crop is usuallay used as food, feed and industrial raw materials of maize flour, refined products, as well as industrial fermentation and distillation (Wada et al. 2008). In Indonesia, maize is an important food commodity after rice and soybean.
Maize productivity is strongly influenced by biotic and abiotic stresses (Rakshit et al. 2009). Genetic improvement of maize to increase its productivity can be conducted by conventional hybridization between popular variety with other variety containing desired genes. These desired genes will be used for plant improvement through non-conventional technique or genetic engineering by transformation of homologous or heterologous genes which have been used for varied purposes (Carneiro and Carneiro 2013). Inserted genes (foreign genes) can be derived from the relative taxon or cross-taxon such as bacteria, fungi, viruses or distant relatives of crops regarding the genes of interest (Herman 1997).

The conventional plant transformation is a biological transfer method through Agrobacterium and physical or chemical gene transfer like gene gun to produce plants containing desired traits. Utilization of A. tumefaciens in plant genetic engineering is based on its ability to transfer gene that causes crown gall disease in plant cells. This phenomenon is exploited by researchers to replace “the crown gall disease” genes with genes encoding the desired properties so that these genes can be integrated into the plant genome and expressed by plants. The main step in A. tumefaciens method is based on co-incubation of plant vegetative organ or tissue (seeds, leaves, roots, stems or meristems) in bacterial-cell suspension (Chumakov 2007). These methods have been successfully used for transformation of important genes to many plants whose tissue culture system has already been established (Hiei et al. 1994; Ishida et al. 1996; Gelvin 2003; Carneiro and Carneiro 2013; Martins et al. 2015; Jan et al. 2016). Some advantages of transformation via A. tumefaciens have been reported such as higher transformation efficiency with a single copy gene and can be carried out with simple laboratory equipment and low-cost technique (Supartana et al. 2005). Transformants with a single copy gene are preferable as it is a easier analysis process and the segregation usually follows Mendelian inheritance pattern (Rahmawati 2006). But, the tissue culture-based transformation methods are time-consuming and lead some mutations from clonal variation that affect both qualitative and quantitative characters of plants (Martins et al. 2015; Jan et al. 2016).

The direct transformation method without any tissue culture steps is termed as in-planta transformation (Chumakov et al. 2006). Floral dip method has been used in genetic engineering strategy as it directly produces genetically modified transformant without the laborious tissue culturing procedures (Martins et al. 2015). The production of a large number of uniform plants in a short time, less labor efforts and minimal reagent requirements are some of the main advantages of in-planta transformation system (Bent 2000). Production of transformants in-planta seems especially promising for monocotyledonous plants, including maize since plant regeneration from suspension and callus cultures is difficult (Moisseeva et al. 2014). This method also proved to be faster, simpler and cheaper (Mu et al. 2012).

The development of a simple and rapid transformation method for Setaria viridis as a C4 model for monocotyledonous plants was time-saving and cost-effective compared with traditional methods in plant genetic engineering (Martins et al. 2015). In-planta transformation techniques have been succeeded in buckwheat plant (Fagopyrum esculentum M.), mulberry (Morus alba L.) and kenaf (Hibiscus cannabinus L.) (Ping et al. 2003; Kojima et al. 2004). The transformation efficiency of all plants transformed by such method was extremely high compared with the general in vitro transformation method (Kojima et al. 2000).

Several studies reported the success of transformation of nptII gene. Supartana et al. (2005) obtained the levels of efficiency of in-planta transformation of 40% (PCR) and 34% (GUS histochemical test) by inoculation of embryonic apical meristem rice plants. In-planta transformation using filament pistil (female flowers) of maize with plasmid pTd33 containing gus and nptII genes showed that 6.8% sprouts from the seeds were resistant to kanamycin and 60.3% of the resistant gene nptII were positive (Chumakov et al. 2006). Other study revealed that all tested genotypes of sugarcane cv. Co86032, Co90014, CoC671, Co94012 and Co86056 responded favorably, though CoC671 proved to be the best responding cultivar with 45.4% transformation efficiency. The results concluded that the protocol is cost-effective, efficient and genotype-independent without an involvement of any tissue culture procedures and can generate a relatively large number of transgenic plants in a short period, approximately 2 months (Mayavan et al. 2013).

In Indonesia such transformation method has not been reported yet on maize. Therefore, the aims of this study were to confirm pIG121Hm-Cs plasmid in A. tumefaciens, and to estimate the efficiency of A. tumefaciens-mediated in-planta transformation of Indonesian maize using pIG121Hm-Cs plasmid containing nptII and hpt genes.
MATERIALS AND METHODS

Plant Materials and Plasmid

Four lines of maize, Ultra Genjah, ST-08, ST-18 and ST-27, were used as plant materials. *A. tumefaciens* EHA101 containing the binary pIG121Hm-Cs plasmid was used as gene material to be transformed (Fig. 1). The T-DNA of the pIG121Hm-Cs plasmid containing nptII gene with nos promoter (Pnos) and nos terminator (Tnos), CsNtir-1 gene, was driven by 35s promoter and stopped by Tnos terminator, also *hpt* gene was driven by 35s promoter and stopped by Tnos terminator. The binary pIG121HM-Cs plasmid was the modification of pIG121Hm plasmid by replacing *gus* gene with CsNtir-1 gene.

Confirmation of pIG121Hm-Cs Plasmid in *A. tumefaciens*

A single colony of *A. tumefaciens* EHA101 containing pIG121Hm-Cs plasmid was multiplicated on yeast extract and peptone (YEP) medium containing 50 mg l\(^{-1}\) kanamycin and incubated for two days in an incubator shaker at room temperature. Plasmid was isolated using a modified alkaline lysis method (Sambrook et al. 1989). PCR analyses were performed using specific primers for the nptII gene (nptF 5’-GAGGCT ATTCGGCT ATGACTG-3’ and nptR 5’-ATCGGGAGCGGCGATACGTA-3’) to amplify a 700 bp DNA fragment, and specific primer of *hpt* gene (hptF 5’-CGCACAATCCACTATCTTGGCGA-3’ and hptR 5’-GCGATGGGGATCTGGCAATT-3’) to amplify a 500 bp DNA fragment. PCR reaction was performed according to the protocols of Anuradha et al. (2006) and Listanto et al. (2009) with modification. Total volume of PCR reaction was 25 microliter (µl) per reaction containing 1 PCR buffer, 1.5 mM MgCl\(_2\), dNTPs (each 0.2 mM dNTPs); 2 pmol primer F and R each, 0.25 units Taq DNA polymerase (Invitrogen), and 50 ng DNA template. The amplification reaction was done on PCR machine (MJ-Research PCT-100). Amplification cycle was started in initial denaturation at 94° C for 5 minutes followed by 30 cycles consisting of a denaturation at 94° C for 1 minute, annealing at 53° C for 1 minute and an extension at 72° C for 1 minute, then final extension at 72° C for 5 minutes. The amplification products were electrophoresed on a 1.2% agarose gel and then visualized under UV transilluminator (Chemidoc).

Agrobacterium tumefaciens-Mediated In-Planta Transformation on Maize

In-planta transformation was done on four maize lines (Ultra Genjah, ST-08, ST-18 and ST-27) according to the method of Chumakov et al. (2006). The whole pistil of a single ear of maize on 50–60 days after planting and has not pollinated was rubbed with wetted cotton containing the suspension of *A. tumefaciens* with pIG121Hm-Cs plasmid. The treated pistil was then dusted with pollen from the same plant and covered with paper bags. A number of maize ears were harvested on 12–16 days after pollination. The whole embryos were isolated from each maize ear and grown on MS medium without growth regulator. The embryos were then planted on MS medium in Petri dishes containing 20–30 embryos each dish and incubated at 24 °C room. After one week incubation, the embryos were transferred to MS selection medium.

Fig. 1. T-DNA construction on the binary pIG121Hm (A) and pIG121Hm-Cs (B) plasmids: RB = right border; LB = left border; Pnos = promoter of the nopaline synthase gene; nptII = gene for neomycin phosphotransferase; Tnos = terminator of the nopaline synthase gene; P35S = promoter of the CaMV 35S RNA gene; gus = gene for β-glucuronidase; hpt = gene for hygromycin phosphotransferase, CsNtir-1 = gene for nitrites transporter (Sato and Takamizo 2006).
Transformants which were able to grow on the selection medium (MS medium containing hygromycin) were acclimatized by transferring from in vitro medium into water medium. Selected transformant was chosen from a plant which was survived in the water medium and the number of survival plants in the water medium were counted.

DNA was isolated using modified method of Dellaporta et al. (1983). A small part of leaf (size about 0.5 cm\(^2\)) of each plant was taken, ground and homogenized in a 1.5 ml tube. Four hundred microliters (µl) of extraction buffer (Tris-HCl, pH 8.0 50 mM EDTA, pH 8.0 10 mM NaCl 100 mm, and 1% SDS) was heated to 65°C and added to the tube and shaken for 10 minutes. About 400 µl of Chisam (chloroform : isoamylalkohol = 24 : 1) solution was added and mixed thoroughly. Subsequently, the solution was centrifuged at 12,000 rpm for 10 minutes. About 250 µl of the aqueous solution was transferred to the new 1.5 ml tube, added 150 µl isopropanol and mixed by inverting the tubes for several times. DNA was precipitated by centrifugation at 12,000 rpm for 10 minutes to obtain a DNA pellet. Supernatant was discarded and the DNA precipitate was washed with 70% ethanol and dried. Dried DNA was dissolved using ddH\(_2\)O for PCR analysis.

Molecular analysis of the transformant was done by PCR amplification for the presence of nptII gene (one part of T-DNA component inside pIG121Hm-Cs plasmid) using specific primers (nptIIF and nptIIR). PCR results were electrophoresed on a 1.2% agarose gel and visualized under UV transilluminator (Chemidoc). The positive transformant was detected by the presence of DNA fragment of about 700 bp lengths in the agarose gel under UV transilluminator.

**RESULTS AND DISCUSSION**

**Confirmation of pIG121Hm-Cs Plasmid in A. tumefaciens**

Selection of bacteria strain and binary plasmid determined the success of plant transformation using Agrobacterium system. Agrobacterium EHA101 is a highly virulent strain and therefore, it is expected to increase the rate of infection on plants. While the use of binary plasmid pGI121Hm-Cs can expand the infected host plants (Rahmawati 2006). In our study, A. tumefaciens strain EHA101 containing the binary plasmid pGI121Hm-Cs expectedly improved the efficiency of in-planta transformation on maize.

The presence of selectable marker (nptII and hpt) genes in the pGI121Hm-Cs plasmid in A. tumefaciens was confirmed by PCR using specific primers. The PCR amplification products of pGI121Hm-Cs plasmid in A. tumefaciens showed the presence of nptII and hpt genes with fragment sizes of 700 and 500 bp, respectively (Fig. 2). This confirmation result indicates that the colony of A. tumefaciens used for transformation contains nptII and hpt genes.

Three maize ears have been harvested from Ultra Genjah and ST-08 lines, while from ST-18 and ST-27 lines we got two ears on 12-16 days after pollination. After ear isolation, the number of embryos that can be isolated were 293 embryos of Ultra Genjah line, 190 embryos of ST-18, 27 embryos of ST-27 and 233 embryos of ST-08. The percentage of the number of embryos selected on MS medium containing hygromycin were 26.8% of Ultra Genjah line, 32.6% of ST-18 line, 35.9% of ST-27 line and 26.6% of ST-08 line (Table 1). The resulted germination rates were lower compared with the previous research on maize using Agrobacterium-mediated transformation by in-planta method. In planta floral dip transformation on maize using pCambia 1302 plasmid showed that of 79 T0
Agrobacterium tumefaciens-mediated in-planta transformation of Indonesian maize... (Edy Listanto et al.)

Table 1. The results of Agrobacterium tumefaciens transformation on four maize lines by in-planta method.

<table>
<thead>
<tr>
<th>Line</th>
<th>Average number of planted embryos</th>
<th>Average number and percentage of survived embryos to hygromycin</th>
<th>Average number and percentage of plants to acclimatization</th>
<th>Average number and percentage of positive plants containing nptII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultra Genjah</td>
<td>97.7</td>
<td>26.0 (26.6)</td>
<td>2.7 (10.4)</td>
<td>1.0 (3.8)</td>
</tr>
<tr>
<td>ST-18</td>
<td>95.0</td>
<td>29.3 (30.8)</td>
<td>5.9 (20.1)</td>
<td>1.7 (5.8)</td>
</tr>
<tr>
<td>ST-27</td>
<td>22.5</td>
<td>14.8 (65.8)</td>
<td>5.0 (33.8)</td>
<td>1.9 (12.8)</td>
</tr>
<tr>
<td>ST-08</td>
<td>77.7</td>
<td>20.7 (26.6)</td>
<td>4.3 (20.7)</td>
<td>1.7 (8.2)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are percentages.

seeds used for screening hygromycin resistance, the germination rate was 38% on hygromycin solution at a concentration of 20 mg l⁻¹ (Mu et al. 2012). The low germination rate was possibly due to the seed maturity, pathogen on seed and mutation.

The pIG121HM-Cs plasmid had T-DNA containing selectable marker of nptII and hpt genes. A selectable marker was introduced to differentiate transformed cells from a population of untransformed cells and typically cotransformed with the gene of interest. Most selectable markers were antibiotics, such as kanamycin and hygromycin, or herbicides, such as glyphosate and phosphinothricin acetyl transferase (PAT). Kanamycin or hygromycin selection system has been used in the early step of maize transformation. Maize cells are less sensitive to kanamycin and the typically used concentration of kanamycin is 50 to 200 mg l⁻¹ on maize selection, but more sensitive to hygromycin. One of the factors that contributed to the ineffectiveness of this system might be the high endogenous resistance to kanamycin of maize tissues (Ji et al. 2013). The hygromycin concentration used in this research was 15 mg l⁻¹ for transformant selection. The concentration of hygromycin was based on the result of preliminary study that the LC₉₀ (lethal concentration that kills the test embryo) was 15 mg l⁻¹ hygromycin.

Acclimatization of Transformants and Molecular Analysis

Transformants which are survived on selection MS medium containing hygromycin was transferred to aqueous media for acclimatization step before being transferred into soil media. The number of transformants survived from the acclimatization process is shown in Table 1. Ultra Genjah line had 2.7 plants (10.4 % of survived embryos to hygromycin) survived in aqueous media from 97.7 planted embryos. The ST-18 line revealed 5.9 survived plants (20.1 % of survived embryos to hygromycin) from 95.0 planted embryos, and the ST-27 line showed 5.0 survived plants (33.8 % of survived embryos to hygromycin) from 22.5 planted embryos (20.4%). Approximately 4.3 plants of the ST 08 line survived out of 77.7 planted embryos (20.7 % of survived embryos to hygromycin). Based on the percentage of survival plants on acclimatization step, ST-27 line had the highest transformation efficiency about 33.8 % from an average of 22.5 planted embryos and Ultra Genjah line showed the lowest efficiency of 10.4 % from the mean average 97.7 planted embryos. The result of transformation and acclimatization were showed in Fig. 4.

The success of acclimatization was influenced by internal and external factors. Internal factors included less development of wax/cuticle layer, slight number of palisade cells, less development of vascular tissue from the roots to the shoots and minimum lignification of stem, while external factors were growth medium, humidity, light, temperature and pathogens (Gunawan 1992). The other factors affecting acclimatization were medium treatment, pot type and humidity (Pathi et al. 2013). A small pot composed of a mixture of peat moss, vermiculite and sand in the ratio of 1:1:1, and the increasing humidity by covering with polyethylene bags with small hole resulted in good plant regeneration.

One technique for early detection of transformation was the PCR amplification of the presence of the nptII and hpt genes that inserted on T-DNA of the pIG121Hm-Cs plasmid. T-DNA as a part of a binary plasmid (pIG121Hm-Cs) will be transferred through A. tumefaciens into plant cells by a special mechanism that involves some vir genes on the plasmid (Gelvin 2003). The nptII and hpt genes were introduced to differentiate transformed cells from a population of untransformed cells (Ji et al. 2013). Molecular analysis of transformants was done by PCR amplification of selectable marker gene using the specific primer of the nptII gene. Positive plants
containing \textit{nptII} gene were observed by the appearance of DNA fragment of 700 bp (Fig. 5). The T-DNA construction of pIG121Hm-Cs plasmid is composed of RB, \textit{nptII} gene cassette, \textit{CsNtr-1} gene cassette and \textit{htp} gene cassette. The high frequency of gene fusions observed after T-DNA integration into plant genome, the right T-DNA border were preferentially integrated into potentially transcribed genomic loci in different plant species (Mayerhofer \textit{et al.} 1991). Based on this study, PCR analysis for the existence of \textit{nptII} gene fragment proved T-DNA integration of pIG121Hm-Cs plasmids and the other gene should be inserted into plant genom.

The percentage and number of transformants containing \textit{nptII} gene which survived on hygromycin and acclimatization were one plant of Ultra Genjah line (3.8%), two plants of ST-18 line (5.8%), two plants of ST-27 line (12.8%) and two plants of ST-08

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{\textit{In planta} transformant of maize: (a) \textit{in planta} transformant corn cob, (b) selected transformant on MS medium containing hygromycin (14 days), (c) survival plantlets on water medium (7 days), where plantlet with normal leaves was survived acclimatization.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{PCR products of transformant using \textit{nptII} primer (700 bp): M = 1 Kb DNA ladder, A = ddH\textsubscript{2}O, lines 1–45 = transforms, K - = negative control (nontransformant plant), K + = positive control (containing \textit{nptII} gene).}
\end{figure}
line (8.2%). Previous study on in-planta transformation showed that 60.3% lines were positive of the total of survived plants on kanamycin selection or 6.8% of the total plants tested (Chumakov et al. 2006). In this study, ST-27 line produced 2 plants (12.8%) containing positive PCR nptII gene out of 15 plants survived on hygromycin selection (Table 1). The ST-27 line had the highest level of transformation efficiency, about 12.8% from 22.5 embryos planted on selection medium. On wheat, 26% PCR positive plants were obtained of the total of kanamycin-resistant plants (Razzaq et al. 2011). While on rice, the efficiency of transformation of T1 generation was 40% (Supartana et al. 2005).

Transformation efficiency resulted in this study was lower than that of other studies, and possibly caused by genetic factors of plant genotype and other factors such as temperature when treated with Agrobacterium. Among four maize lines studied, ST-27 had an efficiency level of 12.8% from 22.5 planted embryos. The increasing number of ST-27 line demonstrated the linear correlation with the number of transformant plants. It is assumed that the genetic background of Indonesian maize lines differs from the maize line as reported by Cumakov et al. (2006). Therefore, to increase the number of transformants of Indonesian maize lines, the number of embryos should be increased.

The temperature range of 19 to 22°C is optimal for the expression of T-vir genes in Agrobacterium (Fullner and Nester 1996) and 28°C is critical for excretion and assemble of vir-dependent Agrobacterium T-pili for successful T-DNA transfer (Fullner 1998). The treatment with Agrobacterium suspension on the silk of maize needs specific temperature particularly above 25°C (about 09.00 AM), in constrast, temperature of 22–25°C were less favorable for Agrobacterium-mediated transformation. These conditions seem related to the low efficiency of in-planta transformation. Moiseeva et al. (2014) stated that temperature was the most factor in Agrobacterium-mediated plant transformation, including on maize with antisense suppressor of the proline dehydrogenase gene (ASPG).

Based on the results, in-planta transformation through A. tumefaciens can be done successfully on Indonesia maize using pistil flower with minimum tissue culture step, simple and faster than regular transformation. The T1 plants successfully survived on the selection medium containing hygromycin, showed positive PCR using primers for nptII gene and successfully acclimatized. It proved that maize transformation using A. tumefaciens can be done by in-planta with minimum tissue culture steps for plants those are difficult to be in vitro regenerated.

CONCLUSION

Maize plant transformation can be conducted using A. tumefaciens-mediated in-planta method with minimum tissue culture step. The ST-27 line had the highest level of transformation efficiency of 12.8% of the total number of embryos based on PCR analysis using specific primer for nptII gene. Enhancement of the number of planted embryos can increase the number of transformant plants. A. tumefaciens-mediated in-planta transformation on maize using flower pistil was faster, simpler and low-cost method.

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