T-DNA Transfer and GUS Expression in Agrobacterium-mediated Transformation of C. annuum under a Range of in vitro Culture Conditions

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Abstract: Several factors that affect the survival rate and the effectiveness of Agrobacterium-mediated transformation which resulted in the expression of gus gene in transgenic C. annuum were investigated. The initial investigation on seed treatment has shown that intact seeds exhibited the highest rate of generation with 96.33%. Analysis of preculture treatment revealed that two days incubation on wet filter paper resulted in highest number of explants with GUS expression. No significant differences on percentage of GUS expression were observed on preculture with shaking at 120 rpm and exposure at 4°C which showed the highest of only 17% of explants with GUS activity recorded. Analysis of Agrobacterium and mature seeds interaction involved three Agrobacterium concentration for the inoculation phase, three co-culture and two incubation regimes. No differences in explant survival and GUS activity were observed between the two incubation time tested. The OD reading of 0.55 was shown to be the optimum inoculation condition for both explants survival and GUS activity. Analysis of co-culture regimes indicated that the shorter co-culture times resulted in higher explant survival and higher GUS activity, whereas the co-culture time of five days severely reduced the survival of explants and lowered the GUS potential. Selection of regenerated transgenic plants was performed on hygromycin-containing medium. The presence of the T-DNA was confirmed by Polymerase Chain Reaction (PCR) amplification of the internal fragment of gus and hpt genes. Enzymatic GUS assay confirmed the expression of the GUS-intron gene in the primary transformants as well as the progeny. The Chi-square analysis for segregation of Hyg-B resistance on at least 10 T progenies revealed that the transgene was inherited as a single or two mendelian locus.

Key words: T-DNA, Agrobacterium tumefaciens, Capsicum annuum, β-glucuronidase

INTRODUCTION

Plant genetic transformation provides a significant approach for modifying important traits of crop species by transferring specific exogenous DNA molecules into the plant genome. In recent decades, gene transfer technology and recombinant DNA has refurbished the plant biotechnology industry, spawning countless new plant based products and generation of modified plants with plenty of new characters such as resistance to foreign substances (etc. insect, pest, pathogen, virus), biotic/abiotic stress, improved yield quality and possible metabolite engineering for secondary products. Apart from that, the application of the technology has been expanded towards production of molecular pharming molecules in plants such, medicine, vaccines, proteins and other important compounds. Agrobacterium tumefaciens provides a reliable and well documented means for introducing exogenous DNA molecules into plant cells.

A. tumefaciens-mediated delivery of foreign gene into numerous plant species has been extensively described since Horsch et al. (1985) first demonstrated the utility of the system with subsequent documentation by other workers (Wordlagon and Dons, 1992; Fisk and Danekar, 1993; Ishida et al., 1996; Hiei et al., 1994).

A. tumefaciens-mediated transformation in many recaileant plant species such as C. annuum has far been from routine. During the past decades, researchers worldwide have made great deal of efforts and progress towards pepper regeneration and transformation for genetic improvement of pepper (Wang et al., 1991; Ye et al., 1993; Christopher et al., 1997; Jayashankar et al., 1997; Subhash and Christopher, 1997; Manoharan et al., 1998). Transgenic pepper plant expressing the cucumber mosaic virus (CMV) coat protein gene (Zhou et al., 1991; Zhu et al., 1996) and plant express CMV satellite RNA (Dong et al., 1992; Kim et al., 1997) were obtained, but with low regeneration and
transformation efficiencies and the published protocol could not repeated in other laboratories. Despite those recent successful results, pepper are still considered to be recalcitrant group for tissue culture and transformation. Some other promising alternative transformation methods using Agrobacterium have also been carried out in some other species such as seedling meristem (e.g., in Glycine max: Chee and Slighton, 1995; in Lotus Japonicus; Oger et al., 1996) and electroporation of apical meristem (e.g., in Pisum sativum and Vigna unguiculata: Chowraja et al., 1995, 1996). With these new methods, the tissue culture step is almost completely avoided, the process is faster and somaclonal variation is minimized.

For these reasons, we decided to re-explore the use of Agrobacterium tumefaciens as a vehicle for transformation using mature dried seeds as an explant source. Preliminary work on C. annuum using this technique (Ismail et al., 2005) has shown the possibility of transforming the T-DNA and recovery of transgenic lines. The data obtained have reported that the transgenes were transmitted to the progeny in a mendelian fashion even though some of the generated plants were believed to be chimeric. So far, there have been almost no report on the stable transformation and regeneration of fertile transgenic pepper plants by Agrobacterium transformation with mature dried seeds. Due to its potential as an alternative transformation system, we initially focused our effort on several parameters which we did not take into account in our previous work in order to increase the efficiency of the T-DNA transfer and its expression. In the study reported here, we tested the effect of varying seed treatments, precultured conditions, Agrobacterium concentration during inoculation, period of infection and co-cultivation. These biological parameters were expected to have strong effect on the survivability of the explants and GUS expression.

MATERIALS AND METHODS

Plant material: Dried mature seeds of pepper (Capsicum annuum) cultivar MC 11 were obtained from Malaysian Agriculture Research and Development Institute (MARDI). These seeds were surface sterilized with ethanol (1 min), 0.1% Hg Cl₂ (10 min) and commercial sodium hypoiodite (0.4% active chlorine) 20 min with gentle shaking at 120 rpm. Seeds were then thoroughly rinsed with sterile water four times. Seeds were allowed to germinate on half-strength Murashige and Skoog (1962) Medium in three difference conditions to test the general survivability response: i) dissection of the seeds into 2 halves ii) gently injured the seeds with forceps iii) intact seeds. All seeds cultures were sealed with Handi-Wrap and incubated at 24°C under a 16/18 h (day/night) photoperiod with light provided by cool-white fluorescence tubes at an average light intensity of 40 μmol m⁻²s⁻¹. The generated explants were observed after two weeks in culture.

Pre-culture condition: Before Agrobacterium infection, the following pre-culture conditions were applied to sterilized mature seeds; i) Incubation on wet filter paper and kept in the culture room at 25°C under a 16 h day photoperiod ii) Incubation on MS liquid media at 25°C and gently shake at 120 rpm iii) Incubation on wet filter paper and kept at 4°C. All the steps above were carried out for zero to seven days to analyse the optimum condition for transformation which will base on the number of explants with GUS activity.

Agrobacterium suspension culture: Agrobacterium tumefaciens strain LBA 4404 (Hoekema et al., 1983) containing the binary vector pCAMBIA 1301 (kindly supplied by Dr. Jeffery) was used for transformation. The pCAMBIA 1301 plasmid contained a gusA reporter gene and hygromycin phosphotransferase (hpt) gene which confers resistance to hygromycin. Both genes are under the regulatory control of the CaMV promoter but with CaMV 35 and nos terminator, respectively. The coding region of the reporter gene contains an intron that prevents translation by A. tumefaciens. The bacteria was cultured overnight at 29°C / 200 rpm in 20 mL of Luria Burtani (LB) media containing 100 μg mL⁻¹ rifampicin, 300 μg mL⁻¹ streptomycin and 50 μg mL⁻¹ kanamycin. The Agrobacterium suspension was pelleted by low-speed centrifugation using a table centrifuge and then resuspended to the desired OD₆₀₀ as indicated in the text for the Agrobacterium concentration experiments in liquid MS media with 100 μM acetosyringone being added immediately before seeds infection.

Transformation experiments: The transformation procedures were as described previously (Ismail et al., 2005), with modifications. Co-cultivation of mature dried seeds was carried out on MS medium that contained basal constituent with different concentration of Agrobacterium tumefaciens suspension (OD₆₀₀ = 0.4, 0.55 and 0.8) for 2 min and 30 min and co-cultivation on MS medium (solidified with 0.8% bacto agar) for 2, 4 and 5 days in the dark at 25°C. After co-cultivation, explants were washed three times with sterile distilled water and one time with liquid MS medium containing 500 μg mL⁻¹ cefotaxime (Duchefa) in 125 mL flask and shaken at 125 rpm. The explants were then blot-dried on filter paper and placed on MS medium containing 10 μg mL⁻¹ hygromycin and 250 μg mL⁻¹ cefotaxime for selection. All
cultures were maintained in growth chamber at 25°C with 16 h photoperiod under a photon flux of 80 μmol m⁻²s⁻¹ provided by a cool white fluorescence. After 4 weeks of selection, explants survival rate and the frequency of explant with GUS positive were visually determined. The optimized protocol which take into account the previous experiment was used for transformation and subsequent generation of T₁, progenies. After 2 days of co-cultivation (after treating with A. tumefaciens suspension; OD₆₀₀ = 0.55) the explants were transferred to MS medium containing hygromycin and maintained in condition as indicated above. After the plantlets reached 20-25 cm, they were transferred to soil in covered boxes for 7 days. They were then transferred to the green house, left to grow until reach maturity, self fertilized and produced seeds for T₁ progeny.

Segregation analysis of T₁ progenies: To test the functional expression of the hpt gene in the T₁ progenies, a germination test was performed. At least 180 seeds collected from T₀ plants and those from untransformed were germinated on medium containing 10 μg mL⁻¹ hygromycin. PCR analysis were carried out on germinated seedlings randomly selected among those survived on hygromycin-containing medium to check for the presence of transgenes.

β-Glucuronidase (GUS) histochemical analysis: Histochemical assay of GUS was performed on hygromycin resistant seedlings of T₀ and T₁ generation according to the modified methods of Jefferson (1987). The explants were incubated at 37°C overnight in 100 μL to 300 μL in X-Glc A (Sigma-Aldrich) solution with a composition modified to 50 mM Na₂HPO₄, 10 mM K₂, Fe (CN), 0.3% (v/v) Triton X-1, 0.5 mM K₂Fe(CN)₆, 0.5 mM sodium ascorbate (pH 7.0). In order to facilitate the penetration of the substrate, before reaction, the tissue were incubated in 90% acetone for 20 min at -20°C. The plant materials were incubated for 16 h at 37°C and then immersed in 70% (v/v) ethanol to bleach the chlorophylls in the case of cotyledons to reduce background and then rehydrated before observing under a dissecting microscope.

Polymerase chain reaction (PCR analysis): Molecular analysis was performed on the selected putative transgenic plants of T₀ and T₁ using polymerase chain reaction (PCR). Genomic DNA was extracted from whole transgenic seedling using “Genomic PrepTM Cells and Tissues DNA isolation kit” (Amersham, United kingdom) following the manufacturer’s protocol. The primer sequences created for amplification were as follows: 5’- CGCCGGATGCGAGATATTGGTA -3’ and 5’- ATTAATGCGTGCTGCTGCA -3’ for the 789 bp fragment of the gus gene; 5’- ACAGCGTCTCCGACCTGATGCA -3’ and 5’- AGTCAATGACCGGAGTTATGCG -3’ for the 591 bp fragment of the hpt gene. The final volume of PCR reaction mixture was 50 μL containing approximately 25 ng of genomic DNA, 20 pmol of primer, 0.5 U Taq polymerase (GIBCO-BRL Life Technologies Inc., Auckland New Zealand), 1 X PCR buffer, 1.5 mM MgCl₂, 10 mM Tris-HCL (pH 8.3), 50 mM KCL and 0.1 mM of each deoxynucleotide triphosphate (dNTPs). The reaction mixture was covered with mineral oil and denatured at 94°C for 5 min using a thermal controller (B. Braun, USA) followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 2 min with a final 10 min extension at 72°C. After the PCR reaction, 10 μL of each sample were separated by electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide (0.5 μg mL⁻¹).

RESULTS

Seed treatments for germination: We have shown that genetic transformation of pepper through in planta transformation could be an alternative method to the conventional tissue culture-based procedure. This has lead to presence work which looked into important varying parameters in order to optimize the gene transfer and its expression. One of those factors is the optimum condition of the mature seeds for Agrobacterium infection. The ability of the regeneration and its frequency was first to be determined in this method as these significantly affects the efficiency of seed transformation. In this experiment, we assayed three different types of treatment on seeds followed by germination on MS medium. In type I treatment, following sterilization the seeds were cut according to Ezura et al., (1993) method. After two weeks in culture, some seeds formed only the cotyledons or hypocotyls, whereas the other seeds produced incomplete hypocotyls and cotyledons (figure not shown). The overall percentage of germination is about 40% (Table 1). In type II treatment, the seeds were wounded using scalp. The survival rate of the seeds after two weeks of culture is considerably high with 77% (Table 1). Nevertheless, 48.1% of the germinating seeds showed necrotic symptom specifically at the hypocotyls and roots site. Furthermore, the medium and site of wounding turned into brown (figure not shown). In type III treatment, intact seeds were used and resulted in 96.33% of generation rate. The seeds germinated and developed into complete and normal plantlets in 2-3 weeks in MS medium without any plant growth regulators. Based on the survival and generation rate, we then used type III-treated seed as explants for our transformation experiments.
Fig. 1: Percentage of explants with GUS expression following different precultured treatments; a) Incubation on MS liquid media at 25°C and gently shake at 120 rpm, b) Incubation on wet filter paper and kept at 4°C, c) Incubation on wet filter paper and kept in the culture room at 25°C under a 16 h day photoperiod.

Table 1: Seeds treatments and percentage of germination. Data represents the mean values ± standard error (SE) of three replicates.

<table>
<thead>
<tr>
<th>Seeds treatments</th>
<th>Number of seeds for germination (3 replicates)</th>
<th>Percentage of seeds germinated</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>300</td>
<td>49.3 ± 12.5</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>77.0 ± 16.7</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>96.3 ± 8.9</td>
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Effect of preculture treatments on Agrobacterium infection: The influence of preculture condition on the capability of the seeds resistance towards Agro infection and GUS expression can be analysed in Fig. 1a–c. Seeds in this experiment were first precultured accordingly before infection with Agrobacterium suspension culture. In experiment 1, seeds were cultured in MS media at 25°C and shake at 120 rpm for 0-7 days. Based on Sunil Kumarr (1999), tobacco tissue produced high level of vir induction compound after shaking process due to active cells divisions thus increased the efficiency of transformation. Figure 1a showed that seeds shaked in the MS media for one day resulted in highest number of explants with GUS positive (17%). Nevertheless the GUS expression reduced with time after that. The rate of GUS expression in seeds cultured on wet filter paper and kept at 4°C was analysed. Cold treatment was reported to enhance synchronized germination and GUS expression of A. thaliana seeds (Feldmann and Marks, 1987). However, based on our observation, cold treatment is not
suitable for pepper seeds. The percentage of plantlet with GUS positive was considerably low (less than 10%). For explants that have been precultured for more than 3 days, the seeds turned to brown colour and the germination percentage was only 3% (Fig. 1b). However, the GUS expression in pepper seedlings was enhanced by preculturing on wet filter paper at 25°C. Result from Fig. 1c has showed that 2 and 5 days preculture period has resulted in 46 and 30% of GUS positive respectively. For 3 and 4 days period, the GUS positive was 0 and 12% and this may due to Agrobacterium contamination as observed around the seeds in the medium.

Factors affecting the survival and GUS activity of the plantlets: To test the parameter that might be responsible for the efficient seeds transformation of pepper, we evaluated three Agrobacterium concentrations (OD600=0.4, 0.55 and 0.8), two infection period in the Agrobacterium suspension (2 and 30 min) and three co-cultivation times (2, 4 and 5 days). Explants were scored for survivability and frequency of GUS expression (Fig. 2a-c). Concentration of OD600 = 0.55 was the most suitable for infection of seeds with an average of 45% explants expressing the GUS activity. Even though higher number of explants (48%) with GUS activity was obtained with Agrobacterium concentration at OD600=0.8, the level of survivability is lower (53%) as compared to 92% and 94% at OD=0.4 and 0.55, respectively. One of the main reason was due to high density of Agrobacterium suspension, thus make it difficult to be eliminated even though with higher antibiotic concentration. This contamination remarkably affected the generation rate by facilitating the development of necrotic symptoms which finally reduced the germination rate (Fig. 2a).
Regeneration of To Transgenic Chili and its T₃ Progenies: Following the protocol and optimization of parameters as mentioned above, the intact mature seeds were used as explant for initiating experiments on genetic transformation, subsequent regeneration of T₃ progeny and analysis of T-DNA expression. Over 130 independently putative transgenic plants identified through hgy-B selection and PCR analysis were maintained in the glasshouse for further analysis. Transgenic plants appeared phenotypically normal but not all plants set viable seeds. No apparent undesirable genetic change due to transformation process has been observed in the glasshouse grown plants. This process has been tested with variety Bangi-4 and Bangi-2 for transformation response and a number of putatively transformed shoots were rescued. Molecular test have confirmed their transgenic status (Ismail et al., 2005). All the seeds collected from self fertilized of an individual transformant were dried and stored at 5-10°C. So far about 66 independently putative transformed lines have successfully been transplanted to the glasshouse and their T₃ generation seed were collected. Several independent transformant lines were selected randomly and their seeds collected for germination on selection MS media containing hygromycin antibiotic for transgene segregation analysis.
Table 2: Genetic analysis of the progeny from 15 transgenic plants for the hygromycin resistance trait and GUS analysis.

<table>
<thead>
<tr>
<th>To Lines</th>
<th>No. of T1 seeds</th>
<th>Hpt*</th>
<th>Hpt</th>
<th>Ratio</th>
<th>Number of Explants with GUS positive</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>germinated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>148</td>
<td>52</td>
<td>3.1</td>
<td>0.11 16/20</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>154</td>
<td>46</td>
<td>3.1</td>
<td>0.43 17/20</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>189</td>
<td>11</td>
<td>15:1</td>
<td>0.19 17/20</td>
</tr>
<tr>
<td>4</td>
<td>180</td>
<td>138</td>
<td>40</td>
<td>3.1</td>
<td>0.27 15/20</td>
</tr>
<tr>
<td>5</td>
<td>189</td>
<td>135</td>
<td>54</td>
<td>3.1</td>
<td>1.28 13/20</td>
</tr>
<tr>
<td>6</td>
<td>175</td>
<td>136</td>
<td>39</td>
<td>3.1</td>
<td>0.69 17/20</td>
</tr>
<tr>
<td>7</td>
<td>200</td>
<td>158</td>
<td>42</td>
<td>3.1</td>
<td>1.71 18/20</td>
</tr>
<tr>
<td>8</td>
<td>190</td>
<td>169</td>
<td>21</td>
<td>15:1</td>
<td>7.47 15/20</td>
</tr>
<tr>
<td>9</td>
<td>200</td>
<td>160</td>
<td>40</td>
<td>3.1</td>
<td>2.67 17/20</td>
</tr>
<tr>
<td>10</td>
<td>185</td>
<td>142</td>
<td>43</td>
<td>3.1</td>
<td>0.31 12/20</td>
</tr>
<tr>
<td>11</td>
<td>190</td>
<td>62</td>
<td>128</td>
<td>-</td>
<td>- 3/20</td>
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<tr>
<td>12</td>
<td>180</td>
<td>0</td>
<td>180</td>
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<tr>
<td>13</td>
<td>200</td>
<td>0</td>
<td>160</td>
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<td>- 0/20</td>
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<tr>
<td>14</td>
<td>200</td>
<td>38</td>
<td>162</td>
<td>-</td>
<td>- 3/20</td>
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<tr>
<td>15</td>
<td>150</td>
<td>17</td>
<td>133</td>
<td>-</td>
<td>- 0/20</td>
</tr>
<tr>
<td>Control K2a</td>
<td>200</td>
<td>24</td>
<td>186</td>
<td>-</td>
<td>- 0/20</td>
</tr>
</tbody>
</table>

Indicator: a) Degree of freedom (df) = 1, p = 0.05, \( \chi^2 = 3.84 \) 
b) Degree of freedom (df) = 1, p = 0.05, \( \chi^2 = 7.82 \)

Table 3: PCR analysis on the presence of gus and hpt genes in T1 population.

<table>
<thead>
<tr>
<th>To Lines</th>
<th>No. of progeny analysed</th>
<th>gus Hpt*</th>
<th>Hpt GUS</th>
<th>Hpt* GUS*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>9</td>
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<tr>
<td>3</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
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<td>9</td>
<td>1</td>
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<tr>
<td>Control K2a</td>
<td>5</td>
<td>-</td>
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<td>-</td>
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GUS expression and PCR analysis: GUS analysis was carried out after 3-4 weeks in culture medium in order to allow a complete formation of the plantlets. Both putative transgenic To and T1 lines which were previously screened as hygromycin insensitive were randomly selected and incubated in GUS solution. In most cases, the primary transformants and its progenies exhibited GUS staining either at certain parts or at whole plantlet. The intensity of GUS staining observed in the progeny were generally similar to that observed in the initial transformants. Meristem tissues displayed blue staining with higher frequency compared to the other parts. Due to a very thin layer of young plantlet tissue, GUS expression can be observed throughout the vascular tissue of the plantlet (Fig. 3). A few progenies, however differ from their parent in term of GUS expression level. In some of the lines, the level of GUS expression reduced with the increased of the plantlet's age. In extreme case, the expression was completely lost in tissue obtained from transgenic plant grown in the glasshouse. These lines have been previously screened as hygromycin insensitive and positive with PCR analysis.

PCR and transgene segregation analysis: A total of 150-200 T1 progenies obtained after self pollination of randomly selected 15 T0 plants were studied for inheritance of hygromycin resistance. 10 of the 15 T0 plants exhibited the mendelian inheritance pattern based on the Chi-square analysis at the confidence level of 95%. Most of these progenies showed 3:1 segregating ratios whereas 2 T1 plants (T1a and T1b) have their progenies with 15:1 ratios. Progenies from 2 independent transgenic plant (T1a and T1b), however, were all hygromycin sensitive. From all the lines that showed mendelian segregation, 65% of their resistance T1 progeny tested were GUS positive. 15% of GUS positive was recorded from plantlets which did not follow the mendelian segregation pattern. No GUS activity was observed in control plant (Table 2). DNA amplification was carried out on To and T1 transgenic plants to confirm and analyse the presence and pattern of the transgenes. Genome DNA from young leaves of both generations were prepared and then analysed by PCR. Expected 783 and 591 bp fragments corresponding to the internal fragments of gus and hpt gene coding sequence was observed. These PCR results indicated that both linked marker gus and hpt genes were present in both To and T1 plants thus suggesting stably inheritance of the genes between generation (Fig. 4). In another experiment, 10 hygromycin resistance T1 plantlets were selected at random from each of To plants for PCR analysis to determine the pattern of transgenes distribution. Most of the To lines which followed the mendelian segregation (T1 to T0) have shown the presence of both genes in their progenies (Table 3). About 5 progenies of 4 T0 plants (T1a, T1b, T1c, and T1d) inherited only one transgene (either hpt+ gus- or hpt- gus+).

DISCUSSION

Various factors with possible influences on the efficiency of T-DNA delivery and its expression were evaluated and transgenic plants of pepper with its progeny were obtained. Previously, the procedure of pepper transformation (Ismail et al., 2005) has established our effort to use dried mature seeds as plant material for transferring marker gene and subsequent generation of transgenic pepper. In this work, by measuring the expression frequency of transgene in young plantlets/seedlings, we showed that the choice of preculture treatments on intact seeds is a prerequisite for Agrobacterium-mediated transformation and the further culture development. The study also demonstrates the importance of interaction effect between the Agrobacterium and explant that need to be taken into account when designing transformation protocol.
Explant is the basic component in genetic transformation as it contains cells capable of generation and can be further treated to better receive the foreign DNA molecules (Birch 1997; Satyavathi et al., 2002). Normally, only a small number of cells in a target explant receive the foreign DNA and thus reduces the frequency of transformation and survivability. This is due to the fact that very low probability of occurrence for both DNA transfer and regeneration of a particular cell (Chee et al., 1989; Donaldson and Simmonds, 2000). In type I treatment, the seeds were excised according to Ezura et al. (1993). The hypocotyls proximal and radicle excised from intact seeds were able to form adventitious shoots and roots MS media without plant growth regulators. The chromosome number of the generated plantlets is normal (2n=24) with mature plant showed no difference in morphology and seeds production with its parental. In contrast, our attempt using this treatment to produce complete plantlet was not successful. The reason for the failure was unclear but we suspected that the excision was not carried out correctly as the internal structure of pepper seeds was difficult to be figured out and this may lead to severe injury to the explants. In type II treatment, seeds showed necrotic symptom and cause the medium turned into brown color. This phenomenon reduced the regeneration rate and about 77% formed complete plantlets. The use of intact seeds has been the best option for pepper transformation. The regeneration rate was extreme highly at 96% and complete plantlet was formed within 2-3 weeks in MS medium without plant growth regulators. Alejo and Malagon (2001) has reported higher regeneration rate and with shorter time for complete plantlet formation using this techniques compared to other protocols. In other experiment, Grave and Goldman (1986) had successfully transformed the mesocotyl region of the germinating corn seed eventhough no inheritance analysis was reported. This was then followed by successful regeneration of transgenic A. thaliana (Feldman and Marks, 1987) and soya beans (Chee et al., 1989) using mature seed transformation. Other than seeds, cotyledon (McKently et al., 1995; Oger et al., 1996), lateral cotyledon meristem (Bean et al., 1997) and embrio axis (Polowick et al., 2000; Rohini and Rao, 2000) have also been used for successful transformation. The use of these explants has something in common where the plantlet was formed directly without going through callus formation thus, avoiding possible somaclonal variation.

Two days preculture period of explants on wet filter paper positively affect vir gene expression, as indicated by more rapid and greater induction of transient GUS expression from the explants (Fig. 3). The preculture treatment is hypothesized to create a window of competence phase for efficient transformation and to promote cell division and growth of explant. It was also suggested that during preculture treatment, the seeds may have produced a sufficient amount of vir gene inducers. In addition, we found that wounding treatment was not necessary in this work even though previous data have shown the significant of the step for vir gene induction (Tsukazaki et al., 2002; Weber et al., 2003). In fact, our observation with wounded seeds treatment (Type I and Type II) has recorded lower generation rate of 40.33 and 77%, respectively as compared to those untreated seeds. This is consistent with the result obtained by Trifonova et al. (2001) and Popelka and Altpeter (2003) which demonstrated that wounded treatment explant lead to excessive tissue damaged and reduced survivability rate of the transformed cells. However, the question whether seed treatments in our work are producing the appropriate type and amount of wounding signals for induction of the Agrobacterium infection machinery is still open. It is generally accepted that only plants with an appropriate wound response will develop large populations of wound-adjacents cells that are competent for transformation (Potrykus, 1991). The analysis on the co-cultivation protocol within the 2 days regime has indicated the enhancement of A. tumefaciens infection of seeds and facilitated stable transformation. Two days of co-cultivation has been applied in several chili variety and resulted in substantially high frequency of transgenic chili plants (Liu et al., 1990; Li et al., 2003; Manoharan et al., 1998). Such a period was also consistency with the optimum time required for T-DNA transfer and tumor formation in Agrobacterium infection which actually lies between 24 to 48 h (Binnis, 2002) and optimum vir genes induction at 25°C (Salas et al., 2001).

Variation in gene expression level in genetically modified plants is a general phenomenon. In most of the cases, variable GUS expression in transgenic plants is depend on types of tissue, development stage, surrounding environment and transformation event. The variation may due to some genetic factors such as promoter sequences, differential mutilation, gene copy number, co-suppression and phenolic compound (Serres et al., 1997). In this study such a variation was observed in both T0 and T1 plants eventhough the blue staining was highly concentrated at the young meristem tissue where the cells were actively divided and differentiated. We did not carry out the gene copy number analysis, so its connection and effect on the expression level cannot be confirmed in this work. Nevertheless, Trifonova et al. (2001) in their work has reported variation in gene expression level in transgenic barley plants but found it was not seem to be correlated with gene copy numbers. The underlying mechanism for uid A gene variation in expression and silencing in rice were discussed by Kohl et al. (1999). Suprisingly, we also
observed that detectable GUS activity in some of the transgenic plants grown in the glasshouse was reduced significantly or totally lost. The same observation was also reported by Cote and Rutledge (2003) which indicated that plant grown in the field has 10 times lower the GUS activity as compared to those maintained in in vitro culture. This phenomenon was strongly supported by earlier finding which suggested the remarkable negative effect of endogenous inhibitors and stages of plant development cycle on GUS expression (Seres et al., 1997). Another possibility could be the effect of environmental factors on the function of 35 CaMV sequences which resulted in decreased or loss of GUS activity (Schurr and Guerra, 2000).

We have shown the transformation procedure yields reproducible result even though with some variation in efficiency. Molecular and Chi-square analysis confirmed that the plants had carried both transgenes and most often in one locus as indicated by 3:1 segregation ratio in most T1 families while 2 lines showed the segregation at 2 locus in which the 1:5:1 ratio was observed. Probably because selection was performed on hygromycin-containing medium, the internal hpt sequence was detected in all 15 T0 plants and expression was confirmed for majority of the plants. From PCR analysis, most of the T0 plants progenies which follow the mendelian segregation carried both transgenes suggesting efficient selection by 10 μg mL⁻¹ hygromycin. In summary, the seeds transformation and regeneration system proved to be a potential vehicle for the production of a large number of transgenic pepper over relatively short periods. Our study emphasizes on different conditions for transformation efficiency and demonstrate that by monitoring the GUS expression, different factors leading to efficient and reproducible transformation of pepper by means of Agrobacterium can be optimized. While this procedure was only tested on one strain of A. tumefaciens and with one chili cultivar, it is believed that the results will be applicable to other Agrobacterium strains and chili cultivars.

ACKNOWLEDGEMENT

The study was supported by a Universiti Kebangsaan Malaysia grant (320316005) and in part by IRPA grant (09-02-02-0081-EA205) from the Ministry of Science, Technology and Innovation (MOSTI) awarded to Ismanian Ismail. Financial assistance to Miss Sophia Kent and Mr Dinesh a/l Natarajan in the form of National Science Fellowship (NSF) from MOSTI is gratefully acknowledged. We are very thankful to CSIRO Australia for providing the pCAMBIA 1301.

REFERENCES


